2020-06

Oxidative stree management is essential for anopheles mosquito survival post plasmodium infected blood meal ingestion

Tarimo, Brian B.

NM-AIST

OXIDATIVE STREE MANAGEMENT IS ESSENTIAL FOR *ANOPHELES* MOSQUITO SURVIVAL POST *PLASMODIUM* INFECTED BLOOD MEAL INGESTION

Brian B. Tarimo

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology

Arusha, Tanzania

June, 2020

ABSTRACT

Anopheles mosquitoes like other dipterans lack the flavoenzyme glutathione reductase (GR) of the GSH pathway and instead utilize the Thioredoxin (Trx) system for management of oxidative stress. *Anopheles gambiae* (*An. gambiae*) and *Anopheles stephensi* (*An. stephensi*) mosquitoes have been shown to regulate genes and proteins of the Trx system to protect midgut epithelial cells against reactive oxygen/nitrogen species (ROS/RNS) associated with *Plasmodium berghei* (*P. berghei*) infection. However, this mosquito vector-parasite combination is not natural and may not necessarily reflect human malaria transmission biology in the field. Despite its importance, a complete understanding of the Trx pathway at the molecular level is missing.

Mosquito feeding assays were used to examine the Trx response pathway following midgut exposure to ROS/RNS, by measuring first the protein expression level of Thioredoxin-1 (*Ag*Trx-1) post exposure to tert-Butyl hydroperoxide (tBHP) by quantitative immunoblot analysis. This was followed by measuring the global proteomic response to Paraquat (Pqt) and tBHP exposure. The proteomic response was then compared to a spectrum of Trx- and GSH-dependant transcripts 24 hours post-infected bloodmeal ingestion in the more natural vector-parasite combination of *An. gambiae-Plasmodium falciparum* (*P. falciparum*) to assess for: (a) concordance between protein and transcript under different oxidative conditions and (b) similarity to the unnatural vector-parasite (*An. gambiae*/*An. stephensi-P. berghei*). It was observed that protein levels of *Ag*Trx-1 remained unchanged in midgut epithelial cells exposed to different concentrations of tBHP. Moreover, proteomics profiles of midgut epithelial cells under tBHP-and Pqt-induced oxidative stress showed cells that are undergoing redox regulation through ribosomal/nucleolar and ER-stress responses, respectively. This response is contrary to the canonical antioxidant response previously described. Furthermore, transcript data showed an absence of significant upregulation in the Trx- and GSH-dependent genes. This is consistent with the concept that *P. falciparum* does not induce marked midgut destruction in *An. gambiae*, therefore its invasion process is associated with reduced oxidative stress.

The ribosomal/nucleolar and ER stress responses to oxidative stress suggest additional response mechanisms to the canonical antioxidant responses. These additional responses could be translated to develop strategies that could lead to unmanageable levels of ROS/RNS exposure to the parasite

in the midgut, yet still allowing the mosquito to survive the dysregulation. This would lead to a stop in parasite development leading to blocking of transmission.

DECLARATION

I, (BRIAN B. TARIMO) do hereby declare to the Senate of Nelson Mandela African Institution of Science and Technology that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Brian B. Tarimo _____ **Date**: _____

Name and signature of candidate

The above declaration is confirmed

Prof. Joram J. Buza _____ **Date:** _____

Supervisor 1

Prof. Rhoel R. Dinglasan _____ **Date:** 08 APRIL 2020

Supervisor 2

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology the dissertation titled: "Oxidative Stress Management is Essential for *Anopheles* Mosquito Survival Post *Plasmodium* infected Blood Meal Ingestion" by Brian B. Tarimo in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Life Sciences and Bioengineering of the Nelson Mandela African Institution of Science and Technology.

Prof. Joram J. Buza _____ Date: _____

Supervisor 1

Prof. Rhoel R. Dinglasan _____ **Date:** 08 APRIL 2020

Supervisor 2

ACKNOWLEDGEMENT

Exactly 30 years ago, I embarked on my formal education journey when my parents enrolled me for Grade I at Muhimbili Primary School. It has been a remarkable journey, one characterized by deep lows and super highs. There is an African proverb that says, “It takes a village to raise a child”. Many people have played a role in my education, whether directly or indirectly, and I would like to extend my gratitude to them as I near completion of my PhD.

First, I would like to thank my parents for their love, support, and encouragement that they have shown to me and my siblings. Thank you for instilling in me the enthusiasm for knowledge and the values of hard work and determination towards accomplishing my goals. Furthermore, I would like to thank my siblings for their love and friendship, and for filling my life with happiness and joy from a child up to now. And to the many other relatives: grandparents, aunties, uncles, cousins, in-laws, nieces and nephews I would like to say thank you very much.

My passion for science, particularly Biology, stems from very early in my education. Thanks to the many teachers throughout primary school, high school, and university who taught me, and encouraged me to pursue this subject as a profession.

I thank my employer, the Ifakara Health Institute (IHI), who solely sponsored my studies. Thank you for granting me a study leave so I can pursue my studies. Thank you for the patience and tolerance you have extended to me as I’ve tried to finalize and hand-in my thesis.

To my PhD supervisors, Profs. Joram J. Buza and Rhoel R. Dinglasan, I would like to say thank you very much. Rhoel, thank you for granting me the opportunity to spend time in your laboratory to carry out my research. Thank you for the financial support and the mentorship that you have extended to me towards my studies. I thank you for your continued and invaluable support as I embark on my own scientific career. Furthermore, I would like to thank all the members of the Dinglasan lab who made my stay at Johns Hopkins pleasant and memorable. Thank you for teaching me valuable techniques and concepts, and for your patience and tolerance when I screwed up experiments or didn't understand basic concepts. Your friendship has been extremely valuable. Also, thanks to the members of the NM-AIST community who made studies and life at campus pleasant and enjoyable.

Last but certainly not the least, I would like to thank my wife Jane Flora Mgone. I was your friend when I started this journey and now, I am your husband and a father to our little girl as I finish this. Thank you for being that one constant support throughout this journey. Thank you for believing in me when I didn't and for the encouragement to persevere when times were tough. Thank you for tolerating my absence and for gracefully handling it. I love you very much.

DEDICATION

To my wife, Jane Flora Mgone, and our beloved daughter Ava Imani Tarimo.

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LIST OF ABBREVIATIONS AND SYMBOLS

$^{\cdot}OH$	Hydroxyl radical
2D	2 Dimension
ACT	Artemisinin-Combination Therapy
Ag	*Anopheles gambiae*
*Ag*APN-1	*Anopheles gambiae* Aminopeptidase 1
*Ag*RpL32	*Anopheles gambiae* 60S Ribosomal Protein L32
*Ag*Trx-1	*Anopheles gambiae* Thioredoxin 1
AMA1	Apical Membrane Antigen 1
ANOVA	Analysis of Variance
AP2-G	Apetala2-G
APN1	Aminopeptidase 1
ATP	Adenosine Triphosphate
ATQ	Atovaquone
ATSB	Attractive Toxic Sugar Baits
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
CAT	Catalase
CelTOS	Cell Traversal Protein for Ookinetes and Sporozoites
$CO3^{\cdot-}$	Carbonate anion radical
CPS	Chemoprophylaxis with Sporozoites

CQ	Chloroquine
CSP	Circumsporozoite protein
CYP	Cytochrome P450
CYT	Cytochrome
DDT	Dichlorodiphenyltrichloroethane
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DNA	Deoxyribose Nucleic Acid
ER	Endoplasmic Reticulum
FAD	Flavin Adenosine Dinucleotide
FASP	Filter-Aided Sample Preparation
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
FP	Ferri/ferroprotoporphrin IX
G6PD	Glucose-6-phosphate Dehydrogenase
GAP	Genetic Attenuated Parasites
GLURP	Glutamate-rich Protein
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
Grx	Glutaredoxin
GS	Glutathione Synthase

GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-Transferase
GTS	Global Technical Strategy
H_2O_2	Hydrogen peroxide
$HO_2^{\cdot}$	Hydroperoxyl radical
HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
IgG	Immunoglobin G
IHI	Ifakara Health Institute
IPTi	Intermittent Preventive Treatment in infant
IPTp	Intermittent Preventive Treatment in pregnancy
IRS	Indoor Residual Spray
ITNS	Insecticide Treated (bed)nets
JNK	c-Jun N-Terminal Kinase
JHSPH	Johns Hopkins Bloomberg School of Public Health
JHMRI	Johns Hopkins Malaria Research Institute
K-13	Kelch propeller domain
LC-MS/MS	Liquid Chromatography-tandem mass spectroscopy
LSM	Larval Source Management
lysoPC	lysophosphatidylcholine

mAb	monoclonal Antibody
MB	Methylene Blue
MC	Maurer's Cleft
MDM2	Mouse Double Minute 2
MFA	Membrane Feeding Assay
MMV	Medicine for Malaria Ventures
MSP 1	Merozoite Surface Protein
MVIP	Malaria Vaccine Implementation Programme
NADPH	Nicotinamide dinucleotide phosphate
NAI	Naturally Acquired Immunity
NM-AIST	Nelson Mandela African-Institution of Science & Technology
NO	Nitric oxide
NO_2	Nitrogen dioxide
$O_2^{-\cdot}$	Superoxide anion
OH^-	Hydroxide ion
$ONOOCO2^-$	Nitrosoperoxy carbonate
$OONO^-$	Peroxynitrite
Pb/P. berghei	*Plasmodium berghei*
PBS	Phosphate Buffered Saline
Pf/P. falciparum	*Plasmodium falciparum*
Pfcrt	*Plasmodium falciparum* CQ Resistance Transporter

*Pf*EMP-1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
*Pf*GDV1	*Plasmodium falciparum* Gametocyte Development Protein 1
*Pf*Hda2	*Plasmodium falciparum* Histone Deacetylase 2
*Pf*HP1	*Plasmodium* falciparum Heterochromatin Protein 1
Pfmdr1	*Plasmodium falciparum* Multidrug Resistance 1
*Pf*NEK4	*Plasmodium falciparum* Nima-related Kinase 4
*Pf*SPZ	*Plasmodium falciparum* Sporozoite
Pk/P. knowlesi	*Plasmodium knowlesi*
PM	Peritrophic Matrix
Pm/P. malariae	*Plasmodium malariae*
Po/P. ovale	*Plasmodium ovale*
PQ	Primaquine
Pqt	Paraquat
PV	Parasitophorous Vacuole
Pv/P. vivax	*Plasmodium vivax*
Py/P. yoelii	*Plasmodium yoelii*
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
QTOF	Quadrupole Time of Flight
RAS	Radiation Attenuated Sporozoites
RBC	Red Blood Cell
Rh5	Reticulocyte-binding Protein homolog 5

RNS	Reactive Nitrogen Species
RON 2	Rhoptry Neck Protein 2
ROS	Reactive Oxygen Species
RP	Ribosomal Protein
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SLARP	Liver stage Asparagine-rich Protein
SMFA	Standard Membrane Feeding Assay
SOD	Superoxide dismutase
SP	Sulfadoxine-Pyrimethamine
SSA	sub-Sahara Africa
TBD	Transmission-blocking Drug
tBHP	tert-Butyl Hydroperoxide
TBV	Transmission-blocking Vaccine
TCP	Target Candidate Profile
TPx	Thioredoxin Peroxidase
TRAP	Thrombospondin-related Adhesion Protein (TRAP)
Trx	Thioredoxin
$Trx(SH)_2$	Reduced Thioredoxin
TrxR	Thioredoxin Reductase
$TrxS_2$	Thioredoxin disulphide

UPR	Unfolded Protein Response
WHO	World Health Organisation
XA	Xanthurenic Acid

CHAPTER ONE

GENERAL INTRODUCTION AND BACKGROUND

1.1 Malaria Overview

Malaria is a disease caused by a blood cell infection by a protozoan parasite of the genus *Plasmodium*. Malaria is transmitted from an infected to an uninfected human by female mosquitoes of the genus *Anopheles* (Carter & Mendis, 2002). Historically, four species of *Plasmodium* parasites are known to infect humans, these are *Plasmodium malariae (P. malariae)*, *Plasmodium ovale (P. ovale)*, *Plasmodium vivax (P. vivax)* and *Plasmodium falciparum (P. falciparum)* (Carter & Mendis, 2002). Currently, six species of *Plasmodium* are known to infect humans and these are *P. falciparum, P. vivax, P. malariae, Plasmodium knowlesi (P. knowlesi)* (evidence of only zoonotic transmission) and the recently recognized species of *P. ovale curtisi* (former classic type) and *P. ovale wallikeri* (former variant type) (Cox-Singh *et al.*, 2008; Cox-Singh & Singh, 2008; Fuehrer & Noedl, 2014; Singh *et al.*, 2004; Sutherland *et al.*, 2010).

The widest global distribution has almost been achieved by *P. vivax* and *P. malariae* having been identified and characterized in Europe since historical times as benign tertian and quartan periodic fevers, respectively (Celli, 1933; Jones, 1909). Today, *P. vivax* and *P. falciparum* are the most widespread malaria parasites with *P. falciparum* being the most prevalent on the African continent, while *P. vivax* has a wider geographical distribution across Asia as well as Central and South America (Carter & Mendis, 2002). Not all existing species of *Anopheles* mosquitoes transmit malaria; out of about 400 existing species only 30 are vectors of major importance (Global Malaria Programme, 2015b; Sinka *et al.*, 2012). In Africa, four species of *Anopheles* mosquitoes are the dominant vectors: *Anopheles coluzzii*, *Anopheles gambiae s.s.*, *Anopheles arabiensis*, and *Anopheles funestus* (Sinka *et al.*, 2011; Sinka *et al.*, 2012).

About 3.2 billion people, that is nearly half of the world's population, are at risk of malaria. In 2017 alone, there were an estimated 219 million new cases of malaria and 435 000 deaths (Global Malaria Programme, 2018). Most of the malaria cases occurred in Sub-Saharan Africa and thus *P. falciparum* was responsible for most of the malaria cases and deaths (Global Malaria Programme, 2018). Despite the high mortality rate, substantial progress in malaria control has been made. There

were an estimated 20 million fewer cases in 2017 compared to the year 2010, which registered an estimated 239 million cases globally as shown in Fig. 1 below.

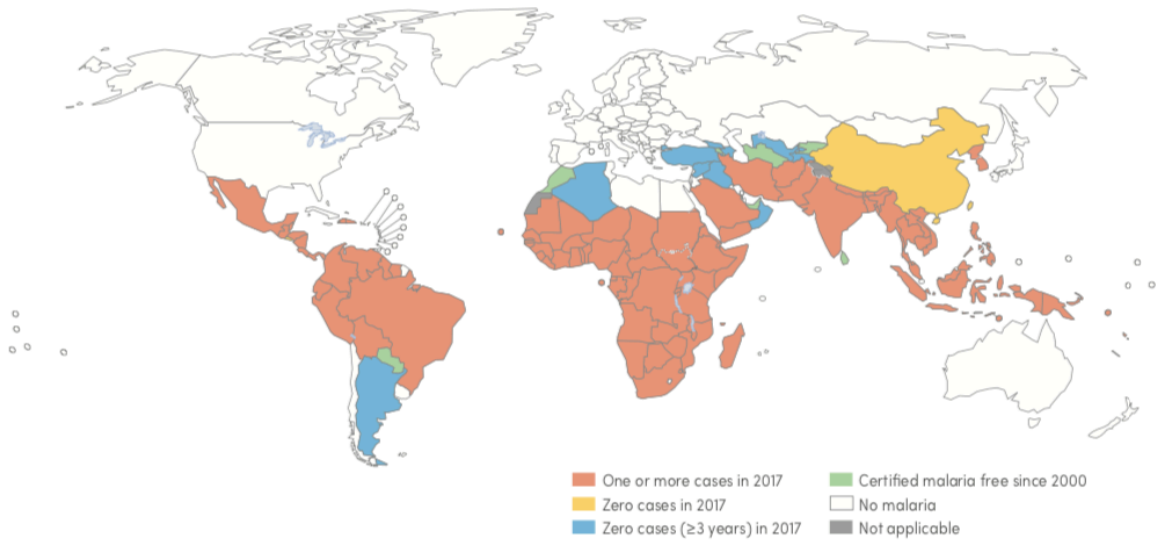

Figure 1. Global malaria cases as of 2017. The figure shows countries with indigenous Malaria cases in 2000 and their status in 2017 (Global Malaria Programme, 2018)

Only a minimal (if not slightly upward) change in the global malaria cases was reported between 2015-17, suggesting that progress had generally stalled (Global Malaria Programme, 2018). This is a massive obstacle in the vision of a world free of malaria through the WHO Global Technical Strategy (GTS) 2016-30, that aims for at least a 40% reduction in malaria cases and deaths by the year 2020, at least a 90% reduction in cases and deaths by the year 2030, and elimination of malaria in at least 35 countries by the year 2030 (Global Malaria Programme, 2015a). In 2017, annual funding for malaria control and elimination were estimated at US$ 3.1 billion per year (Global Malaria Programme, 2018).

Annual funding for malaria control and elimination will have to increase to at least US$ 6.6 billion per year by 2020 if we are to meet the 2030 GTS goals (Global Malaria Programme, 2018). With this in mind, it's clearly evident that the current tools for malaria control are reaching or have reached their maximum protective levels. Novel strategies for malaria control that will

complement the existing tools will have a profound effect on the number of malaria cases and deaths, and thus shifting the balance in favour of malaria control and elimination efforts. For this to happen, there is an urgent need for detailed understanding of the basic biology of *Plasmodium*, *Anopheles*, and their host-parasite interactions in order to develop novel strategies against malaria.

1.1.1 *Plasmodium* Life Cycle

Plasmodium leads an intricate life cycle that involves development in the invertebrate female *Anopheles* mosquito and a vertebrate (e.g. humans). In each of these divergent hosts, *Plasmodium* undergoes development in different body compartments of the host.

1.1.2 Pre-erythrocytic (Liver) Stages

The life cycle in the vertebrate host begins when *Plasmodium* sporozoites residing in mosquito's salivary glands are injected into their skin as it probes for blood (Sinnis & Zavala, 2012). Using its proboscis (a prominent mouthpart), *Anopheles* mosquitoes repeatedly thrust (probe) into a host's dermal network of blood vessels searching for a blood vessel and during this process of probing, saliva is deposited to prevent the blood from coagulating while simultaneously introducing the sporozoites within it into the skin of the host (Matsuoka, Yoshida, Hirai, & Ishii, 2002; Sidjanski & Vanderberg, 1997).

Sporozoites use gliding motility until they encounter a blood vessel and are then transported passively to the liver (Amino *et al.*, 2007; Amino *et al.*, 2006; Hopp *et al.*, 2015; Vanderberg & Frevert, 2004). Upon reaching the liver, sporozoites cross through the liver sinusoidal cells and Kupffer cells (resident macrophages) before finally reaching the hepatocytes (Mota, Hafalla, & Rodriguez, 2002; Mota *et al.*, 2001; Pradel & Frevert, 2001; Pradel, Garapaty, & Frevert, 2002, 2004). The sporozoites migrate through several hepatocytes until reaching a final one where they multiply and grow within a parasitophorous vacuole (PV) as shown in Fig. 2 (Frevert, 2004; Pradel *et al.*, 2002). Each sporozoite develops into a schizont containing tens of thousands merozoites (Amino *et al.*, 2006; Kebaier, Voza, & Vanderberg, 2009). The duration of development of sporozoites in the liver lasts for an average of 7 days with variability depending on the *Plasmodium* specie (Vaughan, Aly, & Kappe, 2008). Matured merozoites are packed into budding vesicle called merosomes , evident in *Plasmodium* species that infect rodents *P. bergehi* and *P. yoelii*, leave the

hepatocytes and the liver intact entering the blood stream where it ruptures to releases the merozoites as shown in Fig. 2 (Baer, Klotz, Kappe, Schnieder, & Frevert, 2007; Sturm *et al.*, 2006). It is not evident whether *P. falciparum* merozoites also leave the hepatocytes through merosomes, however merosome-like structures have been observed in the liver of humanized mice (Soulard *et al.*, 2015; Vaughan *et al.*, 2012).

In *P. vivax* and *P.ovale*, some of the early liver stages may remain dormant for some time after sporozoite invasion of hepatocytes (Battle *et al.*, 2014). These forms are known as hypnozoites and may develop into schizonts later after some latent period. Hypnozoites are responsible for relapse of clinical infection after the initial infection (Cogswell, 1992; Collins, 2007; Krotoski, 1989; Lover & Coker, 2013). It is worth mentioning that the infection of hepatocytes by a *Plasmodium* sporozoite and subsequent development is silent and doesn't bring about any clinical symptoms of malaria to the infected individual.

1.1.3 Erythrocytic (Blood) Stages

The ejected merozoites contact a red blood cell (RBC) and invade it to initiate the erythrocytic (blood) stage as shown in Fig. 2. Invasion of RBC by merozoites is facilitated by molecular interactions between distinct ligands on the surface of the merozoite and host receptors on the RBC membrane (Alaganan, Singh, & Chitnis, 2017; Cowman & Crabb, 2006; Cowman, Tonkin, Tham, & Duraisingh, 2017). It has been observed that the duration from the release of merozoites from the liver to invasion of RBCs is extremely quick occurring in few seconds (Dvorak, Miller, Whitehouse, & Shiroishi, 1975; Gilson & Crabb, 2009; Glushakova, Yin, Li, & Zimmerberg, 2005; Winograd, Clavijo, Bustamante, & Jaramillo, 1999). This is a very fast process because the merozoite is one of the few stages in *Plasmodium* life cycle in which the parasite is extracellular and therefore must minimize the window of exposure of the antigens on its surface, and thereby avoiding detection by the host immune response (Wright & Rayner, 2014).

The merozoites are enclosed in a PV inside the RBC and undergoes cyclic development through the ring, trophozoite, and schizont stages over the duration of 24-72 hours depending on *Plasmodium* species (Hanssen, McMillan, & Tilley, 2010). Shortly after invasion (~0-18 hrs) the central region of the parasite becomes quite thin while the peripheral regions thicken due to presence of the nucleus and other organelles. This gives infected RBC its characteristics "ring"

appearance when smears are stained with Giemsa as shown in Fig. 2 (Hanssen *et al.*, 2010). Also, finger-like extensions of the PV membrane are observed forming the basis for exportation of proteins from the parasite to RBC cytoplasm and membrane (Boddey & Cowman, 2013; De Koning-Ward, Dixon, Tilley, & Gilson, 2016; Goldberg & Cowman, 2010). The parasite begins to ingest and digest haemoglobin that is abundantly present in the RBC cytoplasm through a cell mouth known as cytostome (Elliott *et al.*, 2008; Lazarus, Schneider, & Taraschi, 2008; Smythe, Joiner, & Hoppe, 2008). The digestion of haemoglobin releases free haem and globin; globin is degraded into its constituent amino acids which are used by the parasite for growth, while haem is toxic and is sequestered into a crystalline form known as hemozoin (malaria pigment) (Abu Bakar, Klonis, Hanssen, Chan, & Tilley, 2010; Scholl, Tripathi, & Sullivan, 2005; Wunderlich, Rohrbach, & Dalton, 2012). The ring stage is thought to serve as a lag phase during which the host membrane undergoes modifications necessary for subsequent export and surface expression of parasite proteins involved in immune evasion and virulence (Spielmann *et al.*, 2006).

During the trophozoite stage (~20-38 hrs post invasion) the parasite continues to ingest on haemoglobin and grow as shown in Fig. 2. The parasite is at its metabolically most active stage, characterized by the large number of free rough ER, a Golgi complex, and an increase in size of the mitochondrion, signifying an increase in protein synthesis (Hanssen *et al.*, 2010). Exportation of parasite's proteins to the RBC membrane continues to happen and modification of this membrane is evident with structures such as the Maurer's cleft (MC) and knobs (Boddey & Cowman, 2013; De Koning-Ward *et al.*, 2016; Goldberg & Cowman, 2010). These structures on the RBC membrane associate with other peripheral proteins and act as platforms for the presentation of parasite's adhesion proteins, such as the *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) (Haldar & Mohandas, 2007).

During the schizont stage (~38-48 hrs post invasion) the parasite undergoes multiple rounds of mitotic DNA replication to produce 16-20 merozoites each with their own complete set of organelles and invasion machinery as shown in Fig 2 (Hanssen *et al.*, 2010). Similarly, haemoglobin ingestion by the parasite continues and at this stage approximately 80% of RBC's haemoglobin has been consumed (Loria, Miller, Foley, & Tilley, 1999). The type of division that the parasite undergoes is termed as schizogony, characterized by multiple rounds of nuclear divisions happening prior to cell division (Gerald, Mahajan, & Kumar, 2011; Matthews, Duffy, &

Merrick, 2018). The result is multiple daughter merozoites tightly packed/stacked within a schizont as shown in Fig. 2 (Bannister, Hopkins, Fowler, Krishna, & Mitchell, 2000). When the infected RBC ruptures it releases the merozoites into the bloodstream to invade other RBCs restarting the blood stage cycle of the parasite as shown in Fig.2. The release of merozoites into the blood stream is accompanied by malaria paroxysm (bouts of illness alternating with symptom free periods), which is brought about by extremely high levels of pro-inflammatory cytokines from splenic macrophages in their attempt to remove parasitized or altered RBCs (Clark, Budd, Alleva, & Cowden, 2006; Haldar, Murphy, Milner, & Taylor, 2007; Schofield & Grau, 2005). In *P. vivax* and *P. ovale* malaria, fever occurs after every 48 hours (tertian malaria), whereas in *P. malariae* malaria fever occurs after every 72 hours (quartan malaria). The fever in *P. falciparum* malaria may occur after every 48 hours and usually severe and often fatal (Carter & Mendis, 2002).

1.1.4 Gametocyte Formation (Sexual) Stages

A sub-population of blood stage parasites will produce gametocyte progeny and initiate the sexual cycle as shown in Fig. 2. Gametocyte development (gametocytogenesis) happens in the intermediate vertebrate host until maturation before they are taken up by a mosquito vector (Nilsson, Childs, Buckee, & Marti, 2015). Commitment to gametocytogenesis is believed to occur at a low "baseline" rate during each asexual blood stage cycle (Kafsack *et al.*, 2014; Sinha *et al.*, 2014). However, several environmental cues collectively termed as "stress" have been reported to alter this baseline rate of conversion from asexual to sexual parasites (Nilsson *et al.*, 2015). These environmental cues include antimalarial drugs, anaemia, elevated reticulocytes levels, and host immune factors (Talman, Domarle, McKenzie, Ariey, & Robert, 2004).

The first step in gametocytogenesis begins with the sexually committed schizont. The molecular switch responsible for the commitment from asexual to sexual development is the activation of the transcription factor Apetala2-G (AP2-G), found in both *P. falciparum* and *P. berghei* (Kafsack *et al.*, 2014; Sinha *et al.*, 2014). In *P. falciparum*, AP2-G has been shown to be under the epigenetic control of histone deacetylase 2 (*Pf*Hda2) and heterochromatin protein 1 (*Pf*HP1) (Brancucci *et al.*, 2014; Coleman *et al.*, 2014). Expression of AP2-G during blood stage development has been shown to be silenced by *Pf*Hda2 and *Pf*HP1. In *P. falciparum*, the schizont expressed proteins gametocyte development protein 1 (*Pf*GDV1) and Nima-related kinase 4(*Pf*NEK4) have been

identified as potential upstream activators of AP2-G (Eksi *et al.*, 2012; Filarsky *et al.*, 2018; Reininger, Garcia, Tomlins, Muller, & Doerig, 2012). Furthermore, a recent study in *P. falciparum* has shown presence of lysophosphatidylcholine (lysoPC), a class of phospholipids abundant in serum, which modulates gametocytes commitment (Brancucci *et al.*, 2017). However, it remains unclear how lysoPC translates into *Pf*GDV1 and AP2-G activation. Activation of AP2-G in a subpopulation of blood stage parasites in early schizogony results in the expression of gametocyte specific genes in all their daughter merozoites. These daughter merozoites become sexually committed and develop into gametocytes instead of merozoites on subsequent reinvasion of RBCs (Ngotho *et al.*, 2019; Nilsson *et al.*, 2015).

Development of *P. falciparum* gametocytes proceeds through five morphologically discernible stages (I-V) over the course of 9-12 days (Hawking, Wilson, & Gammage, 1971). Stage I gametocytes are similar in appearance to asexual trophozoites and morphologically indistinguishable. Stages II-V gametocytes are different in appearance to asexual blood stage parasites. They transition from a lemon or oat grain shape with pointed end in stage II, to the letter D resemblance in stage III, to a banana shape in stage IV, and finally to a crescent shape with rounded ends in stage V (Sinden, 1982). Development of stage I-IV gametocytes takes place in the extravascular compartments of the bone marrow and spleen, while mature stage V gametocytes are present in peripheral blood circulation (Aguilar *et al.*, 2014; De Niz *et al.*, 2018; Joice *et al.*, 2014; Lee, Waters, & Brewer, 2018). Mature stage V gametocytes pass through the microvasculature in the dermis in order to increase the likelihood of ingestion by feeding mosquitoes (Bousema *et al.*, 2012; Nixon, 2016). Male and female gametocytes present in the ingested blood meal undergo fertilization and proceed through other developmental stages inside the mosquito.

1.1.5 Sporogonic (Mosquito) Stages

Female *Anopheles* mosquitoes require a blood meal, breaking it down to its constituent amino acids, and using them for egg production. Blood feeding and reproduction can be repeated every 3–4 days for the duration of the female *Anopheles* mosquito's lifespan and *Plasmodium* utilizes this cyclic feeding behaviour for its transmission from one vertebrate host to the next (Smith & Jacobs-Lorena, 2010). Between the next 8 -15 days, depending on the *Plasmodium* specie, the

parasite develops in the mosquito midgut then leaving this compartment and making its way into the salivary glands, ready for transmission to another human host in subsequent mosquito bites as shown in Fig. 2 (Baton & Ranford-Cartwright, 2005).

Ingested gametocytes pass to the midgut lumen of the mosquito and within ~ 5-20 minutes gametocytes egress from their host erythrocytes and initiate gametogenesis to produce male and female gametes, microgamete and macrogamete respectively as shown in Fig. 2 (Baton & Ranford-Cartwright, 2005). Gametogenesis is triggered by the fall in temperature from ~37^{o}C in the vertebrate host to ambient temperature in the mosquito, and the presence of a mosquito derived factor called xanthurenic acid (XA) (Billker *et al.*, 1998; Garcia, Wirtz, Barr, Woolfitt, & Rosenberg, 1998; Nijhout, 1979; Sinden, Butcher, Billker, & Fleck, 1996). *In vitro*, gametogenesis can be triggered by a decrease in temperature and an increase in pH from 7.4 to 8.2 (Billker, Shaw, Margos, & Sinden, 1997; Nijhout & Carter, 1978). Gametocytes emerge from the ingested RBCs where female gametocytes (macrogametocytes) produce a single non-motile spherical female gamete, while male gametocytes (microgametocytes) undergo exflagellation, a process of 3 rounds of endomitosis, to produce 8 motile microgametes as shown Fig. 2 (Baton & Ranford-Cartwright, 2005; Smith & Jacobs-Lorena, 2010). Within ~1 hour of blood meal ingestion into the mosquito midgut, the haploid micro- and macrogametes fuse to form a diploid zygote as shown in Fig. 2 (Janse, Van der Klooster, Van der Kaay, Van der Ploeg, & Overdulve, 1986; Sinden, 1983). The diploid zygote initiates the first round of endomeiotic division within 2-3 hours after fertilization (Janse *et al.*, 1986; Sinden, Hartley, & Winger, 1985). The zygote then transforms into a motile ookinete during the next 10-30 hours as shown in Fig. 2, and the second round of endomeiotic division occurs during this period (Aikawa, Carter, Ito, & Nijhout, 1984; Mehlhorn & Peters, 1980; Robert *et al.*, 1998).

Ookinetes asynchronously begin to leave the blood bolus at ~20-36 hours after blood meal ingestion, traverse the peritrophic matrix (PM) and invade the midgut epithelium (Baton & Ranford-Cartwright, 2004). Peritrophic matrix is an extracellular envelope composed of chitin, glycoproteins and proteoglycans that lines the digestive tract of most insects (Shao, Devenport, & Jacobs-Lorena, 2001; Tellam, Wijffels, & Willadsen, 1999). In hematophagous insects, including *Anopheles* mosquitoes, the PM is secreted by midgut epithelial cells caused by distension of mosquito midgut following ingestion of a blood meal, and completely surrounds the blood meal

compartmentalizing the midgut lumen into ecto-(outer) and endo(-inner) peritrophic spaces (Baton & Ranford-Cartwright, 2004; Shao *et al.*, 2001; Tellam *et al.*, 1999). The PM serves as molecular sieve that mediates the movement of molecules (enzymes, metabolites, and digested products) to and from the midgut lumen, and acts as a barrier to pathogen invasion of midgut epithelium (Billingsley & Rudin, 1992; Dinglasan *et al.*, 2009; Langer, Li, Popov, Kurosky, & Vinetz, 2002; Langer, Li, & Vinetz, 2002; Li *et al.*, 2004; Shahabuddin, Lemos, Kaslow, & Jacobs-Lorena, 1996; Shahabuddin, Toyoshima, Aikawa, & Kaslow, 1993). Ookinete invasion of midgut epithelium is rapid and happens within ~30 mins, with the ookinete traversing through several epithelial cells before settling into the basal lamina space (Han, Thompson, Kafatos, & Barillas-Mury, 2000; Zieler & Dvorak, 2000). There is successive reduction in parasite numbers due to various factors happening in the mosquito midgut from gamete formation and fertilization, zygote transformation into the ookinete and ookinete migration across the midgut epithelium (Graca-Souza *et al.*, 2006; Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar, Gupta, Han, & Barillas-Mury, 2004; Lensen, Bolmer-Van de Vegte, Van Gemert, Eling, & Sauerwein, 1997; Naotunne, Karunaweera, Mendis, & Carter, 1993; Peterson, Gow, & Luckhart, 2007).

Within ~24-48 hours after blood meal ingestion, the ookinete initiates the process to transform into a sessile spherical oocyst in the basal lamina as shown in Fig. 2 (Baton & Ranford-Cartwright, 2004; Han *et al.*, 2000). Mitotic replication immediately ensues after oocyst formation and continues throughout the period of sporogony, followed by sporoblast formation during middle-aged oocysts (~5-10) days, and last with sporozoites budding off from the sporoblast bodies during the remaining days of oocyst development (Howells & Davies, 1971; Sinden & Strong, 1978). At this stage, *Plasmodium* parasites experiences a resurgence in their numbers with ~1 x 10^3–10^4 sporozoites being produced per oocyst (Beier, 1998).

Sporozoites then egress from a mature oocyst and traverse the basal lamina into the mosquito haemolymph. In the haemolymph, the sporozoites are passively transported to the salivary glands and invade them as shown in Fig. 2. During subsequent blood feeding by an infectious mosquito, the sporozoites in the salivary glands are injected with saliva into the vertebrate (human) host dermal tissues, thus initiating the next stage of *Plasmodium* life cycle as shown in Fig. 2 (Frischknecht *et al.*, 2004; Vanderberg & Frevert, 2004).

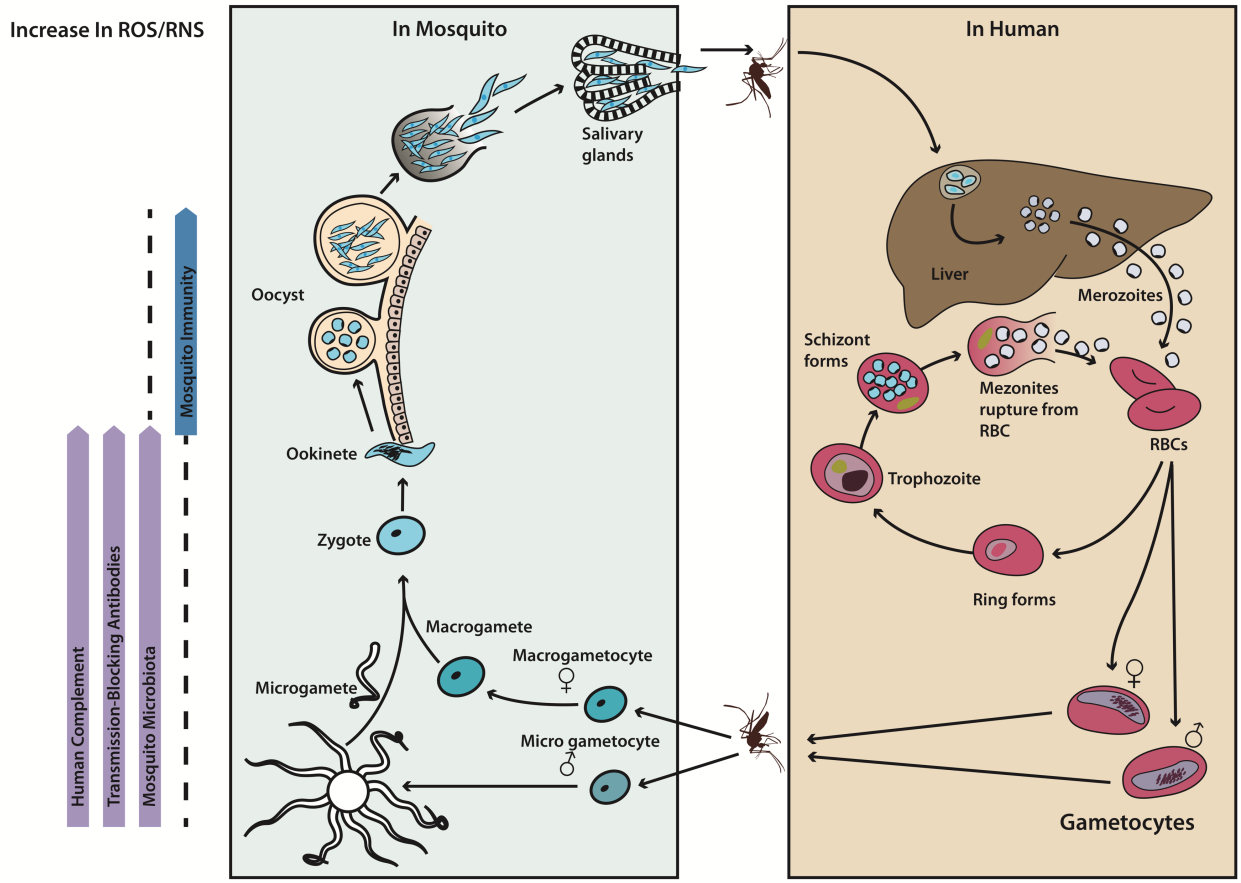

Figure 2. Life cycle of common human malaria parasites. (Right panel) *Plasmodium* development in the vertebrate host (human). (Left panel) *Plasmodium* development in the vector (mosquito). The diagram is reproduced with minor amendments from (Smith, Vega-Rodriguez, & Jacobs-Lorena, 2014) and (Bousema & Drakeley, 2011)

1.1.6 Control and Treatment of Malaria

Between 2000-2015 a total of 663 (542-753 credible interval) million clinical cases of malaria were averted cases translating into 18%, and ~50% decline in cases and number of deaths (Bhatt *et al.*, 2015; Global Malaria Programme, 2015b). This substantial reduction in both the number of cases and deaths globally is due to the widescale roll out of control and treatment measures for malaria (Bhatt *et al.*, 2015). Control of malaria has mainly been achieved by targeting the mosquito vector, while treatment has focused on case management with antimalarials against the *Plasmodium* parasite and its effective diagnosis. One of the greatest challenges in malaria control and treatment has been the development of resistance in *Anopheles* and *Plasmodium* against

insecticides and antimalarials, respectively; reducing the effectiveness of the current control and treatment interventions against malaria.

1.1.7 Vector Control

The primary vector control interventions include the use of insecticide-treated (bed)nets (ITNs) and indoor residual spraying (IRS) of houses with insecticides. These interventions have had massive success in the control of mosquito vectors in Africa (Bhatt *et al.*, 2015; Lengeler, 2004). This is predominantly due to the fact that the principal malaria vectors, *Anopheles gambiae* and *Anopheles funestus* complexes (Gillies & Coetzee, 1987; Gillies & De Meillon, 1968; White, 1973), primarily feed on humans (anthropophagy) while indoors (endophagy) at night and rest indoors (endophily) (Gillies & De Meillon, 1968; Killeen *et al.*, 2006; Pates & Curtis, 2005) as explained in Table 1. Insecticide-treated nets have been shown to not only offer personal protection against infectious bites but also reduce the survival, feeding frequency, feeding success and density of mosquito vector populations if reasonable levels of community-wide coverage are achieved, with approximately half of the population using them each night (Hawley *et al.*, 2003; Killeen & Smith, 2007; Killeen *et al.*, 2007). Therefore, ITNs not only prevent malaria in protected persons (those who frequently use them) but can also reduce the exposure of unprotected person by suppressing transmission across entire communities.

Extensive use of ITNs and IRS has resulted in a dynamic shift in the malaria vector population (Killeen, 2014). In the recent past, vectors that feed predominantly outdoors (exophagic), early at night, and on animals (zoophily), as explained in Table 1, have been observed in Africa (Geissbuhler *et al.*, 2007; Lwetoijera *et al.*, 2014; Oyewole & Awolola, 2006; Pates & Curtis, 2005; Russell *et al.*, 2010). This shift in malaria vectors population dynamics has resulted from a response to the use of ITNs and IRS (Killeen, 2014). This has the consequence of drastically reducing the level of personal protection conferred by ITNs and IRS (Mutuku *et al.*, 2011; Okumu, Kiware, Moore, & Killeen, 2013; Russell *et al.*, 2010). It is believed that this population of zoophilic and exophagic mosquitoes that can bite outdoors, early at night, and on livestock in the absence of human blood source, are sustaining malaria transmission in areas of low endemicity in Africa (Finda *et al.*, 2019; Monroe *et al.*, 2019). Furthermore, effectiveness of ITNs and IRS has severely been impacted by the development of insecticide resistance in mosquito populations

across many settings (Alout, Labbe, Chandre, & Cohuet, 2017; Benelli & Beier, 2017; Hemingway, 2018). In that regard, vector control interventions that will complement existing tools are needed. A number of other interventions such as larval source management (LSM), attractive toxic sugar baits (ATSB), endoectocides, spatial repellents, and mosquito-proofed housing have been shown to be effective and sustainable as complementary interventions to ITNs and IRS (Ferguson *et al.*, 2010; Killeen *et al.*, 2017; Williams *et al.*, 2018).

Table 1. Definition of mosquito host seeking behaviour and preference

Mosquito Behaviour	Definition
Anthropophagy	is a tendency of mosquitoes to prefer feeding on human hosts
Endophagy	is a tendency for mosquitoes to prefer biting indoors
Endophily	is a tendency for mosquitoes to prefer resting indoors
Exophagy	is a tendency for mosquitoes to prefer biting outdoors
Exophily	is a tendency of mosquitoes to prefer resting outdoors
Zoophagy	is a tendency of mosquitoes to prefer feeding on animal hosts

1.1.8 Antimalarials

Historically, antimalarials have played a significant part in combating malaria alongside vector control interventions explained above. The earliest records of antimalarial treatment come from ancient China in the year 340 AD, where extracts from the mugwort herb were shown to have fever-reducing properties (Klayman, 1985). It should be noted that the mugwort herb belongs to the genus *Artemisia*, and the fever reducing properties of this herb is based on the plasmodicidal properties of the ingredient artemisinin, which is synthetically produced today and used as the first line treatment against malaria in many parts of the world (Kong & Tan, 2015). Other records of historical antimalarial treatment come from the 15th century in Peru, where the bark of a local tree, called "quina" by the natives, was used to treat the wife of the Spanish viceroy of Peru. The fever reducing properties of this bark were later studied by Spanish Jesuits who imported the bark back to Europe and name it Cinchona, after the name of the wife of the Spanish Viceroy of Peru (Lee, 2002). In the early 19th century, two French chemists isolated quinine from the cinchona bark and it immediately became the favoured treatment against malaria throughout the world (Meshnick, 1998). Prior to World War II, German scientists were able to synthesize Chloroquine, a 4-

aminoquinoline, as a replacement for quinine (Coatney, 1963; Honigsbaum, 2002; Loeb, 1946). Due to inexpensive production, availability, and effectiveness, chloroquine became the first-line treatment against malaria in many parts of the world from 1943 until the later years of the 20th century.

(i) Chloroquine

Chloroquine (CQ) is described as a 4-aminoquinoline compound. CQ acts by interfering with the process of haem (ferri/fferroprotoporphyrin IX; FP) detoxification (Tilley, Loria, & Foley, 2001). As the parasite is growing inside the RBC (intraerythrocytic development) it ingests on the abundantly available amounts of haemoglobin releasing free FP and globin in its digestive food vacuole (Francis, Sullivan, & Goldberg, 1997; Goldberg *et al.*, 1991). Globin is broken down by the parasite's proteases into constituent amino acids, some of which are used by the parasite for its growth. However, free haem is toxic and has deleterious effects on the parasite and has to be immediately removed (Foley & Tilley, 1998). *Plasmodium* lacks the enzyme haem oxygenase needed to degrade the free haem and disposes of it in part by polymerizing it into an inert polymer called hemozoin or degradation through redox pathways (Eckman, Modler, Eaton, Berger, & Engel, 1977; Egan *et al.*, 2002; Ginsburg, Famin, Zhang, & Krugliak, 1998; Slater, 1993; Slater & Cerami, 1992). CQ binds to free FP and also adsorbs to the growing faces of the hemozoin crystals, disrupting the detoxification process and poisoning the parasite (Dorn *et al.*, 1998; Pagola, Stephens, Bohle, Kosar, & Madsen, 2000; Sullivan, Gluzman, Russell, & Goldberg, 1996). This is the reason why CQ is only effective against stages of the parasite (trophozoites and schizonts) that are actively ingesting haemoglobin and not against other stages of the parasite, such as the pre-erythrocytic or gametocytes (Butcher, 1997). *Plasmodium* parasites resistant to CQ survive by reducing the accumulation of the drug in the digestive food vacuole (Verdier, Le Bras, Clavier, Hatin, & Blayo, 1985).

The Global Malaria Eradication Programme was launched by WHO in 1955 and focused on two aspects: CQ for treatment and prevention and DDT for vector control (Greenwood *et al.*, 2008). The campaign achieved some considerable successes, especially in areas of low malaria transmission such as North America and Europe (Jensen & Mehlhorn, 2009). However, the global campaign against malaria experienced major hurdles, one being the development of CQ resistance

noticed as early as 1957 in Southeast Asia along the Thai-Cambodia border and its spread to the rest of the world (Grimmond, Donovan, & Riley, 1976; Harinasuta, Suntharasamai, & Viravan, 1965; Lobel, Campbell, Schwartz, & Roberts, 1985; Moore & Lanier, 1961; Talisuna, Bloland, & D'Alessandro, 2004). The campaign was officially abandoned as a goal in 1972. In Tanzania, the earliest accounts of chloroquine resistance were observed among non-immune travellers in 1978 (Lobel *et al.*, 1985). But as early as 1990, the proportion of malaria parasite that were resistant to chloroquine had increased to 52% (Premji, Minjas, & Shiff, 1994). In 2001 the Tanzania national malaria control program guidelines changed from the required use of chloroquine to sulfadoxine-pyrimethamine as the first line treatment for malaria (Taverne, 2001).

(ii) Sulfadoxine-Pyrimethamine

Sulfadoxine was developed in the early 1960s while Pyrimethamine was developed a few years prior in the 1950s (Laing, 1965; Russell & Hitchings, 1951). The combination of sulfadoxine and pyrimethamine (SP) was approved for use for the treatment of malaria in 1971. Both drugs are known to target *Plasmodium* folate biosynthesis pathway by competitively inhibiting the enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), respectively (Nzila *et al.*, 2005; Sibley *et al.*, 2001). Actively dividing and growing cells, such as *Plasmodium* parasites, depend on folate biosynthesis for generation of folate derivatives essential for DNA replication and protein synthesis (Hyde, 2005). By inhibiting *Plasmodium* DHPS and DHFR, SP deprives the parasite with necessary nutrients required for development and thus lethal to the parasite (Ferone, Burchall, & Hitchings, 1969; Hitchings & Burchall, 1965; Hurly, 1959; Rollo, 1955; Watkins, Mberu, Winstanley, & Plowe, 1997; Zhang & Rathod, 2002).

As SP replaced CQ as the first line treatment against malaria in many parts of the world, its widespread use eventually led to the development of resistance against it. Resistance was first noted in South America and Southeast Asia as early as 1970s, a short time after they were introduced and later on it spread to Africa (Global Partnership to Roll Back Malaria, 2005; Heinberg & Kirkman, 2015; Le Bras & Durand, 2003). A few years after its introduction as the first line treatment against malaria in Tanzania, increasing parasite resistance was observed, and in 2006 the country made a second change in its first line treatment against malaria from SP to artemisinin-based combination therapy (Mugittu *et al.*, 2005; Mugittu *et al.*, 2004). By the year

2005 most malaria endemic countries had made a shift from SP to artemisinin-based combination therapy as their first line treatment against malaria (Eastman & Fidock, 2009). Despite widespread resistance against it, SP is currently being used for intermittent preventive treatment in pregnancy (IPTp) and in infants (IPTi) (Bardaji, Bassat, Alonso, & Menendez, 2012; Peters, Thigpen, Parise, & Newman, 2007).

(iii) Artemisinin-based Combination Therapy

Artemisinin was isolated by Chinese scientists in 1972 from *Artemisia annua* (sweet wormwood), better known to Chinese herbalists from more than 2 000 years as qinghaosu (Klayman, 1985; Kong & Tan, 2015). Artemisinin is a potent, quick-acting and very effective antimalaria drug, especially when it's used in combination with another drug that has a longer half-life, widely known as artemisinin-based combination therapy (ACT) (Ezzet, Mull, & Karbwang, 1998; Hassan Alin, Bjorkman, & Wernsdorfer, 1999; Jansen *et al.*, 2007). The principle behind ACT is based on the assumption that given short plasma half-life artemisinin but high potency of artemisinin combined with a longer acting drug would eliminate any chances of parasites not cleared within the few hours of the drug action and prevent chances of developing resistance (Global Partenrship to Roll Back Malaria [RBM], 2001). Artemisinin and other artemether-group drugs have become the front-line treatment against malaria in many endemic countries. Due to the great positive impact of Artemisinin in combating Malaria, the 2015 Nobel Prize in Physiology and Medicine was jointly awarded to Tu Youyou, a Chinese scientist who is credited for the isolation of Artemisinin in 1971.

Artemisinin is a sesquiterpene lactone compound with an endoperoxide bridge. The mode of action of artemisinin has been the subject of debate as several theories have been proposed (O'Neill, Barton, & Ward, 2010). The most accepted theory is bioactivation of artemisinin by haem (Tilley, Straimer, Gnadig, Ralph, & Fidock, 2016; Wang, Zhang, *et al.*, 2015). This theory states hypothesizes that endoperoxide bridge of Artemisinin is cleaved in the presence of free haem forming an O-centred radical that self-arranges to form a C-centred radical (Olliaro, Haynes, Meunier, & Yuthavong, 2001; Wu *et al.*, 1998). The generated radicals can further react with several cellular targets, including proteins and membrane lipids in their vicinity, bringing about deleterious effects to the parasite (Cui & Su, 2009).

Another theory on mode of action is through haem polymerization. Haem is generated from haemoglobin digestion and must be immediately removed due to its toxicity to the parasite. *In vitro* experiments have shown that artemisinin-haem adducts inhibit haem polymerization in a mechanism that involves parasite's histidine-rich proteins-II and III, and that this inhibition most likely leads to free accumulation of the toxic FP (Wang, Zhang*, et al.*, 2015). Other suggested theories include the direct inhibition of *Pf*ATP6, a sarco/endoplasmic reticulum calcium dependent ATPase in *P. falciparum*, and respiratory chain of the mitochondria through the production of ROS (Eckstein-Ludwig *et al.*, 2003; Krungkrai, Burat, Kudan, Krungkrai, & Prapunwattana, 1999; Li *et al.*, 2005).

Despite its widespread use as first line antimalarial, cases of resistance against ACT have been reported along the Thai-Cambodia border (Noedl *et al.*, 2010; Samarasekera, 2009). Initially, the resistance was confined to the non-artemisinin partner drug and were associated with various haplotypes of either *P. falciparum* multidrug resistance 1(*Pf*mdr1) or *P. falciparum* CQ resistance transporter (*Pf*crt) (Pickard *et al.*, 2003; Price *et al.*, 2004; Sidhu *et al.*, 2006; Witkowski *et al.*, 2017). It's only recently that resistance to artemisinin was directly associated with the kelch propeller domain (K13-propeller) polymorphisms in laboratory parasites and subsequently proven in field isolates from Southeast Asia. Furthermore, these mutations and several others were as well found in more than 18 countries in sub-Saharan Africa though in low frequencies (Ariey *et al.*, 2014; Fairhurst & Dondorp, 2016). The fear is that artemisinin resistance will spread to other parts of the world such as Africa from Southeast Asia, as it was the case for the other two widely used antimalarials (CQ and SP) and severely hampering the efforts against Malaria (Takala-Harrison & Laufer, 2015).

(iv) Transmission-blocking Drugs

Transmission-blocking interventions are tools that are capable of inhibiting the transmission of *Plasmodium* parasites to the mosquito. The intervention can be either a transmission-blocking drug (TBD) or transmission blocking vaccine (TBV) against the parasite at either gametocyte, gamete, zygote, or ookinete stage (Delves, Angrisano, & Blagborough, 2018). This section will solely focus on TBDs and how oxidative stress can be used as a transmission-blocking strategy.

The strategy of targeting the transmission of *Plasmodium* parasites to mosquitoes is particularly opportune. The parasite experiences a population bottleneck during the transmission from human to mosquito such that less than five parasite per mosquito are present at the oocyst stage (Gouagna *et al.*, 1998; Rosenberg, 2008; Sinden & Billingsley, 2001; Whitten, Shiao, & Levashina, 2006). Although the figure of less than five oocyst per mosquito is widely variable, it is considerably less than the number of parasites (~ typically 10^9) circulating in the bloodstream of malaria-infected humans (Baton & Ranford-Cartwright, 2012; Medley *et al.*, 1993; Sinden, 2017). This presents an advantage for the targeted killing of parasites during the transmission stages of the life cycle. Furthermore, *Plasmodium* parasites are extracellular during the first 24 hours in the mosquito compared to ~30 seconds in humans during merozoite invasion of erythrocytes (Gilson & Crabb, 2009). This results into a larger window of opportunity for immune/pharmacological destruction of the parasite (Delves *et al.*, 2018). Lastly, targeting of parasites during transmission stages is conceptually associated with reduction in development of resistance by: (a) the number of genes expressed by the parasites during the transmission stages are invariant to those expressed during blood/liver-stages in the human resulting in reduced polymorphism and (b) TBDs adds to the arsenal of drugs against the parasite, therefore increasing the number of targets the parasite has to develop resistance against and substantially slowing the process of resistance development in a similar manner to how multi-target drug approach works (Escalante, Lal, & Ayala, 1998; Niederwieser, Felger, & Beck, 2001).

Examples of TBDs in use for malaria treatment

There have been tremendous efforts in the discovery and development of transmission-blocking drugs over the years. This endeavour has received an additional boost by the recent decision by Medicines for Malaria Venture (MMV) to formalise the development of compounds specifically targeting malaria parasite transmission, e.g. target candidate profile-5 (TCP-5) (Burrows *et al.*, 2017). A vast number of compounds have subsequently been identified through high-throughput transmission screening assays following this decision (Almela *et al.*, 2015; Bolscher *et al.*, 2015; Delves *et al.*, 2016; Lucantoni, Duffy, Adjalley, Fidock, & Avery, 2013; Miguel-Blanco *et al.*, 2015; Plouffe *et al.*, 2016; Ruecker *et al.*, 2014; Tanaka *et al.*, 2013). Currently, there are three clinically approved antimalarials that show well supported transmission-blocking efficacy: primaquine (PQ), methylene blue (MB), and atovaquone (ATQ) (Delves *et al.*, 2018).

Primaquine is an 8-aminoquinoline that is derived from plasmoquine (plasmochin, pamaquine). Plasmoquine is one of the first generally available synthetic anti-malarial, used predominantly in the cure of *P. vivax* and *P. ovale,* relapsing infection by eliminating the dormant liver hypnozoite stage of these parasites (Ashley, Recht, & White, 2014). It has an additional effect of killing mature gametocytes by unknown mechanism that translates in accelerated gametocyte clearance and cumulative impaired development of subsequent mosquito stages (Burgess & Bray, 1961). However, PQ causes haemolytic anaemia in individuals who are deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PD) deficient (Ashley *et al.*, 2014). The deficiency in this enzyme is an X-linked mutation that is widespread in malaria endemic areas with gene frequencies typically ranging from 3-30%, and thus limiting the use of PQ (Howes *et al.*, 2012). Nevertheless, with renewed interest in malaria elimination, single low dose of PQ (0.25mg/kg) is recommended for use by the WHO for transmission-blocking (Goncalves *et al.*, 2016).

Interestingly, MB was the first synthetic anti-malarial to be used, which occurred in a German hospital some 120 years ago (Gutttman & Ehrlich, 1891). Its global use in malaria endemic areas is well documented for the late 19th and early 20th centuries (Frost, 1908; Marshall, 1920; Marshall & Gee, 1893). However, its global use faded with the discovery of new synthetic antimalarials (Schirmer, Adler, Pickhardt, & Mandelkow, 2011). Interest in MB as an anti-malaria was renewed when GR was identified as a new drug target, although this concept is still contested (Buchholz *et al.*, 2008; Farber, Arscott, Williams, Becker, & Schirmer, 1998; Pastrana-Mena *et al.*, 2010; Sarma *et al.*, 2003; Schirmer *et al.*, 2003). Methylene blue appears to perturb the redox balance within the parasite, and like 4-aminoquinolines, it also interacts with the polymerisation of haem to hemozoin (Buchholz *et al.*, 2008). MB is effective against asexual blood stage parasites, gametocytes, and mosquito stages (Lu *et al.*, 2018).MB has been considered as a potential useful partner drug for ACT, particularly when elimination is the final goal (Muller, Sie, Meissner, Schirmer, & Kouyate, 2009).

Atovaquone is a hypoxaphthoquinone that selectively inhibits mitochondrial electron transport chain at the cytochrome bc1 complex of malaria parasites (Barton, Fisher, Biagini, Ward, & O'Neill, 2010). This drug is given in combination with Proguanil as Malarone® and is now considered as a first-line prophylaxis for travelers to prevent the development of liver-stage parasites (Staines *et al.*, 2018). Potent transmission-blocking activity of ATQ has been shown

against ookinete and oocyst formation in the mosquito when carried across in the bloodmeal (Butcher & Sinden, 2003; Fowler, Sinden, & Pudney, 1995). Although not tested yet, the concept of atovaquone-combination therapy as an effective and long-lasting transmission blocking drug is very appealing (Delves *et al.*, 2018).

The future of transmission-blocking strategy for development of antimalarials looks very promising. Cipargamin (KAE609/NITD609), SJ733, and KAF156 are several of transmission-blocking antimalarials at various stages in clinical development (Dennis, Lehane, Ridgway, Holleran, & Kirk, 2018; Jimenez-Diaz *et al.*, 2014; Kuhen *et al.*, 2014; Lim *et al.*, 2016; Van Pelt-Koops *et al.*, 2012; White *et al.*, 2016; White *et al.*, 2014). However, development of TBDs to date, has been regarded as adding transmission-blocking activity to schizonticides. This approach has the possibility of developing TBDs that are minimally effective by: (a) *in vitro* data of most advanced transmission-blocking molecules shows that they require drug concentrations about an order of magnitude higher than the schizonticidal dose to be efficacious and (b) if resistance mechanisms in asexual parasites also translate to resistance in gametocytes which are already sensitive to the particular schizonticide, there will likely be preferential transmission of resistance alleles (Delves *et al.*, 2018). An alternative approach is the development of TBDs that would specifically target biological pathways specific to gametocytes and/or mosquito stages with no activity against blood stage parasites (Delves *et al.*, 2018).

1.1.9 Vaccines

Vaccines have been readily developed for many bacterial and viral infections, and they have had dramatic impact on the eradication of infectious diseases, such as smallpox and polio (Hoffman, Vekemans, Richie, & Duffy, 2015). Vaccines are considered the most cost-effective single intervention for control, prevention, elimination, and eradication of infectious diseases due to such great previous success (Hoffman *et al.*, 2015). However, the development of an effective and safe vaccine against malaria has been one of the greatest challenges that have vexed malariologists for a very long time. This is primarily due to the complex biology of *Plasmodium* parasites compared to the bacteria and viruses for which we have vaccines. The genome of *Plasmodium* is much larger than those of bacteria or viruses (*Pf*> 5000 genes), with multiple stages in their life cycle (Carlton *et al.*, 2008; Gardner *et al.*, 2002; Hall *et al.*, 2005; Pain *et al.*, 2008). This greater number of genes

translates into an extensive antigenic diversity across the multiple life stages of the parasite (Ouattara *et al.*, 2015; Riley & Stewart, 2013). Such that, protective antibodies against sporozoites injected by mosquitoes do not recognize the asexual blood stages that cause the disease (Hoffman *et al.*, 2015). Furthermore, natural malaria infection does not induce much immune protection. It is only after repeated and prolonged exposure to *Plasmodium* parasites that immunity is acquired, which is short-lived ad highly stage- and strain-specific (Waters, 2006). This immunity is unable to eradicate all the parasites, nor does it provide complete protection against future challenge (Waters, 2006). Instead, it only results in a mild, sometimes asymptomatic infection with the persistence of parasites (Waters, 2006).

Despite these challenges, several vaccine candidates have been tested over the years, many without much success. Due to the complexity in *Plasmodium* life cycle, some of these candidate vaccines target the pre-erythrocytic stages, some the erythrocytic stages, some on the sexual stages, some on mosquito stages and some are multi-stage targeting the parasite at more than one stage in its life cycle (Hoffman *et al.*, 2015). However, steady progress is being made, especially with regards to breakthroughs in our understanding of the cellular and molecular mechanisms mediating protection in animal models and humans (Draper *et al.*, 2018). This recent success coupled with the renewed global optimism for achieving malaria elimination and eradication by the year 2030 have led to a revised Malaria Vaccine Technology Roadmap to 2030, which has called for a next-generation vaccine to achieve 75% efficacy over 2 years against *P. falciparum* and/or *P. vivax*, while also retaining its original 2015 "landmark" goal of a first-generation vaccine with protective efficacy of >50% lasting more than a year (Draper *et al.*, 2018; Moorthy, Newman, & Okwo-Bele, 2013). Herein, we provide an overview on some of the vaccine candidates, which were successful to progress to human trials, and touch base on future prospects for vaccine candidates across the different stages of *Plasmodium* life cycle.

(i) Pre-erythrocytic Vaccines

Pre-erythrocytic malaria vaccine development encompasses both the invading sporozoite and liver stage of *Plasmodium* infection. Vaccines for this stage have been the major focus for several reasons: (a) low numbers of inoculated sporozoites and therefore low numbers of infected hepatocytes (b) human parasites like *P. falciparum* and *P. vivax* take nearly a week to complete

development in hepatocytes, providing sufficient time for an effective immune response to eliminate them; (c) parasite infection at this stage is clinically silent (completely asymptomatic); and (d) effective killing of parasite at this stage will prevent the subsequent symptomatic stage (Lindner, Miller, & Kappe, 2012). However, apart from the above-mentioned advantages, development of pre-erythrocytic vaccine has been quite challenging. The pre-erythrocytic vaccine must provide sterile immunity from sporozoite infection to protect against malaria infection (Doolan & Martinez-Alier, 2006; Silvie, Amino, & Hafalla, 2017). Escape by a single parasite form pre-erythrocytic development results in full-blown symptomatic blood stage infection (Alonso *et al.*, 2005; Waters, 2006).

CSP is an immunodominant pre-erythrocytic antigen and a suitable candidate for a vaccine (Arnot *et al.*, 1985; Dame *et al.*, 1984; Enea *et al.*, 1984). The most advanced vaccine candidate is based on the circumsporozoite protein (CSP) and is known as RTS,S (Casares, Brumeanu, & Richie, 2010; Cohen, Nussenzweig, Nussenzweig, Vekemans, & Leach, 2010). It has shown sub-optimal results in the field by reducing clinical malaria by 30-50% (Agnandji *et al.*, 2012, 2014, 2015; Agnandji *et al.*, 2011; Neafsey *et al.*, 2015). Despite this low efficacy, it is argued that RTS,S will substantially save lives if used in complement with other malaria control and treatment tools. It is for this reason that RTS,S is now branded as "Mosquirix" and is going to be deployed in Kenya, Malawi, and Ghana in what is known as malaria vaccine implementation programme (MVIP) (Global Malaria Programme, 2016; World Health Organization, 2016, 2018). Other immunodominant pre-erythrocytic antigens with vaccine candidates in clinical trial stages include, the sporozoite surface protein-2/thrombospondin-related adhesion protein (SSP2/TRAP) and cell traversal protein for ookinetes and sporozoites (CelTOS) (Bergmann-Leitner, Legler, Savranskaya, Ockenhouse, & Angov, 2011; Bergmann-Leitner *et al.*, 2010; Ewer *et al.*, 2013; Hodgson *et al.*, 2015). Further research work on pre-erythrocytic subunit vaccines has largely focused on achieving higher vaccine efficacy by enhancing the $CD8^+$ T cells responses through several strategies such as viral-vectored or DNA-based (Duffy, Sahu, Akue, Milman, & Anderson, 2012; Hill *et al.*, 2010; Kazmin *et al.*, 2017; Ockenhouse *et al.*, 2015; Schuldt & Amalfitano, 2012).

Seminal studies on immunization of rodents, nonhuman primates, and humans with radiation-attenuated sporozoites (RAS) showed that they develop sterile immunity from an infected

mosquito bite challenge (Nussenzweig, Vanderberg, & Most, 1969; Nussenzweig, Vanderberg, Most, & Orton, 1967; Vanderberg, Nussenzweig, & Most, 1969; Vanderberg, Nussenzweig, Most, & Orton, 1968). These studies developed the rationale for a whole sporozoite vaccine and introduced a challenge model that has become the gold standard of assessing immune protection after vaccination (Clyde, 1975; Clyde, Most, McCarthy, & Vanderberg, 1973). However, it was generally thought that, scaling up RAS vaccines for clinical use was too challenging, so attention was turned to developing subunit malaria vaccines. This was until the company Sanaria® embarked on a mission to translate the mosquito-based immunization approach into injectable whole *P. falciparum* sporozoite (*Pf*SPZ) malaria vaccines, and to use the vaccine(s) to eliminate malaria from geographically defined areas through mass immunization ad to prevent malaria in non-immune visitors to malarious areas (Hoffman *et al.*, 2015). A breakthrough came in 2009 when the company was able to isolate a purified, aseptic, cryopreserved, and RAS (*Pf*SPZ vaccine) for clinical trials (Hoffman *et al.*, 2010). This vaccine has undergone clinical trials in USA, Europe, and Africa to assess its safety, immunogenicity, route of administration, and protective efficacy (Epstein *et al.*, 2017; Epstein *et al.*, 2011; Jongo *et al.*, 2019; Jongo *et al.*, 2018; Lyke *et al.*, 2017; Olotu *et al.*, 2018; Seder *et al.*, 2013; Sissoko *et al.*, 2017; Zenklusen *et al.*, 2018).

Other techniques have been developed for attenuating the sporozoites to be used as vaccines. These include targeted gene deletion and chemoprophylaxis known as genetic attenuated parasites (GAP) and chemoprophylaxis with sporozoites (CPS), respectively. Targeted genes in GAP include P52, P36, or B9 (involved in liver stage PV formation or sporozoite) and liver stage asparagine-rich protein (SLARP; involved in transcriptional regulation for liver stage replication) (Annoura *et al.*, 2014; Mikolajczak *et al.*, 2014; Van Schaijk *et al.*, 2014; VanBuskirk *et al.*, 2009). While, CPS delivers wild-type sporozoites to allow complete development of pre-erythrocytic stages and controlling the symptomatic blood stages with chemoprophylaxis, particularly chloroquine (Bijker *et al.*, 2013). Both GAP and CPS have undergone clinical trials to assess their safety, immunogenicity, route of administration, and protective efficacy (Kublin *et al.*, 2017; Mordmuller *et al.*, 2017; Roestenberg *et al.*, 2009; Roestenberg *et al.*, 2011; Spring *et al.*, 2013). Whole sporozoite vaccines have shown variable and short-lived protective efficacy such that they are not close to deployment for malaria control and treatment (Itsara *et al.*, 2018).

(ii) Blood-stage Vaccines

Naturally acquired immunity (NAI) to malaria arises through repeated exposure to blood-stage parasite diversity, generation of a broad antibody repertoire against merozoites and infected erythrocytes, and a complex interplay of inflammatory and immuno-regulatory cellular responses (Draper *et al.*, 2018). Blood-stage vaccines have generally focused on a handful of well-studied merozoite antigens that are required for parasite invasion of erythrocytes. Many potential blood-stage candidate antigens have been identified but some of the most extensively studied candidates in recent clinical trials include apical membrane antigen-1 (AMA1), merozoite surface protein-1 and -3 (MSP1 and MSP3), rhoptry neck protein 2 (RON2), and glutamate-rich protein (GLURP) (Wilson, Flanagan, Prakash, & Plebanski, 2019). However, vaccines based on these antigens have failed to attain convincing protective efficacy in clinical trials (Genton *et al.*, 2002; Jepsen *et al.*, 2013; Laurens *et al.*, 2013; Ogutu *et al.*, 2009; Payne *et al.*, 2016; Sheehy *et al.*, 2012; Sheehy *et al.*, 2011; Sirima *et al.*, 2016; Srinivasan *et al.*, 2017; Srinivasan *et al.*, 2011; Srinivasan *et al.*, 2014; Srinivasan *et al.*, 2013; Theisen, Adu, Mordmuller, & Singh, 2017; Thera *et al.*, 2011). Some of the challenges faced by these vaccine candidates include: (a) the high antigen specific antibody titres required for protection due to fast kinetics of merozoite invasion or (b) antibody responses which cannot cover substantial levels of antigenic polymorphism and/or redundant invasion pathways (Draper *et al.*, 2018; Wilson *et al.*, 2019).

This led to the search of other blood-stage antigens that can overcome these challenges and led to the discovery of reticulocyte-binding protein homolog 5 (Rh5) as a vaccine candidate. Rh5 is part of a complex that forms an essential interaction with basigin (CD 147) on the erythrocyte surface during invasion (Crosnier *et al.*, 2011). There is high level of sequence conservation in *P. falciparum* Rh5 (*Pf*Rh5) and natural acquired antibodies to the *Pf*RH5 complex have been described in naturally-exposed human populations in Africa and Papua New Guinea (Partey *et al.*, 2018; Richards *et al.*, 2010; Weaver *et al.*, 2016). Trials in animal models and healthy human volunteers showed that *Pf*Rh5 has a well-tolerated safety profile that is associated with reduced levels of acquiring malaria, with high level of antigen specific antibodies that were inhibitory towards multiple parasite strains in *vitro* (Douglas *et al.*, 2015; Patel *et al.*, 2013; Payne *et al.*, 2017). Currently, *Pf*Rh5 is undergoing phase I clinical trial to assess safety, tolerability, and immunogenicity at the Ifakara Health Institute in Bagamoyo, Tanzania.

(iii) Transmission-blocking vaccines

A key factor to the eradication of malaria will be the ability to break the cycle of transmission of *Plasmodium* parasites between its human and mosquito hosts. An approach, such as a drug or vaccine, capable of preventing the transmission of the parasite that cause malaria are known as transmission-blocking interventions. The concept of transmission-blocking vaccines (TBVs) emerged in the 1970s from two seminal studies using animal models (Carter & Chen, 1976; Gwadz, 1976). Unlike canonical vaccines that directly protect the individual from infection, TBVs aim at preventing the spread of the parasite from an infected individual to a noninfected individual by targeting the transmission stages of *Plasmodium* (Dinglasan & Jacobs-Lorena, 2008). There are two types of TBVs with regards to its target: (a) those that interrupt transmission from infected humans to mosquitoes by targeting gametocytes, gametes, or ookinetes prior to invasion of mosquito midgut wall and (b) those that interrupt transmission from mosquito to humans by targeting the sporozoite in the recipient host (Chaudhury *et al.*, 2016; Chowdhury, Angov, Kariuki, & Kumar, 2009; Kapulu *et al.*, 2015; Mordmuller *et al.*, 2017; Tachibana *et al.*, 2011; Wu *et al.*, 2008).

Research and development on transmission-blocking vaccines has in recent years made significant progress. Vaccines that target the gametocyte/gamete surface proteins (such as Pfs230, Pfs48/45, HAP2) and macrogamete and ookinete surface protein such as Pfs25, are showing promising results (Angrisano *et al.*, 2017; Blagborough *et al.*, 2013; Datta *et al.*, 2017; Kapulu *et al.*, 2015; Lee *et al.*, 2016; Tachibana *et al.*, 2011; Talaat *et al.*, 2016). Furthermore, vaccines that interrupt the interaction between ookinetes and mosquito midgut ligands such as Alanyl aminopeptidase N (APN-1) have also shown promising results (Armistead *et al.*, 2014; Atkinson *et al.*, 2015; Dinglasan *et al.*, 2007; Mathias *et al.*, 2012).

Direct protection of immunized individuals is unlikely the direct outcome of TBVs, rather they could have substantial impact in an endemic population of asymptomatic and/or submicroscopic carriers. Such vaccines will serve to arrest onward transmission of malaria and thus provide protection to the community as other vaccines do through herd immunity (Draper *et al.*, 2018). Therefore, current emphasis in TBV development has been on increasing immunogenicity against the lead candidate antigens without unduly raising safety concerns (Hoffman *et al.*, 2015).

1.1.10 Oxidative Stress

(i) Oxidative Stress in General

Oxidative stress can be defined as a disturbance in the balance between the production of free radicals (oxidants) and antioxidant defences (Gutteridge & Halliwell, 2018; Sies, 2015; Sies, Berndt, & Jones, 2017). A free radical is a molecule that contains one or more unpaired electron(s) in its outer orbit (Betteridge, 2000). The unpaired electrons alter the chemical reactivity of an atom or molecule, therefore increasing the chemical reactivity of free radicals compared to non-radicals (Betteridge, 2000). The free radicals in question here include reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced as by-products of cellular metabolism, as well as naturally occurring environmental agents (Forrester & Stamler, 2007).

Under normal physiological conditions, cells are capable of counterbalancing the production of oxidants with antioxidants (Klaunig & Kamendulis, 2004). The majority of cellular antioxidants are enzymatic in nature such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), thioredoxin peroxidase (TPx), or thioredoxin reductase (TrxR) (Betteridge, 2000). However, there are some that are non-enzymatic in nature, including compounds such as vitamin E, vitamin C, and β-carotene (Clarkson & Thompson, 2000). In certain instances, a cell would overproduce ROS and RNS to levels that the capacity of its own antioxidant defences is diminished and unable to maintain equilibrium between oxidants and antioxidants. A cell that is in such a state is under oxidative stress, which may lead to damage and injury to cellular components if it's not immediately mitigated (Sies, 2015; Sies *et al.*, 2017).

(ii) Sources of ROS and RNS

Superoxide anion ($O_2^{\cdot-}$) produced by one electron reduction of molecular oxygen, is considered as the ''primary'' ROS because it can further react with other molecules in a cell and form ''secondary'' ROS either through enzyme- or metal- catalysed reactions (Fridovich, 1986). Protonation (addition of H^+) of $O_2^{\cdot-}$ results in the formation of hydroperoxyl radical ($HO_2^{\cdot}$), a much stronger radical than $O_2^{\cdot-}$ (Yu, 1994). Upon further protonation, $HO_2^{\cdot}$ is converted to hydrogen peroxide (H_2O_2), which can also be formed by dismutation of $O_2^{\cdot-}$ catalysed by the enzyme SOD (Yu, 1994). In the presence of reduced metal, particularly Iron (Fe), H_2O_2 generated from the above

reactions can be converted to hydroxyl radicals (˙OH) through the Fenton and Haber-Weiss reactions (Table 2.1) (Betteridge, 2000). The hydroxyl radical is an extremely potent radical and rapidly interacts with cellular components such as nucleic acids, lipids, and proteins (Betteridge, 2000).

Nitric oxide (NO) plays a dual role in the generation of RNS in biological systems; first it's the main RNS produced by cells, and on the other hand, it is the main source for other RNS (Martinez & Andriantsitohaina, 2009). It is produced from L-arginine and oxygen. The reaction is catalysed by the enzyme nitric oxide synthases (NOS) (Squadrito & Pryor, 1998). Three isoforms of NOS exist and these are NOS 1 or neuronal NOS (nNOS), NOS 2 or inducible NOS (iNOS), and NOS 3 or endothelial NOS (eNOS) (Dedon & Tannenbaum, 2004; Martinez & Andriantsitohaina, 2009). In biological systems, the dominant reactions of NO will be with another free radical to generate RNS, and the best-known free radical that reacts with NO is superoxide anion to produce peroxynitrite ($OONO^-$) (Radi, 2013, 2018). Under physiological conditions, peroxynitrite is unstable and isomerizes to nitrate (NO_3^-), a chemically inert compound. This reaction was once seen as a way of scavenging and neutralizing superoxide anion (Radi, 2013, 2018). Furthermore, $OONO^-$ is a strong oxidant and has the potential to react with all major classes of biomolecules to produce an array of other free radical molecules capable of mediating further cell damage (Calcerrada, Peluffo, & Radi, 2011). It undergoes oxidation, nitration, and nitrosation to produce NO adducts that can undergo secondary reactions with metals, thiols, and additional targets to give further products, often with biological activity and capable of bringing further oxidative damage to biological systems (Calcerrada *et al.*, 2011).

Biological systems are exposed to ROS and RNS through endogenous sources such as from mitochondria, inflammatory cell activation, peroxisomes, and cytochrome P450 (CYP450) metabolism or exogenous sources such as environmental (xenobiotic) agents, ionizing radiation (IR), industrial and pharmaceutical compounds (Klaunig & Kamendulis, 2004; Klaunig, Wang, Pu, & Zhou, 2011).

Table 2. Pathways for intracellular generation of ROS and RNS

1. Generation of ROS via reduction of molecular oxygen:	
$O_2 + e^- \rightarrow O_2^{-\cdot}$	(1.1)
$O_2^{-\cdot} + H_2O \rightarrow HO_2^{\cdot}$	(1.2)
$HO_2^{\cdot} + e^-H \rightarrow H_2O_2$	(1.3)
$H_2O_2 + e^- \rightarrow OH^- + {}^{\cdot}OH$	(1.4)
2. Production of reactive nitrogen species:	
L-Arginine + $O_2 \rightarrow$ NO (Nitric oxide) + L-Citrulline	(1.5)
$O_2^{-\cdot} + NO \rightarrow OONO^-$ (peroxynitrite)	(1.6)
$OONO^- + CO_2 \rightarrow ONOOCO_2^-$ (nitrosoperoxycarbonate)	(1.7)
$ONOOCO_2^- \rightarrow NO_2$(nitrogen dioxide) + $CO_3^{\cdot-}$(carbonate anion radical)	(1.8)
3. Fenton reaction:	
$Fe^{3+} + O_2^{-\cdot} \rightarrow Fe^{2+} + O_2$	(1.9)
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$	(1.10)
4. Haber-Weiss reaction:	
$O_2^{-\cdot} + H_2O_2 \rightarrow O_2 + HO_2^{\cdot} + OH^-$	(1.11)

Chemical reactions for the generation of various ROS/RNS intermediates. (1) A cascade of chemical reactions for the generation of ROS intermediates in the presence of O_2. (2) A cascade of chemical reactions for the generation of RNS intermediates in the presence of O_2. (3) Chemical reactions between a reduced metal (Fe) and ROS intermediates to generate more ROS intermediates. (4) A net chemical reaction for the Fenton reaction as shown in (3). $O_2^{-\cdot}$= superoxide anion, $HO_2^{\cdot}$= hydroperoxyl radical, H_2O_2= hydrogen peroxide, ${}^{\cdot}OH$ = hydroxyl radical, NO = nitric oxide, $OONO^-$ = peroxynitrite, $ONOOCO_2^-$ = nitrosoperoxy carbonate, NO_2= nitrogen dioxide $CO_3^{\cdot-}$= carbonate anion radical, Fe^{2+} = Iron (II) , Fe^{3+} = Iron (III), OH^- = hydroxide ion

(iii) Management of Oxidative Stress

Substances that reduce the potential ill effect of ROS/RNS are generally grouped in the so-called antioxidant defence system (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Antioxidant defence systems can be divided into non-enzymatic and enzymatic. These antioxidant defence systems are extremely important as they represent the direct removal of ROS/RNS (pro-oxidants), thus providing maximal protection to cells. A good antioxidant should have, some if not all, of the

following properties/characteristics (Sies, Stahl, & Sevanian, 2005; Valko *et al.*, 2006): (a) specifically quench free radicals, (b) chelate redox metals, (c) interact with (regenerate) other antioxidants within the "antioxidant network", (d) have a positive effect on gene expression, (e) be readily absorbed, (f) have a concentration in tissues and biofluids at a physiologically relevant level, and (g) work in both the aqueous and/or membrane domains.

Enzymatic antioxidant defence system include SOD, catalase, GPx, and TPx to mention a few (Mates, 2000); while non-enzymatic antioxidant defence system include vitamin C (ascorbic acid), vitamin E (α-tocopherol), vitamin A (carotenoids), thiol compounds (glutathione (GSH) and thioredoxin (Trx)), natural flavonoids, a hormonal product of the pineal gland, melatonin also to mention a few (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Herein, research was carried out in alignment with the objective of the study to evaluate the thiol compounds (Trx and GSH) as antioxidant defence systems in *An. gambiae* midguts following exposure to ROS inducers.

Thiols (Glutathione and Thioredoxin) Systems

The cellular antioxidant milieu is largely maintained and regulated by the two enzyme-based thiol systems: the GSH and Trx system (Lu & Holmgren, 2014; Schafer & Buettner, 2001). In each system, an NADPH-dependent flavoenzyme—namely glutathione reductase (GR; EC 1.8.1.7a) and TrxR (EC 1.8.1.9) are involved, where both enzymes belong to a family of homodimeric pyridine nucleotide-disulfide oxidoreductases that includes enzymes like lipoamide dehydrogenase, trypanothione reductase, and mercuric ion reductase (Williams, 1992).

These systems exist in the thiol-reduced and the disulfide-oxidized forms, GSH/GSSG and $Trx(SH)_2/TrxS_2$, respectively. These thiol/disulphide couple systems (GSH/GSSG and $Trx(SH)_2/TrxS_2$) interacts with nearly all physiological oxidants, and have therefore proved to be essential cellular antioxidant buffers. The antioxidant capacity of thiol compounds is due to the sulphur atom which can easily accommodate the loss of a single electron (Karoui, Hogg, Frejaville, Tordo, & Kalyanaraman, 1996). In addition, the lifetime of sulphur radical species thus generated, i.e. a thiyl radical $RS^{\cdot}$, may be significantly longer than many other radicals generated during the

stress. Thereafter, the $RS^{\cdot}$ generated may dimerise to form the oxidized non-radical product, GSSG or $TrxS_2$.

Glutathione is a multifunctional intracellular non-enzymatic antioxidant and a major low-molecular weight thiol in cells (Meister & Anderson, 1983). GSH is a tripeptide, L-γ-glutamyl-L-cysteinyl-glycine, and is synthesized from L-glutamate, L-cysteine, and glycine in two consecutive steps catalysed by glutamyl-cysteine synthase and glutathione synthase (Eq. 6.1 and 6.2, respectively) (Meister & Anderson, 1983). It is a suitable cellular thiol "redox buffer" because it lacks the toxicity associated with cysteine and the GSH/GSSG ratio therefore is a good measure of oxidative stress level of a cell (Schafer & Buettner, 2001; Vina *et al.*, 1983). The redox potential for the GSSG/2GSH couple is determined by the redox environment in which the couple is functioning (Jones *et al.*, 2000). Cellular organelles vary in their redox potentials which determines the concentration of GSH compartmentalized in these organelles. The reduced-thiol form, GSH, is the predominant form and accounts for >98% of total GSH (Forman, Zhang, & Rinna, 2009). Eukaryotic cells have three major reservoirs of GSH; where 80–85% is in the cytosol, 10–15% is in the mitochondria, and the remaining small percentage is in the endoplasmic reticulum (Yuan & Kaplowitz, 2009). A shift in the level of GSH caused by either its over consumption or synthesis due to increased oxidative stress will influence the redox potential. The main antioxidant roles of GSH against cellular ROS/RNS are (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005):

(i) GSH is a cofactor of several detoxifying enzymes against oxidative stress, e.g. GPx, GST, and etc.

(ii) GSH participates in amino acid transport through the plasma membrane.

(iii) GSH directly scavenges $^{\cdot}OH$ and $^1O_2$ (Eq. 6.3) and detoxifies H_2O_2 and ROOH by the catalytic action of GPx (Eq. 5.1 and 5.2).

(iv) GSH is able to regenerate vitamins C and E back to their active forms. GSH can reduce the tocopherol radical of Vitamin E directly or indirectly via reduction of semidehydroascorbate to ascorbate.

Thioredoxins are small, ubiquitous thiol proteins with a relative molecular mass of ~12 kDa and a redox active cysteine pair within a conserved active site of amino acids WCGPC (Nakamura et al., 1997). They contain two adjacent sulfhydryl (–SH) groups in its reduced form [$Trx(SH)_2$] that are

converted to a disulphide unit in the oxidised form ($TrxS_2$) in the antioxidant reactions (Eq. 6.4 and 6.5). The oxidized form is recycled back to the reduced form by the transfer of reducing equivalents from NADPH, the reaction catalysed by the enzyme TrxR. The reduction of the disulphide back to the dithiol form is catalysed by the NADPH- dependent flavoprotein TrxR. Cellular Trx levels are much less compared to GSH, about 100- to 1000- fold less. Previously, it was believed that the antioxidant functions of Trx and GSH are clearly separated. However, it has been shown that Trx and GSH systems constitute a balanced redox network in which functionality can be shared between the constituents to some extent (Gromer, Urig, & Becker, 2004). For example, dipterans like *Drosophila melanogaster* and *Anopheles spp* lack the enzyme GR (Bauer *et al.*, 2003; Kanzok *et al.*, 2001) and the cycling of GSSG back to GSH is catalysed by TrxR, ensuring the maintenance of GSH/GSSG ratio. Apart from its antioxidant activity, some of the other functions of Trx includes transcription factor regulation, protein binding, and inhibition of apoptosis (Mustacich & Powis, 2000).

$$\text{L-Glutamate} + \text{L-Cysteine} \rightarrow \gamma\text{-GluCys} \quad (6.1)$$

$$\gamma\text{-GluCys} + \text{L-Glycine} \rightarrow \text{GSH} \quad (6.2)$$

$$2^{\cdot}OH + 2GSH \rightarrow GSSG + 2H_2O \quad (6.3)$$

$$Trx(SH)_2 + H_2O_2 \rightarrow H_2O + TrxS_2 \quad (6.4)$$

$$Trx(SH)_2 + ROOH \rightarrow H_2O + TrxS_2 \quad (6.5)$$

$$TrxS_2 + NADPH^+ + H^+ \rightarrow Trx(SH)_2 + NADP^+ \quad (6.6)$$

(iv) Oxidative Stress during *Plasmodium* Sporogonic Development

Plasmodium development in *Anopheles* mosquito midgut is associated with increased levels of ROS/RNS. The source of excess ROS/RNS in the mosquito midgut is primarily due to either mosquito innate immunity to the developing parasite (endogenous sources) or factors in the ingested vertebrate blood meal, such as immune factors or digestion products of haemoglobin (exogenous sources). Studies using the murine parasite, *P. berghei* infecting *An. gambiae* or *An. stephensi* mosquitoes, have shown that its ookinetes inflicts physical damage to *Anopheles* midgut

epithelial cells while it traverses through them in its journey to reach the basal lamina of the midgut. The *Anopheles* mosquito mounts an immune response to the inflicted physical damage by triggering a cascade of events, which include an excess release of ROS/RNS. The excess ROS/RNS leads to oxidative stress and can be considered as a "time bomb" because they can have fatal consequences to both the invading parasite and epithelial cells (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004). However, the invading parasite and epithelial cells can survive this time bomb if the excess ROS/RNS levels are immediately dealt with by antioxidant mechanisms from both the *Plasmodium* parasite and *Anopheles* mosquito.

Plasmodium parasites and *Anopheles* mosquitoes have in their arsenal a variety of mechanisms to deal with the excess ROS/RNS but use the GSH and Trx systems as their primary line of defence against oxidative stress. Previous research studies on this topic have shown how dependant *Plasmodium* parasites are to GSH and TRx systems for their antioxidant defence. These studies have shown that mutant *Plasmodium* parasites with defective component(s) of either the GSH or Trx systems were not able to fully develop in the *Anopheles* midgut compared to wild type *Plasmodium* parasites due to their diminished ability to reduce oxidative stress in their environment (Pastrana-Mena *et al.*, 2010; Vega-Rodriguez *et al.*, 2009; Yano *et al.*, 2008). Similarly, other studies have shown how parasite's transcripts or proteins of the GSH and Trx system increase when there is an increased ROS/RNS in its environment (Turturice et al., 2013).

Furthermore, *Anopheles* mosquitoes have been shown to use various antioxidant mechanisms in midgut epithelial cells to reduce the oxidative stress associated with *Plasmodium* infection. Overexpression of antioxidant defence enzymes has been observed in several studies following the ingestion of a *Plasmodium* blood meal (Dimopoulos *et al.*, 2002; Molina-Cruz *et al.*, 2008; Peterson & Luckhart, 2006). Other studies have shown how epithelial cells undergo apoptosis and get completely removed from the midgut if the excess ROS/RNS due to invading parasite is not dealt with (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004). However, *Anopheles* mosquitoes like other dipterans lack the flavoenzyme GR of the GSH pathway and therefore utilize instead the Trx system for mitigating oxidative stress (Bauer *et al.*, 2003). Therefore, the Trx system is of major importance when it comes to management of oxidative stress by *Anopheles* mosquitoes.

(v) Oxidative Stress as a Transmission-Blocking Strategy

Studies have shown that mosquitoes with increased oxidative stress in their midguts are resistant to *Plasmodium* transmission (Bahia *et al.*, 2013; Goncalves *et al.*, 2012; Molina-Cruz *et al.*, 2008; Peterson *et al.*, 2007; Peterson & Luckhart, 2006). The concept here is straightforward; oxidative stress in the mosquito midgut is generated post infected blood meal ingestion in anticipation that it will be deleterious to the parasites yet tolerable by the mosquito. The stress therefore blocks parasite transmission to another individual during subsequent blood meals. Furthermore, oxidative stress has been credited as the mode of action of several antimalarials that target blood stage parasite in the human host (refer to section 1.1.8). Therefore, it is very plausible that oxidative stress could be harnessed as a strategy against *Plasmodium* while its developing in the mosquito midgut. Despite the potential of this strategy, very few antimalarials either in use or in clinical development use it. A major challenge is in identifying compounds that would not lead to fitness cost to the mosquito while still generating selective toxicity in the midgut that is deleterious to the parasite yet tolerable to the mosquito. An oxidative stress strategy associated with fitness cost to the mosquito will translate into the mosquito developing compensatory mechanisms to overcome the fitness cost and eventual development of resistance (Wang, Pakpour*, et al.*, 2015). Another challenge is in identifying compounds and their respective dose regimen that are tolerable and not toxic to the human host, and yet maintain its effectiveness in the mosquito host after bloodmeal ingestion. The thesis research work described herein is founded on possible application of oxidative stress concept for transmission-blocking drug development. The ROS inducing compounds, tert-butyl hydroperoxide (tBHP) and Paraquat (Pqt), were used to generate oxidative stress in mosquito midguts and their global proteomic responses was assessed, with particular interest to the Trx and GSH systems. Furthermore, the global proteomics response was assessed to identify pathways that could be used as potential target(s) for future transmission-blocking strategies.

1.2 Statement of the Research Problem

The Trx system is of great importance in the regulation of oxidative stress in *Anopheles* mosquitoes. Despite this importance, a complete understanding of the Trx pathway in *Anopheles* mosquitoes at the molecular level is missing. Basic biological information regarding the regulation

of the components of this pathway under different oxidative conditions is missing. Furthermore, the data supporting the time bomb theory and management of oxidative stress in *Anopheles* mosquitoes were obtained using the *An. stephensi*/*An. gambiae*-*P. berghei* system. Refer to section 1.1.10(iv) for more information on *P. berghei* invasion of *An. stephensi*/*An. gambiae* and associated oxidative stress. This vector-parasite combination is not natural and may not necessarily reflect human malaria transmission biology in the field. The natural, co-evolved, parasite–vector system that is responsible for malaria morbidity and mortality in sub-Saharan Africa is *P. falciparum/An. gambiae*. In this vector-parasite system, *P. falciparum* is known for being less abrasive and more finesse when invading midgut epithelial cells of *An. gambiae*. Thus, the level of epithelial cell destruction associated with their invasion process is low, henceforth, the type and extent of the redox response (transcripts and protein levels) may vary from that of *An. stephensi*/*An. gambiae*-*P. berghei*.

1.3 Rationale of the Study

The absence of GR in *Anopheles* mosquitoes is compensated by the Thioredoxin pathway, where the thiol Trx-1 is pivotal component of it. Due to this importance, the thiol Trx-1 may act as the cellular redox sensor, where a change in the cell's redox status is reflected in its expression at both transcript and protein levels. The study aimed to prove this by looking at the transcript and protein expression levels of Trx-1 and other Trx pathway transcripts/proteins under different oxidative conditions.

Furthermore, different ROS inducers may elicit different responses to oxidative stress. Conversely, different doses of the same ROS inducer may elicit different responses to oxidative stress. The proteomic profile in *An. gambiae* midgut epithelial cells was investigated under different oxidative stress conditions of two ROS inducers, Pqt and tBHP. This would determine if the response to oxidative stress in *An. gambiae* midgut epithelial cells is blanket or tailored to the ROS inducer used. This would shed more light on the translational application of oxidative stress as a transmission-blocking strategy.

Thirdly, *Plasmodium* ookinete invasion of *Anopheles* mosquitoes is quick happening within 15 minutes. Furthermore, *P. falciparum* ookinete invasion of *Anopheles* midgut cells is subtle and not associated with extensive injury to the epithelial cells compared to *P. berghei*. This injury is

responsible for some of the increased oxidative stress observed in *Anopheles* midgut cells at this particular stage. The redox response by *Anopheles* mosquitoes to this oxidative stress is crucial as it could determine whether the mosquito survives this insult or not. The *An. gambiae* mosquitoes' response to *P. falciparum* midgut invasion was investigated by assessing the transcript profile of 9 Trx- and GSH-dependent genes. Furthermore, the *P. falciparum* midgut invasion response was compared for similarity to the response of *An. gambiae* midguts to oxidative stress under different conditions. Concordance in the response (at transcript and protein levels) to oxidative stress from various sources is of great importance as it could lead to the identification of conserved regulatory elements that could be used as targets for development of transmission-blocking drugs.

Therefore, this study aimed to better understand the redox regulation in *An. gambiae* mosquitoes during *P. falciparum* sporogonic development and provide novel insights into the dynamics of the host-parasite interactions between this vector-parasite system. These novel insights can be further be translated into strategies that can developed into drugs or vaccines that target and stop the development of *Plasmodium* in this mosquito eventually leading to a stop in the transmission of Malaria.

1.4 Objectives of the Study

1.4.1 General Objective

To investigate the differential response of *An. gambiae* midgut epithelial cells to oxidative stress by assessing the transcript and protein expression levels under varied oxidative stress conditions, with particular interest to the Trx and GSH pathway.

1.4.2 Specific Objectives

(i) To characterize the transcript and protein expression levels of *Ag*Trx-1 in *An. gambiae* midgut epithelial cells under different oxidative conditions.

(ii) To characterize the proteomic profiles of *An. gambiae* midgut epithelial cells under different oxidative conditions.

(iii) To characterize the Trx- and GSH-dependent transcript profile in *An. gambiae* midgut

epithelial cells during *P. falciparum* ookinete invasion.

1.5 Hypotheses

This study looked at oxidative stress management in the midguts of *Anopheles* mosquitoes during the first 24 hours of *Plasmodium* sporogonic development. The research question investigated was, what is the response of midgut epithelial cells, at transcript and protein levels, to oxidative stress with particular interest to the Trx and GSH systems?

(i) Null hypothesis (H_o): There is no significant difference in the response of the Trx and GSH systems to different oxidative stress conditions.

(ii) Alternative hypothesis (H_a): There is a significant difference in the response of the Trx and GSH systems to different oxidative stress conditions.

1.6 Significance of the Study

In order to move towards malaria elimination and eradication, more and more interventions that reduce the burden of the disease will be of great advantage. Currently, malaria efforts are greatly hindered by the development of resistance in *Anopheles* to insecticides and in *Plasmodium* to antimalarials as described in sections 1.1.7 and 1.1.8, respectively. New classes of insecticides and antimalarials without any resistance to *Anopheles* and *Plasmodium*, respectively are needed in order to shift the balance in favour of malaria control efforts. This study revealed novel insights on redox homeostasis in the *An. gambiae* midgut, which can aid in the development of transmission-blocking strategies, in particular a drug. Transmission-blocking strategies disrupt the development of *Plasmodium* whilst in *Anopheles* and have the advantage of reduced chances of resistance development in the mosquito as they are not associated with any fitness costs. However, the likelihood of development of resistance in the parasite is possible but substantially slowed down as a TBD would be an additional arsenal in the fight against malaria. An additional drug against *Plasmodium* would increases number of drugs that the parasite needs to develop resistance against in order to overcome its deleterious effects.

1.7 Delineation of the Study

In this study, only the expression levels of *Ag*Trx-1 transcript and protein in *An. gambiae* midgut epithelial cells under different conditions of oxidative stress induced by tBHP was considered. Secondly, the global proteomic profile in *An. gambiae* midgut epithelial cells under different oxidative stress conditions of the induced by Pqt and tBHP. This was done by first evaluating enriched proteins in the experimental vs control group with canonical functions in the regulation of oxidative stress. Then, this was followed by an evaluation of enriched proteins in experimental vs. control group with non-canonical functions in the regulation of oxidative stress and determine their association with regulation of oxidative stress and redox homeostasis. Lastly, the profile of Trx- and GSH- dependent transcripts in *An. gambiae* midgut cells at 24 hours post infected blood meal ingestion (i.e. during *P. falciparum* ookinete invasion) was considered.

On the contrary, some of the aspects were not considered in the scope of this study. One, the study focused only on the transcript and protein expression levels of *Ag*Trx-1. Other Trx proteins such as Trx-2, Trx-3, Trx-4 were not considered in this study because the background information from previous studies pointed out on the importance of Trx-1 over the other Trx pathways components in the regulation of oxidative stress in *An. gambiae*. Furthermore, the availability of only *Ag*Trx-1 antiserum necessitated that the focus of the study should be on Trx-1 over the other Trx pathway transcript and proteins. Secondly, only proteins that were enriched following Pqt and tBHP treatment were the ones evaluated under this study. It is entirely plausible that different proteins would have been enriched if different ROS inducers were used. Thirdly, Pqt induced oxidative stress was only evaluated at the 8 hours timepoint as this coincided with essential *Plasmodium* biology during sporogonic development in mosquitoes. Other timepoints prior or after the 8 hours timepoint were not the scope of this study. Lastly, the study considered only the 24 hours timepoint for evaluation of overexpressed Trx- and GSH-dependent transcripts following *P. falciparum* blood meal ingestion. Other time points prior to the 24 hours mark were not included in the scope of this study.

CHAPTER TWO

Paraquat mediated oxidative stress in *Anopheles gambiae* mosquitoes is regulated by an endoplasmic reticulum (ER) stress response[1]

Brian B. Tarimo[1,2,3], Henry Chun Hin Law [4,†], Dingyin Tao [2,†], Rebecca Pastrana-Mena [2], Stefan M. Kanzok[5], Joram J. Buza[1] and Rhoel R. Dinglasan[1,2,4]

[1]Department of Health and Biomedical Sciences, Nelson Mandela-African Institution of Science and Technology, Tengeru, Arusha 23302, Tanzania

[2]W. Harry Feinstone Department of Molecular Microbiology & Immunology & the Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA

[3]Department of Environmental Health & Ecological Sciences, Ifakara Health Institute, Dar es Salaam 14112, Tanzania

[4]Emerging Pathogens Institute, Department of Infectious Diseases & Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA;

[5]Department of Biology, Loyola University Chicago, Chicago, IL 60660, USA;

† These authors contributed equally to this work.

[1]*Proteomes* 2018, 6, 47; doi:10.3390/proteomes6040047, 1-15

2.0 Abstract

Paraquat is a potent superoxide ($O_2^{\cdot-}$)-inducing agent that is capable of inducing an oxidative imbalance in the mosquito midgut. This oxidative imbalance can super-stress the malaria parasite, leading to arrested development in the mosquito midgut and reduced transmission. While several studies have explored the effect of paraquat on malaria parasites, a fundamental understanding of the mosquito response to this compound remains unknown. Here, we quantified the mosquito midgut proteomic response to a paraquat-laced sugar meal and found that *An. gambiae* midguts were enriched in proteins that are indicative of cells under endoplasmic reticulum (ER) stress. We also carried out qRT-PCR analyses for nine prominent thioredoxin (Trx) and glutathione (GSH)-dependent genes in mosquito midguts post *P. falciparum* blood meal ingestion to evaluate the concordance between transcripts and proteins under different oxidative stress conditions. Our data revealed an absence of significant upregulation in the Trx and GSH-dependent genes following infected blood meal ingestion. These data suggest that the intrinsic tolerance of the mosquito midgut to paraquat-mediated oxidative stress is through an ER stress response. These data indicate that mosquitoes have at least two divergent pathways of managing the oxidative stress that is induced by exogenous compounds, and outline the potential application of paraquat-like drugs to act selectively against malaria parasite development in mosquito midguts, thereby blocking mosquito-to-human transmission.

2.1 Introduction

Malaria, caused by the protozoan parasite *Plasmodium*, remains a major global public health problem despite extensive investment in the control and elimination of this disease. A major gap in knowledge in this field is our understanding of the various processes that occur during parasite transmission through its insect vector, the *Anopheles* mosquito. Following *Plasmodium* infected blood meal ingestion by *Anopheles* mosquitoes, the parasite undergoes an intricate developmental stage in the mosquito. *Plasmodium* gametocytes in the ingested blood fuse and undergo sexual reproduction to form a zygote that shortly thereafter transforms into a motile ookinete. The ookinete then leaves the blood bolus to invade and traverse the mosquito midgut epithelium to its basal lamina side where it differentiates into an oocyst. The oocyst grows in size and undergoes sporogony to produce thousands of sporozoites. Once the oocyst matures, it ruptures and releases

the sporozoites into the haemocoel, where they are passively transported to the salivary glands and invade them. The mosquito then re-establishes the cycle in the vertebrate host by transmitting the sporozoites through its saliva as it blood feeds (Baton & Ranford-Cartwright, 2005).

The success of *Plasmodium* is in part due to the fact that the parasite has evolved a balance with its insect host. Disrupting this balance could lead to new interventions that can reduce, if not completely block malaria transmission. *Plasmodium* parasite development in *Anopheles* mosquitoes is associated with excessive amounts of ROS and RNS from several sources. These sources include vertebrate immune factors present in the ingested blood (Lensen *et al.*, 1997; Naotunne *et al.*, 1993), digestion of haemoglobin (Graca-Souza *et al.*, 2006; Peterson *et al.*, 2007), and the mosquito's innate immunity due to invasion of its midgut epithelial cells by the parasite (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004). Oxidative stress could be fatal to cells if not immediately dealt with due to its ability to cause damage to cellular macromolecules such as proteins, cell membranes, and nucleic acids (Avery, 2011).

At the cellular level, most organisms depend on the Thioredoxin (Trx) and Glutathione (GSH) systems as a prominent line of defence against oxidative stress. For example, in *Plasmodium*, the absence or deficiency of an antioxidant gene, e.g. glutathione reductase (GR) severely affects the development of the parasite in the insect host (Pastrana-Mena *et al.*, 2010; Vega-Rodriguez *et al.*, 2009). Interestingly, *Anopheles* mosquitoes, like other dipterans, lack the GR and compensate by utilizing the Trx system to recycle GSSG to GSH as shown in Fig. 3 (Kanzok *et al.*, 2001) This emphasizes the importance of the Trx system in oxidative stress regulation in *Anopheles* mosquitoes. Thus they regulate proteins of the Trx system to protect midgut epithelial cells against oxidative stress, specifically when associated with infection by the malaria parasite (Molina-Cruz *et al.*, 2008; Peterson & Luckhart, 2006).

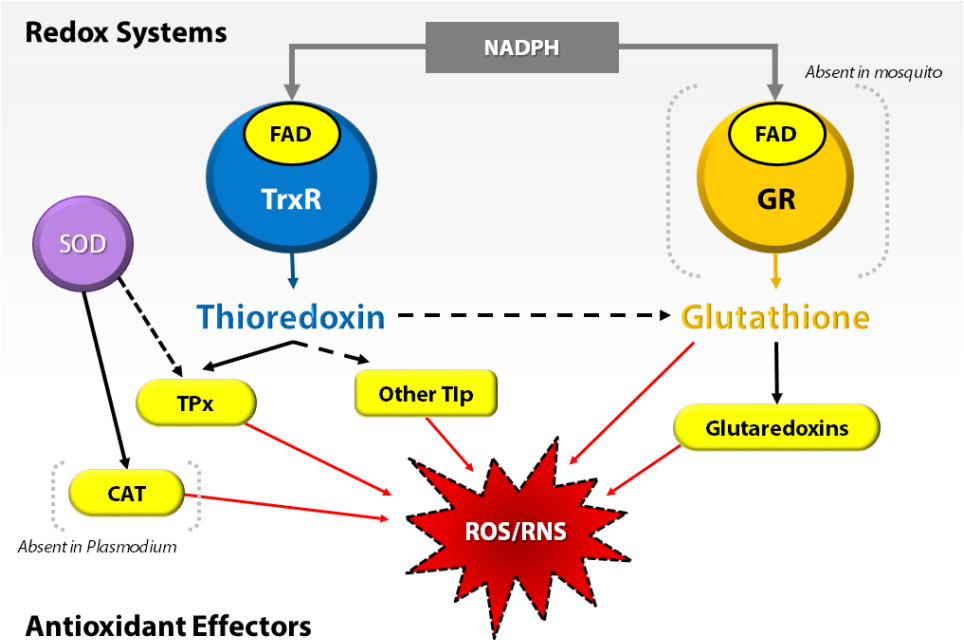

Figure 3. Interplay of redox systems in *Plasmodium* and mosquito. ROS/RNS = reactive oxygen species/reactive nitrogen species, GR = glutathione reductase, FAD = flavin adenosine dinucleotide, NADPH = reduced nicotinamide dinucleotide phosphate, TrxR = thioredoxin reductase, SOD = superoxide dismutase, TPx = thioredoxin peroxidase, CAT = Catalase, and Tlp = thioredoxin-like proteins

A basic understanding of redox homeostasis in *An. gambiae* midgut epithelial cells under different oxidative conditions and during *P. falciparum* ookinete invasion is missing and such information can potentially guide the development of new malaria transmission-blocking drugs. Existing data on redox homeostasis in the *Anopheles* mosquito midgut due to *Plasmodium* ookinete invasion has been largely generated using the *P. berghei-An.stephensi/An. gambiae* parasite-vector model, which is not a natural parasite-vector system of malaria transmission. The natural, co-evolved, parasite-vector system responsible for malaria morbidity and mortality in sub-Sahara Africa (SSA) is *P. falciparum-An. gambiae*. The ROS inducer Pqt (1,1'-dimethyl-4,4'-bipyridylium di-chloride) upregulates antioxidant responses in *P. berghei ookinete in vitro* (Turturice *et al.*, 2013), suggesting that Pqt-associated oxidative stress can be exploited as a transmission-blocking strategy. We hypothesize that the mosquito vector can mitigate the extra ROS produced following ingestion of Pqt with a bloodmeal, permitting selective toxicity against malaria parasites during the first 24 hours following ingestion of infected blood. To explore the feasibility of this proposed transmission-blocking strategy, we used a quantitative proteomic approach to profile the organ

(midgut)-level response of *An. gambiae* mosquitoes to Pqt-induced oxidative stress. To evaluate the concordance between transcripts and proteins under different oxidative conditions, we measured the expression profile of Trx- and GSH-dependent genes in *An. gambiae* midguts 24 hours post *P. falciparum* blood meal ingestion.

2.2 Materials and Methods

2.2.1 Mosquito Rearing, Experimental Treatments, and ROS Induction Assays

An. gambiae (KEELE strain) mosquitoes were used for all the experimental treatments. These mosquitoes were maintained in an insectary at the Johns Hopkins Malaria Research Institute (JHMRI), kept at 26°C and 70% humidity with 12 hours light and dark cycles and supplemented with 10% sucrose solution.

ROS induction (oxidative stress) assays were performed using *in vivo* and *in vitro* studies. Direct membrane feeding assays (DMFA) with 50-75 pre-starved *An. gambiae*female mosquitoes (4-7 days old). An ATP-saline solution [150 mM NaCl, 10 mM NaHCO3 pH 7.0] and 1 mM ATP (Billingsley & Rudin, 1992; Galun, Avi-Dor, & Bar-Zeev, 1963; Moskalyk, Oo, & Jacobs-Lorena, 1996) added as a phagostimulant containing the following treatments: 10% sucrose solution (control group) or 1 mM Pqt (treatment group) was prepared to a 2x concentration. To track uptake, an equivalent volume of colored water (artificial red food color) was added to the experimental groups and then delivered directly into glass, water-jacketed membrane feeders warmed to 37°C. Mosquitoes were allowed to feed for 30-45 minutes. The fed mosquitoes were kept at 26°C, 70% humidity for 8 hours and maintained on sugar (10% sucrose) and water. The colored water in the treatments aided the selection of only those mosquitoes that fed on the solution. The artificial food color used did not contain any ingredients known to either favor or hinder the production of ROS/RNS. The midguts were dissected from 50 fully fed mosquitoes per experimental group and transferred into 200 µL 1x PBS on ice. All of the 1x PBS was removed and the samples were stored at -80°C until further liquid chromatography-tandem mass spectrometry LC-MS/MS analysis. These experiments were replicated three times using independent biological cohorts of mosquitoes to ensure reproducibility.

Standard membrane feeding assays (SMFA) used pre-starved 50-75 *An. gambiae* female mosquitoes (4-7 days old) per treatment group. *P. falciparum* (NF54) gametocyte cultures (15–18 days post-initiation) were pelleted and diluted to 1.0% gametocytemia with human blood that had been washed with RPMI 1640 (Thermo Fisher Scientific) and brought up to 50% hematocrit with normal AB serum. Gametocytemic blood was kept at 37°C until feeding. During feeding, 200 µL of gametocytemic blood (experimental treatment), human blood at 50% hematocrit (blood control treatment), and 10 % sucrose solution (sugar control treatment) were delivered directly into glass, water-jacketed membrane feeders warmed to 37°C. Mosquitoes were allowed to feed for 30-45 minutes. After blood feeding, non-blood fed mosquitoes were removed from each treatment group and the fed mosquitoes were maintained on sugar and water at 26°C and 70% humidity (to assure survival and prevent desiccation) for 24 hours prior to midgut dissections. Midguts were dissected into TRIzol reagent (Life Technologies, Carlsbad, CA) for total RNA extraction. These experiments were replicated three times using independent biological cohorts of mosquitoes.

2.2.2 Extraction, Solubilization, and Digestion of Proteins

Prior to LC-MS/MS analysis, experimental groups (50 midguts/sample) were processed as follows. Total protein lysate was prepared by lysing the midgut samples with 45 µL of SDST-lysis buffer (4% SDS (w/v), 100 mM Tris/HCl, 0.1 M DTT pH 7.6) and boiled at 95°C for 5 minutes. An aliquot of the protein lysate (30 µL) was used for protein digestion according to the Filter-Aided Sample Preparation (FASP) protocol (Wisniewski, Zougman, Nagaraj, & Mann, 2009) using a 10 kDa molecular weight cut-off filter (Tao, King*, et al.*, 2014; Tweedell, Tao, & Dinglasan, 2015) (EMD Millipore, Billerica, MA). Acidified tryptic peptides from following FASP treatment were desalted using an HPLC C18 column on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) (Tao, King*, et al.*, 2014; Tweedell *et al.*, 2015). Concentration of the peptides following FASP was estimated using protein digest standards whose concentrations, as determined by BCA, were known (Tao, King*, et al.*, 2014; Tweedell *et al.*, 2015).

2.2.3 Online 2D LC-MS/MS Analysis

Peptide products desalted and digested by the FASP protocol were dissolved in loading buffer (97.9% water, 2% CAN, and 0.1% formic acid (FA)) and ~ 20 µg was injected to an online 2D

HPLC-MS/MS system, using the exact method as described previously and briefly detailed below (Tao, King*, et al.*, 2014; Tao, Ubaida-Mohien*, et al.*, 2014; Tweedell *et al.*, 2015). The online 2D HPLC-MS-MS system was constructed by integrating one SCX column (150 µm i.d. x 2 cm length PolySULFOETHYL A^{TM}, 5 µm 300 Å, PolyLC INC) into an Agilent LC-MS system comprised of a 1200 LC system coupled to a 6520 QTOF via an HPLC Chip Cube interface. For the online SCX fractionation, in the first dimension peptides were loaded into the SCX column at 1.8 µl/min and the peptides were eluted using the autosampler by injecting 6 µl of each increasing salt concentration (0, 15, 30, 45, 60, 120, 160, and 300mM NaCl in 2% ACN/0.1% FA) followed by one injection of 500 mM NaCl in 2% ACN/0.1% FA to wash the column. The salt elution was captured by a C18 enrichment column integrated into the Agilent Polaris-HR-Chip-3C18 chip (360 nL, 180 Å C18 trap with a 75 µm i.d., 150 mm length, 180 Å C18 analytical column). In the second dimension, with the valve switched and the RPLC gradient started, the peptides were eluted from the enrichment column and separated by a C18 analytical column. Elution of peptides from the analytical column was performed using a gradient starting at 97% A (A: 99.9% water, 0.1% FA) at 300 nL/min. The mobile phase was 3–10% B (B: 90% ACN, 9.9% water, 0.1% FA) for 4 min, 10–35% B for 56 min, 35–99% B for 2 min, and maintained at 99% B for 6 min, followed by re-equilibration of column with 3% B for 10 min. Data-dependent (autoMS2) MS acquisition was performed by an Agilent 6520 QTOF at 2 GHz. Precursor MS spectra were acquired from *m/z* 315 to 1700, and the top four peaks were selected for MS/MS analysis. Product scans were acquired from *m/z* 50 to 1700 at a scan rate of 1.5 spectra per second. A medium isolation width (~4 amu) was used, and a collision energy of slope 3.6 V/100 Da with a 2.9 V offset was applied for fragmentation. A dynamic exclusion list was applied with precursors excluded for 0.50 min after two MS/MS spectra were acquired.

2.2.4 Database Searching and Label-free Quantification Analysis

All the LC-MS/MS raw data were converted to Mascot generic format (.mgf) by Agilent MassHunter Qualitative Analysis B.04.00. The data acquired was used to search the VectorBase *Anopheles gambiae* protein FASTA sequence database (VectorBase, http://www.vectorbase.org, *Anopheles gambiae* PEST, AgamP4.2.) for peptide sequence alignments. The search engine used for the search was MASCOT version 2.5 with the following parameters: precursor ion mass tolerance of 50 ppm, fragment ion mass tolerance of 0.2 Da, carbamidomethylation of cysteine

and oxidation of methionine residues set as fixed and variable modifications respectively. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavage sites were allowed for tryptic digestion. The MASCOT search results were exported as .DAT format and then imported into the Scaffold software (version 4.4.5, Proteome Software) for curation, label-free quantification, analysis, and visualization. Overall, protein false discovery rates of less than 1% and peptide false discovery rates of less than 1% were obtained with Scaffold filters, and each protein had ≥ 2 unique peptides. Identified proteins were clustered to remove redundancy. Proteins were clustered together if there was a peptide identification shared between them, because this indicates substantial sequence similarity, and the protein with the greatest number of peptides identified was considered the unique protein identification from that group. The data analysis pipeline meets all MIAPE standards (Taylor *et al.*, 2007) and the detailed peptide data can be found in Appendix 1.

2.2.5 qRT-PCR

Total RNA was extracted from samples of *in vivo* studies with SMFA using Trizol reagent (Life Technologies) according to the manufacturer's protocol. Extracted RNA was checked for purity and concentration using the Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Complimentary DNA (cDNA) was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Grand Island, NY). Quantitative RT-PCR was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) on a StepOnePlus Real-Time PCR System (Applied Biosystems).

Relative transcript levels of the Thioredoxin system (Trx-1, Trx-2, TrxR, TPx-1, and PrxV) and Glutathione system (Grx-1, GSTD1, GPx, and GS,) were determined using gene-specific primers and cycling conditions as per manufacturer's protocol. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) relative to the *An. gambiae* ribosomal protein RpL32 (*Ag*RpL32; AGAP002122) gene, which was amplified using *Ag*RpL32 F 5'-GCCGAAGATTGTGAAGAAGC-3' and *Ag*RpL32 R5'- GCACCCGATTGTCAATACCT-3'. All qRT-PCR reactions were done in triplicate. Specific primer sequences of the transcripts can be found in Appendix 2

2.2.6 Statistical Analyses

The Student's t-test comparing quantifiable spectral values between treatment groups was used to identify differentially expressed proteins. For qRT-PCR analyses, comparisons of expression levels of targeted transcripts relative to each other were carried out using multiple Student's t-test followed by Holm-Šidak correction of t-scores to adjust for multiple tests. All statistical analyses utilized the software GraphPad Prism (version 6.0e). The *P*-values of < 0.05 were considered statistically significant. All experimental reactions used at least three independent biological replicate samples**.**

2.3 Results

The presence of a bloodmeal itself prevents the specific, accurate mass spectrometry-based proteomic analysis of the midgut response to Pqt alone from a Pqt-laced bloodmeal. Given this limitation, we sought to identify the midgut's response to Pqt alone considering that the bloodmeal induces a specific antioxidant response by the mosquito midgut. The assumption is that the identified processes would be in addition to those predicted to be mounted by the midgut in order to maintain redox homeostasis during blood feeding and digestion. With this in mind, we used a label-free quantitative proteomic approach to determine the midgut-level regulation of the response to Pqt-induced oxidative stress by *An. gambiae* mosquitoes; thereby complementing several studies that have explored the effect of Pqt on malaria parasites.

2.3.1 Global Proteomic Profiles of Midgut Epithelial Cells under Pqt-induced Oxidative Stress are Largely Conserved

We captured the proteomic profiles of *An. gambiae* midguts dissected 8 hours after ingestion of a 1 mM Pqt-laced sugar meal. This concentration of Pqt was found to induce oxidative stress without noticeable fatal damage to the midgut epithelial cells, evident in the absence of loss of tissue structure (data not shown). We analysed the global proteomic profile in midgut epithelial cells treated with Pqt (experimental group) and sugar (control group). A total of 631 quantifiable proteins were identified by label free techniques with a protein false discovery rate of <1% and normalization based on area under the curve as shown in Appendix 3. Of this, 578 proteins (91.6%) were shared between Pqt- and sugar-treated midguts, 24 (3.8%) were found only in Pqt-treated

midguts, and 29 (4.6%) were found only in sugar-treated midguts as shown in Fig. 4A. Antioxidant groups of proteins were organized from the 631 identified proteins, and this included heat shock proteins (HSP), cytochrome P450s (CYP), Trx-dependent, and GSH-dependent as shown in Appendix 3. The quantitative proteomic profiles of Pqt- and sugar treatment groups identified 20 out of the total 631 (0.031%) proteins that were differentially expressed between the groups based on spectral counts ($P \leq 0.05$; Student's t-test) as shown in Fig. 4B and Appendix 3. It was found that 11 out of the 20 (55%) proteins enriched (highly expressed) in Pqt-treated midguts. Annotated functions revealed that 7 (63.6%) of these proteins are involved in ER stress response or cellular detoxification machinery as shown in Table 3 below.

Table 3. Proteins enriched in Pqt-treated midguts that are directly involved in mitigation of the induced oxidative stress

Protein description	Fold change	*P*-value	Function
ABCC8 (AGAP008437) ATP-binding cassette transporter (ABC transporter) family C member 8	4.37	0.015	Upregulated in bendiocarb resistant *An. gambiae*, a detoxification gene (Antonio-Nkondjio *et al*., 2016)
ALDH6A1 (AGAP002499) Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial	4.28	0.035	Classified as environmental and oxidative stress proteins (Sonenshine *et al*., 2011)
Ca-P60A (AGAP006186) Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	5.99	0.039	Function impaired by oxidative stress (Lafleur, Stevens, & Lawrence, 2013; Park, Zhou, Lee, Lee, & Ozcan, 2010; X. Tong, Kono, & Evans-Molina, 2015; XiaoYong Tong, Evangelista, & Cohen, 2010)
CRT (AGAP004212) Calreticulin	1.64	0.017	Ca^{2+} homeostasis (Ihara, Kageyama, & Kondo, 2005; H. Liu *et al*., 1997; Ruddock & Molinari, 2006) and pro-apoptotic protein (Zhang *et al*., 2014)
EIF2S1 (AGAP011190) Eukaryotic translation initiation factor 2 subunit alpha	1.69	0.034	Conserve in eukaryotes, the phosphorylation form of this protein serve as a signal of cell survival by attenuating translation of mRNA (Back *et al.*, 2009; Harding, Zhang, & Ron, 1999; Knutsen *et al*., 2015)
ODC (AGAP011806) Ornithine decarboxylase	2.72	0.043	Upregulated after ivermectin-containing blood meals (Seaman *et al*., 2015)
SDHB (AGAP007309) Succinate dehydrogenase (ubiquinone) iron-sulfur subunit	4.23	0.045	Ferredoxin balance system

The data is summarised into four columns. In the first column, the name of the protein is described with its abbreviated form and accession number. The second column shows the

fold change in enrichment level for each of the described proteins. In the third column, *P*-value ($P < 0.05$) results of Student's t-test on the fold change in enrichment level are reported for each of the described proteins. The fourth column gives a brief summary of the function of the described protein, with any associated references

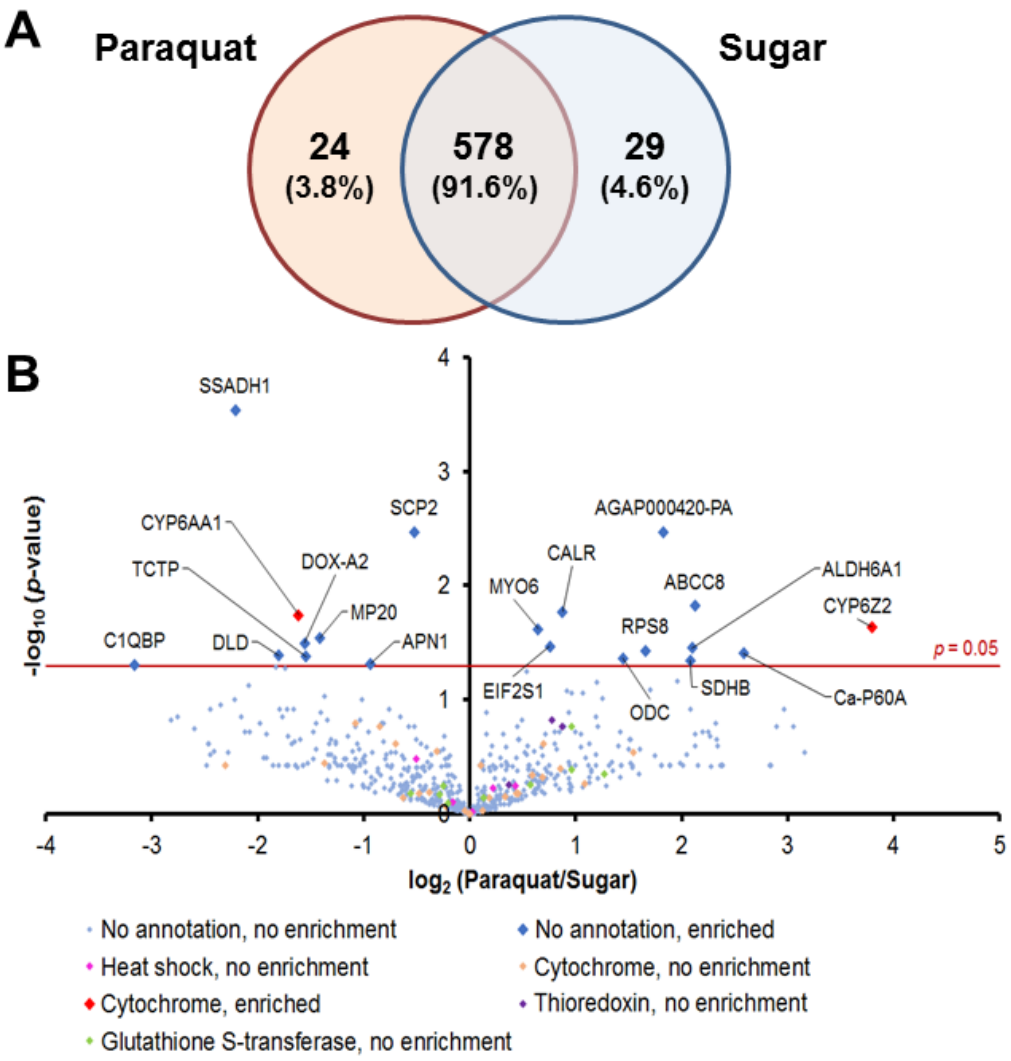

Figure 4. Comparative label-free quantitative proteomic analyses of the *An. gambiae* midgut responses to the ROS-inducer Paraquat (Pqt). (A) Global distribution of proteins in midguts under Pqt and sugar solution treatment. Midgut lysates from female *Anopheles gambiae* mosquito midguts treated with 1mM concentration of Pqt and sugar (10% sucrose) solution were subjected to a LC-MS/MS analysis to identify expressed proteins. Of the 631 proteins quantified, 3.8% were specific to Pqt-treated midguts, 4.6% partitioned to sugar-treated midguts, and 91.6% of the total proteins were conserved in both Pqt and sugar treated midguts. (B) Protein identification comparisons between treatment groups in *An. gambiae* midguts. Midgut lysates from female *An. gambiae* mosquito midguts treated with Pqt were subjected to a LC-MS/MS analysis to identify expressed proteins. Volcano plots of quantifiable protein comparisons in Pqt vs. sugar (10% sucrose) solution treated midguts. Significant fold change was calculated with Student's t-test with *P*-value ≤ 0.05. The red line indicates a *P*-value = 0.05. Annotation of significantly enriched proteins is shown

2.3.2 Antioxidant Proteins are not Involved in the Regulation of Pqt-induced Oxidative Stress in *An. gambiae* Midguts

A detailed examination of proteins identified as antioxidants previously described in section 2.3.1 was performed. Cytochrome P450 6Z2 (CYP6Z2; AGAP008212) was found enriched by 13.86-fold (*P*-value = 0.023) in Pqt-treated midguts and was the only CYP450 protein whose enrichment was statistically significant. We observed that CYP9J4 (AGAP012292) and CYP4H24 (AGAP013490), were also enriched (>1.5-fold), but their enrichment was not deemed statistically significant as shown in Appendix 3. Although thioredoxin reductase (TrxR; AGAP000565) and thioredoxin peroxidase 4 (TPx-4; AGAP011824) were found enriched (> 1.5-fold) in the Pqt-treated mosquitoes their enrichment was not deemed statistically significant as shown in Appendix 3. Glutathione S-transferase epsilon class 3 (GSTE3 AGAP009197), glutathione S-transferase theta class 1 (GSTT1; AGAP000761), and glutathione S-transferase delta class 11 (GSTD11; AGAP004378) were the only GSH-dependent proteins found enriched (>1.5-fold), but their enrichment was also not deemed statistically significant as shown in Appendix 3. We did not identify any significant enrichment in any of the identified HSPs.

2.3.3 Evidence of an Endoplasmic Reticulum (ER) Stress Response are Observed in Pqt-treated Midguts

We noted that ER stress regulating proteins were enriched in Pqt-treated midguts relative to sugar-treated midguts. This includes calreticulin (CRT; *P*-value = 0.017), eukaryotic translation initiation factor 2 subunit alpha (EIF2S1; *P*-value = 0.034) and calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type (Ca-P60A; *P*-value = 0.039), which had fold changes of 1.8-4, 1.69-, and 5.99-fold, respectively as shown in Fig. 4B and Table 3.

2.3.4 Proteins Involved in the Detoxification Process are Enriched in Pqt-treated Midguts

ATP-binding cassette transporter family C member 8 (ABCC8; AGAP008437) was found enriched by 4.37-fold (*P*-value = 0.015) in the Pqt-treated midguts as shown in Fig. 4B and Table 3. ATP-binding cassette (ABC) transporters belong to a superfamily of transport system members that efflux drugs, toxic, endo and xenobiotic compounds from cells (Jones & George, 2004; Ter Beek, Guskov, & Slotboom, 2014). Ornithine decarboxylase (ODC), was another detoxification

protein found enriched in the Pqt-treated midguts (2.72-fold, *P*-value = 0.043) as shown in Fig. 4B and Table 3. OCD catalyses the first-rate limiting step in polyamine synthesis and is upregulated in response to stress.

We also found mitochondrial methylmalonate-semialdehyde dehydrogenase ALDH6A1 (AGAP002499) enriched by 4.28-fold (*P*-value = 0.035) in the Pqt-treated midguts as shown in in Fig. 4B and Table 3. A contig from the male accessory gland/testis vas deferens (MAG/TVD) of tick, *Dermacentor variabilis*, was identified through alignment to be ALDH6A1 and known to protect against environmental stress (Sonenshine *et al.*, 2011). Succinate dehydrogenase (ubiquinone) iron-sulphur subunit (SDHB; AGAP0031) was found enriched in the Pqt-treated midguts (4.23-fold, *P*-value = 0.045) as shown in Fig. 4B and Table 3. SDHB is one of the 4 subunits of the mitochondrial succinate dehydrogenase complex (SDH), a key enzyme that links the tricarboxylic acid cycle (TCA) and electron transport chain (ETC) (Ackrell, 2000; Oyedotun & Lemire, 2004). SDHB transfers electrons from flavin adenosine dinucleotide ($FADH_2$) to ubiquinone (CoQ) in the inner mitochondrial membrane.

2.3.5 *P. falciparum* Ookinete Invasion of *An. gambiae* Midguts does not Upregulate Trx- and GSH-dependent Genes

P. berghei ookinete invasion of *Anopheles* mosquitoes is accompanied by an increased production in ROS/RNS (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004). Since the proteomic profiling suggested that several of the Trx- and GSH-dependent proteins were not upregulated following Pqt treatment we carried out qRT-PCR analysis to look closely at the regulation of nine Trx- and GSH- dependent genes in *An. gambiae* midguts following the ingestion of *P. falciparum* infected blood meal as shown in Table 4. The Trx- and GSH-dependent transcripts chosen were recently identified in various studies to be involved in the regulation of oxidative stress or in the detoxification of xenobiotic compounds. Multiple statistical analyses were carried out to identify transcripts that were significantly upregulated in Trx and GSH pathways 24 hours post infected blood meal ingestion as shown in Appendix 4. We found no significant upregulation of any of the Trx- and GSH-dependent transcripts investigated as shown in Table 4.

Table 4. A list of Trx and GSH-dependent transcripts evaluated following *P. falciparum* infected blood meal ingestion

Transcript/Accession ID	Function/Annotation	Response to *P. falciparum* blood meal ingestion.
Thioredoxin-1 (Trx-1; AGAP009584)	Dithiol-disulfide exchange reaction with GSSG to produce GSH (Kanzok *et al*., 2001)	None (*P*-value = 0.308 8)
Thioredoxin-2 (Trx-2; AGAP007201)	Antioxidative function as electron donor to TPx (Bauer, Kanzok, & Schirmer, 2002)	None (*P*-value = 0.730 9)
Thioredoxin reductase (TrxR; AGAP000565)	Key enzyme of the Trx system responsible for replenishing Trx-1 (Bauer *et al*., 2003)	None (*P*-value = 0.880 6)
Thioredoxin peroxidase-1 (TPx-1; AGAP000396)	Antioxidant enzyme that catalyzes peroxides (Bauer *et al*., 2002)	None (*P*-value = 0.797 6)
Atypical 2-Cys peroxiredoxin (Peroxiredoxin V; PrxV; AGAP001325)	Antioxidant enzyme that protects against ROS/RNS (Peterson & Luckhart, 2006)	None (*P*-value = 0.873 6)
Glutathione synthase (GS; AGAP000534)	Involved in the GSH biosynthesis pathway	None (*P*-value = 0.851 5)
Glutathione peroxidase (GPx; AGAP004247)	Antioxidant enzyme that catalyzes peroxides (Molina-Cruz *et al*., 2008)	None (*P*-value = 0.899 8)
Glutathione S-transferase delta class 1 (GSTD1; AGAP004164)	Implicated in insecticide resistance and detoxifies xenobiotic compounds (Prapanthadara, Hemingway, & Ketterman, 1993; Ranson *et al*., 2001)	None (*P*-value = 0.919 5)
Glutaredoxin-1 (Grx-1; AGAP011107)	Essential component of the GSH system (Giordano, Peluso, Rendina, Digilio, & Furia, 2003; Mercer & Burke, 2016)	None (*P*-value = 0.483 8)

The data is summarised into three columns. In the first column gives the name of the transcript with its abbreviated form and accession number. The second column gives a brief summary of the function of the described transcript with any associated references. In the third column, *P*-value (*P*< 0.05) results of multiple Student's t-test on the expression level for each of the described transcript relative to *Ag*RpL32

2.4 Discussion

We expected that Trx- and GSH-dependent proteins would be significantly enriched in mosquito midguts following Pqt treatment. However, we did not observe any significant enrichment in the antioxidant proteins identified in our proteomic data. The absence of enrichment of these proteins

might be due to either an early read-out time after Pqt treatment (8 hours) or a low Pqt concentration used (1 mM). The 8 hrs time frame was selected as it is critically relevant in the context of gamete-to-ookinete transition as well as in ookinete maturation. Furthermore, our intention was to determine whether the Pqt concentration of 1 mM elicits a Trx- and GSH-dependent protein response in the mosquito. In a study on acute Pqt toxicity in *Drosophila melanogaster*, concentrations between 10-40 mM were used with exposure time of 24 hours resulting in significant elevation in oxidative stress biomarkers and antioxidant enzymes (Hosamani & Muralidhara, 2013). The antioxidant enzymes investigated were not Trx or GSH dependent apart from GSTs. However, it clearly shows that the concentration of Pqt in our experiments might have been too low and the exposure time too short and could explain the lack of enrichment of Trx and GSH dependent antioxidant proteins.

Considering the difficulty in conducting LC-MS/MS analyses of a blood fed midgut at 24 hours (Dinglasan *et al.*, 2009), we utilized qRT-PCR analyses to perform a sensitive, separate assessment of the regulation of nine Trx-and GSH-dependent genes. *P. berghei* ookinetes appear to damage the mosquito midgut epithelium during midgut invasion and traversal, more so than *P. falciparum* ookinetes, due to the destructive nature of its invasion process. Invasion of epithelial cells induces the expression of nitric oxide synthase (NOS), which catalyses the formation of nitric oxide (NO) (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004; Peterson *et al.*, 2007), a highly reactive RNS (Brune, von Knethen, & Sandau, 1998). As Trx and GSH pathways are the primary cellular antioxidant and anti-nitrosative defence we expected to observe an increase in expression of Trx-and GSH-dependent genes following *Plasmodium* infected blood meal ingestion. The absence of significant upregulation of the investigated antioxidant genes could indicate that *P. falciparum* ookinete invasion of the *Anopheles* midgut does not cause significant oxidative stress when compared to that observed for *P. berghei*. This could be due to either or a combination of the following reasons. First, *P. falciparum* has co-evolved with *An. gambiae,* and causes less destruction of midgut epithelial cells during midgut invasion compared to *P. berghei* ookinetes (Han & Barillas-Mury, 2002; Han *et al.*, 2000). The reduced destruction is because the total number of ookinetes that leave the blood bolus and invade the midgut epithelium is less in *P. falciparum* compared to *P. berghei* evident in the reported divergent oocyst intensities for the two *Plasmodium* species (Gouagna *et al.*, 1998; Sinden & Billingsley, 2001; Whitten *et al.*, 2006).

Second, the antioxidant response in the midgut is already activated due to the blood meal content and the presence of *P. falciparum* doesn't result in any significant change (Smith *et al.*, 2014).

Additional experiments are required at earlier time-points, between 0 to 20 hrs, following *P. falciparum* infected blood meal ingestion to detect potential variations in the expression of Trx and GSH dependent genes. Furthermore, different tissues of the mosquito midgut experience increased levels of oxidative stress at different time points depending on the development stage reached by *Plasmodium* parasite. From blood meal ingestion to 15 hours, increased levels of ROS/RNS are present in the blood bolus due to vertebrate immune factors and digestion of haemoglobin in the blood meal (Graca-Souza *et al.*, 2006; Lensen *et al.*, 1997; Naotunne *et al.*, 1993; Peterson *et al.*, 2007). Between 15-24 hours increased levels of ROS/RNS are in the midgut epithelial cells due to the initiation of asynchronous invasion of *Plasmodium* ookinetes (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004) and because blood digestion is reaching its midway point towards completion (~48 hours post-blood feeding) (Graca-Souza *et al.*, 2006). Although *P. falciparum* ookinete invasion of the midgut is at its maximum at 24 hours (Graca-Souza *et al.*, 2006), it is entirely plausible that the levels of ROS/RNS in the midgut have already been reduced at this time-point due to either the advanced progress in digestion of haemoglobin and/or the small number of *P. falciparum* ookinetes involved in the invasion process.

The absence of a significant enrichment in antioxidant proteins following ingestion of a Pqt-laced sugar meal prompted us to closely examine the identified enriched proteins in the context of oxidative stress regulation. The mosquito midgut epithelial cells response to Pqt appears to be mediated primarily through the midgut ER-stress pathway indicated by our finding that detoxification and ER stress-related proteins were enriched in the Pqt-treated midguts. Oxidative stress is known to increase the amount of misfolded or unfolded protein in a cell. The unfolded protein response (UPR) is a cellular surveillance mechanism that identifies misfolded proteins in the ER and then either repairs them or redirects those that are misfolded beyond repair to the degradative pathway (Malhotra & Kaufman, 2007). Therefore, the UPR coordinates the ER protein folding demand and capacity with regards to the homeostatic status of a cell.

At 1 mM concentration of Pqt used in our experiments, misfolding and unfolding of proteins resulted prompting their immediate repair. The enriched levels of CRT, EIF2S1, and Ca-P60A is

a UPR induced due to ER stress in midgut epithelial cells caused by the increase in misfolded and unfolded proteins, and intended to re-establish protein homeostasis. CRT recognizes misfolded proteins and binds to them preventing them from leaving the ER, while EIF2S1 attenuates mRNA translation preventing influx of misfolded and damaged proteins into the ER (Bergeron, Brenner, Thomas, & Williams, 1994; Harding, Zhang, & Ron, 1999; Helenius, Trombetta, Hebert, & Simons, 1997). Enriched levels of CRT and EIF2S1 is evidence of protein repair on-going in midgut epithelial cells following Pqt treatment. Furthermore, oxidative stress is associated with decreased levels of calcium ion (Ca^{2+}) in the ER lumen, which further impairs the ER's ability to function properly. The ER has the highest concentration of Ca^{2+} in a cell compared to the cytosol or other cellular organelles (Miyawaki *et al.*, 1997). This concentration is regulated by transporter and channel molecules involved in the uptake or release of Ca^{2+} between the cytosol and ER lumen (MacLennan, Rice, & Green, 1997; Pozzan, Rizzuto, Volpe, & Meldolesi, 1994). Ca-P60A is a transporter in the ER membrane involved in the uptake of Ca^{2+} from the cytosol into the ER lumen. Enrichment in this protein is evidence of Ca^{2+} imbalance associated with ER stress and intended to increase the concentration of Ca^{2+} in the ER of midgut epithelial cells. CRT is also capable of binding to Ca^{2+} and therefore is involved in regulation of Ca^{2+} homeostasis within the ER (Baksh & Michalak, 1991; Ostwald & MacLennan, 1974). This shows the enriched CRT levels was also involved in ensuring there is high Ca^{2+} levels in the ER of midgut epithelial cells.

A limitation of the study is that direct measurement of the protein profile of *An. gambiae* midgut in response to a Pqt-laced blood meal was not carried out. It's suspected that the unpleasant strong odour of Pqt resulted in mosquito repellence and preventing DMFA from taking place. Therefore, the difference in the expected versus observed protein response profiles could be a result of the route of exposure to Pqt, i.e., ATP-saline solution versus blood meal. A completely different pathway for redox homeostasis was observed than previously described by others, which suggests that the mosquito midgut has in place at least two cellular response mechanisms that partition based on the manner by which ROS are induced in the tissue. The homeostatic regulation of ROS during mosquito blood feeding is well established (Billingsley & Rudin, 1992; Drexler *et al.*, 2014; Galun *et al.*, 1963), and it is likely that the mosquito can tolerate concentrations greater than 1 mM of Pqt due to the mitigating effects of several ROS mediation pathways that are active in concert in the midgut. One reasonable interpretation of these results in the context of our hypothesis and

proposed transmission-blocking drug paradigm is that in a real transmission scenario, complete with Pqt-like drugs, a blood meal, and malaria parasites, the mosquito will likely remain unaffected by high levels of ROS, whereas the parasite will succumb

2.5 Conclusion

The study herein has shown that the *An. gambiae* midgut response to Pqt-mediated oxidative stress initiates an ER stress pathway rather than inducing the canonical Trx- or GSH-dependent antioxidant proteins. Quite the opposite has been shown for Pqt-mediated oxidative stress in *Plasmodium* (in both asexual and sporogonic stages), where it is mainly regulated through Trx- and GSH-dependent proteins (Marva, Chevion, & Golenser, 1991; Turturice *et al.*, 2013). This difference in the regulated response to Pqt between *Anopheles* and *Plasmodium* could be harnessed as an intervention strategy against *Plasmodium* development in *Anopheles* midguts. Pqt at the concentration used in our experiments is known to be effective against the parasite (Turturice *et al.*, 2013), but is not immediately harmful to the mosquito. However, the potential utility of Pqt as a transmission-blocking compound was not explored in this present study. Pqt's high toxicity in different cell systems precludes it from such a translational application. Further studies are needed screen a suite of other Pqt-like drugs that can fulfil this role. Ideally, this screen would identify a shortlist of repurposed, druggable compounds and an appropriate, safe dose that is selectively toxic and deleterious to *Plasmodium*, but yet allowing *Anopheles* and human hosts to remain unaffected. The identified drug compound(s) can then be deployed to a field setting to evaluate for its transmission-blocking effect against locally circulating parasites.

CHAPTER THREE

Ribosomal/nucleolar Stress Induction Regulates tert-butyl hydroperoxide (tBHP) Mediated Oxidative Stress in *Anopheles gambiae* Midguts[2]

Brian B. Tarimo[1,2,3], Bernadette A. Hritzo[2], Henry Chun Hin Law[4,*],Dingyin Tao[2,*], Rebecca Pastrana-Mena[2], Stefan M. Kanzok[5], Joram J. Buza[1], and Rhoel R. Dinglasan[1,2,4,]

[1] Department of Health and Biomedical Sciences, Nelson Mandela-African Institution of Science and Technology, Tengeru, Arusha, 23302, Tanzania.

[2] W. Harry Feinstone Department of Molecular Microbiology & Immunology & the Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205, USA.

[3] Department of Environmental Health and Ecological Sciences, Ifakara Health Institute, Dar es Salaam, 14112, Tanzania

[4] Emerging Pathogens Institute, Department of Infectious Diseases & Immunology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611, USA

[5] Department of Biology, Loyola University Chicago, Chicago, Illinois 60660, USA

*contributed equally

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[2]*BMC Research Notes*, 2019, 12, 1; doi: 10.1186/s13104-019-4196-1, 1-8.

3.0 Abstract

A fundamental understanding of redox homeostasis in *An. gambiae* midgut cells under different oxidative conditions is missing. Such knowledge can aid in the development of new malaria transmission-blocking strategies aimed at disrupting natural homeostatic processes in the mosquito during *Plasmodium* parasite uptake (i.e. blood feeding). The aim of this study was to understand how the *Anopheles gambiae* midgut regulates oxidative stress to ROS, especially to a potent ROS-inducer such as tert-Butyl hydroperoxide (tBHP).

Initial studies using quantitative immunoblot indicated that the expression of the classical antioxidant protein *An. gambiae* thioredoxin-1 (*Ag*Trx-1) remained unchanged across challenges with different concentrations of tBHP suggesting that additional mechanisms to regulate ROS may be involved. We therefore conducted a global proteomic survey, which revealed that *An. gambiae* midguts under low (50μM) and high (200μM) tBHP concentrations were enriched in proteins indicative of ribosomal/nucleolar stress. Ribosomal stress is an inherent cellular response to an imbalance in ribosomal proteins (RPs) due to cellular stress such oxidative stress. The data presented herein suggest that ribosomal/nucleolar stress is the primary cellular response in *An. gambiae* midguts under tBHP challenge. Considering these results, harnessing the ribosomal stress response as a potential malaria transmission-blocking strategy is discussed.

3.1 Introduction

The sporogonic life cycle of *Plasmodium* in the mosquito is primarily extracellular and therefore, the parasites are directly and constantly exposed to reactive oxygen and nitrogen species, ROS and RNS, respectively. ROS and RNS are produced in part by mosquito's immune system in response to invasion of its midgut epithelial cells by the parasite (Han & Barillas-Mury, 2002; Han *et al.*, 2000; Kumar *et al.*, 2004), vertebrate immune factors present in the ingested blood (Lensen *et al.*, 1997; Naotunne *et al.*, 1993), and natural digestion of hemoglobin present in the ingested blood (Graca-Souza *et al.*, 2006; Peterson *et al.*, 2007). This highly oxidative environment, results in a population bottleneck for the parasite during development in the mosquito vector (Sinden, 1999; Sinden & Billingsley, 2001).

To maintain redox homeostasis, organisms possess the Thioredoxin (Trx) and Glutathione (GSH) systems as prominent mechanisms against oxidative stress. The GSH system involves the tripeptide, GSH, and in its antioxidant activity, GSH is converted to glutathione disulfide (GSSG) (Schafer & Buettner, 2001). This oxidized form is converted back to the reduced form by the nicotinamide adenine dinucleotide phosphate-dependent flavoenzyme glutathione reductase (NADPH-GR) (Schirmer, Bauer, & Becker, 2002). The Trx system is comprised of thioredoxins (Trxs), and thioredoxin reductase (TrxR) (Arner & Holmgren, 2000; Holmgren, 1985). Trxs are small (12 kDa) and ubiquitous thiol proteins. Trxs cycle between a disulfide and a dithiol form, catalyzed by TrxR (Mustacich & Powis, 2000). *An. gambiae* and *An. stephensi* mosquitoes regulate Trx- and GSH-dependent antioxidants to protect midgut epithelial cells against ROS and RNS (Molina-Cruz *et al.*, 2008; Peterson & Luckhart, 2006). Notably, *Anopheles* mosquitoes and other dipterans lack the flavoenzyme GR of the GSH pathway and utilize the Trx system to recycle GSSG to GSH as shown in Fig.5A (Kanzok *et al.*, 2001).

Little is known about Trx at molecular level in *Anopheles* mosquitoes despite its importance in redox homeostasis in midgut epithelial cells under different oxidative conditions. In this report, we used an *ex vivo* midgut culture model to first investigate *An. gambiae* Thioredoxin-1 (*Ag*Trx-1) protein expression in response to ROS challenge. Contrary to our expectations, we did not observe an upregulation in *Ag*Trx-1 across various concentrations of a ROS challenge. We then expanded our exploration to other redox homeostasis pathways by capturing the global midgut proteomic expression profile, with the aim of understanding organ-level regulation following exposure to the ROS- inducer, tert-Butyl hydroperoxide (tBHP).

3.2 Materials and Methods

3.2.1. *Ex vivo* Midgut Organ Culture Media

Ex-vivo studies were done using *An. gambiae* midguts maintained in a culture media that contained 3895 µL of RPMI 1640 without L-glutamine or phenol red (Quality Biologicals), 1 mL of heat-inactivated fetal bovine serum (Sigma-Aldrich), 100 µL of 10000U: 10mg/ml PenStrep (Cellgro) to a final concentration of 100U; 10 µg/mL, and 5 µL of 250 µg/ml amphotericin. Due to its sensitivity to light, Amphotericin was added to the media last and the final volume was immediately covered by foil to reduce light exposure. The media was equally split into 1.5 ml

tubes each containing 1 ml and stored at -20ºC until usage.

3.2.2 Mosquito Rearing, Experimental Treatments, and ROS Induction Assays

An. gambiae (KEELE strain) mosquitoes were used for all the experimental treatments. These mosquitoes were maintained in an insectary at the Johns Hopkins Malaria Research Institute (JHMRI), at 26°C and 70% humidity with 12 hours light: dark cycles and supplemented with 10% sucrose solution.

ROS induction (oxidative stress) assays were performed using an *ex vivo* system. In these assays, 50 *An. gambiae* female mosquitoes (4-7 days old) were dissected and individual midguts collected in 1x PBS on ice. In each of the treatment groups the 50 midguts were split into two sub-groups: one containing five midguts (for SDS-PAGE and immunoblot analysis) and the other containing 45 midguts (for LC-MS/MS analysis). Midguts in both groups were submerged in 200 µL containing their respective concentrations of tBHP (Alfa Aesar, Haverhill, MA) in organ culture media and left for 15 minutes at room temperature away from light exposure due to sensitivity of amphotericin to light. Treatment media was then removed from the midgut samples followed by stringent wash of the samples with 1x PBS and storage at -20ºC and -80ºC until further SDS-PAGE/immunoblot and LC-MS/MS analyses, respectively. The treatments groups were: control (organ culture media only), 50 µM, 125 µM, 200 µM, 250 µM, 500 µM, and 1 mM. These experiments were replicated three times using independent biological cohorts of mosquitoes.

3.2.3 SDS-PAGE and Immunoblot Analysis

Midgut lysates (5 midgut equivalents) of experimental groups were thawed on ice to RT and then heated at 95ºC for 10 minutes. Approximately 15 µL of midgut lysates (~2.5 midguts per well) were loaded into a 4-20% Tris-glycine gel. Proteins were separated under reducing conditions at a constant 100V and then transferred to a nitrocellulose membrane. The membranes were blocked in a solution of Odyssey blocking buffer (Li-COR Biosciences, Lincoln, NE) then probed first with rabbit anti-*Ag*Trx-1 antiserum (obtained from S. Kanzok, Loyola University) (44 mg/ml) and second with rabbit anti-*An*APN-1 (RCB-A terminal) mAb used as a loading control diluted 1:200 and 1:1000 in a solution of Odyssey blocking buffer, respectively.

Mouse anti-rabbit IgG secondary antibodies labelled with IRDye 680RD (Li-COR Biosciences, Lincoln, NE) diluted 1: 20,000 in Odyssey blocking buffer was used to detect anti-*Ag*Trx-1 and anti-*An*APN-1 separately. Quantitative immunoblotting was performed by determining *Ag*Trx-1 expression levels relative to *An. gambiae* midgut *An*APN-1 (AGAP004809) using the Li-COR analytical software (version 3.0). All immunoblots were imaged using the Li-COR Odyssey infrared imaging system (Li-COR) and signal intensity was calculated in K counts mm^2.

3.2.4 Extraction, Solubilization, and Digestion of Proteins

Prior to LC-MS/MS analysis, treatment groups (45 midguts/samples) were processed for proteins. Total protein lysate was prepared by lysing the midgut samples with 45 µL of SDST-lysis buffer (4% SDS (w/v), 100 mM Tris/HCl, 0.1 M DTT pH 7.6) and boiled at 95^{o}C for 5 minutes. 30 µL of the protein lysates was taken for protein digestion according to the Filter-Aided Sample Preparation (FASP) protocol previously described by Wiśniewski et al. using a 10 kDa molecular weight cutoff filter (EMD Millipore, Billerica, MA) as previously described (Tao, King*, et al.*, 2014; Tweedell *et al.*, 2015). Acidified tryptic peptides from FASP approaches were desalted using an HPLC column and their concentrations determined by BCA as previously described (Tao, King*, et al.*, 2014; Tweedell *et al.*, 2015).

3.2.5 Online 2D LC-MS/MS

Peptide products desalted and digested by the FASP protocol were dissolved in loading buffer (97.9% water, 2% ACN, and 0.1% formic acid (FA)) and ~ 20 µg was injected to our previously constructed online 2D HPLC-MS/MS system, using the exact method as previously described (Tao, King*, et al.*, 2014; Tao, Ubaida-Mohien*, et al.*, 2014; Tweedell *et al.*, 2015).

3.2.6 Database Searching and Label Free Quantification

All the LC-MS/MS raw data were converted to Mascot generic format (.mgf) by Agilent MassHunter Qualitative Analysis B.04.00. The data acquired was used to search the VectorBase*Anopheles gambiae* protein FASTA sequence database (VectorBase, http://www.vectorbase.org, *Anopheles gambiae* PEST, AgamP4.2.) for peptide sequence alignments. The search engine used for the search was MASCOT version 2.5 with the following

parameters: precursor ion mass tolerance of 50 ppm, fragment ion mass tolerance of 0.2 Da, carbamidomethylation of cysteine and oxidation of methionine residues set as fixed and variable modifications respectively. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavage sites were allowed for tryptic digestion.

The MASCOT search results were exported as .DAT format and then imported into the Scaffold software (version 4.4.5, Proteome Software) for curation, label-free quantification, analysis, and visualization. Overall, protein false discovery rates of less than 1% and peptide false discovery rates of less than 1% were obtained with Scaffold filters, and each protein had ≥ 2 unique peptides. Identified proteins were clustered to remove redundancy. Proteins were clustered together if there was a peptide identification shared between them, because this indicates substantial sequence similarity, and the protein with the greatest number of peptides identified was considered the unique protein identification from that group. The data analysis pipeline meets all MIAPE standards (Taylor *et al.*, 2007) and the detailed peptide data can be found in Appendix 5.

3.2.7 Statistical Analyses

For quantitative immunoblot analyses, relative expression of *Ag*Trx-1 was calculated using a one-way multi-variable analysis of variance (ANOVA). Proteins quantification to identify enriched proteins between treatment groups was carried out by Student's t-test. All statistical analyses were carried out using the software GraphPad Prism (version 6.0e). The *P*-values of ≤0.05 were considered statistically significant. All experimental reactions were carried using at least three independent replicate samples.

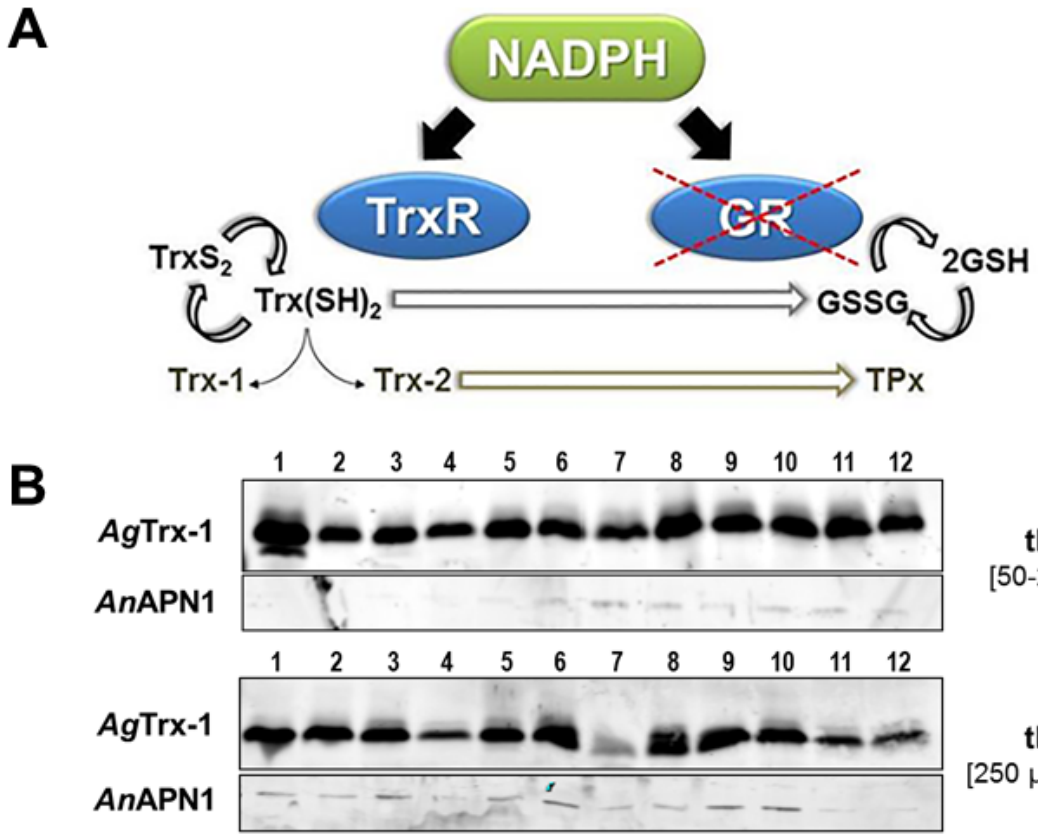
3.3 Results

3.3.1 *Ag*Trx-1 Protein Expression Levels

The lack of GR and instead utilization of the Trx system for GSSH recycling underscores the importance of Trx system in an antioxidant response in dipterans. As Trx-1 is one of main the components of the Trx system, it therefore must play an essential role in this antioxidant response (Bauer *et al.*, 2003; Kanzok *et al.*, 2001). Quantitative immunoblot analysis was carried out for

*Ag*Trx-1 protein expression in midguts that were previously exposed to the ROS producing agent tBHP.

A distinct clear band was observed at M_r of ~12 kDa across all the treatment groups and biological replicates, which corresponds to the M_r of *Ag*Trx-1 as shown in Fig. 5B. Protein doublets observed in the Western blot may reflect multimer of *Ag*Trx-1 or another cellular target of the antiserum used (Lee, Kim, & Lee, 2013). *Ag*Trx-1 protein expression level (K-counts) measured as relative expression to the loading control *Anopheline* aminopeptidase-1 (*An*APN1), did not exhibit any significant difference in *An. gambiae* midguts incubated with different concentrations of tBHP when compared to untreated controls (*P*-value = 0.169 5) as shown in the lower panel of Fig. 5B and Appendix 6. There was no significant change in the *Ag*Trx-1 expression when the tBHP concentration was increased from 250 μM to 1 mM (*P*-value = 0.452 5) as shown in the lower panel of Fig. 5B and Appendix 6.



Biological Replicate	I.I Kcounts: tBHP [50 -200 µM]											
	*Ag*Trx-1 (12 KDa)				*An*APN-1 (135 KDa)				Ratio-(*Ag*Trx-1/*An*APN-1)			
	Control	50 µM	125 µM	200 µM	Control	50 µM	125 µM	200 µM	Control	50 µM	125 µM	200 µM
1	1.00	0.34	0.36	0.24	1.00	26.19	0.22	2.09	1.00	0.01	1.66	0.11
2	1.00	0.67	0.40	1.05	1.00	6.70	6.58	5.00	1.00	0.10	0.06	0.21
3	1.00	0.92	1.15	0.63	1.00	1.25	2.01	2.59	1.00	0.74	0.57	0.24

Biological Replicate	I.I Kcounts: tBHP [250 µM-1mM]											
	*Ag*Trx-1 (12 KDa)				*An*APN-1 (135 KDa)				Ratio-(*Ag*Trx-1/*An*APN-1)			
	Control	250 µM	500 µM	1 mM	Control	250 µM	500 µM	1 mM	Control	250 µM	500 µM	1 mM
1	1.00	0.92	0.68	0.37	1.00	0.90	1.89	0.49	1.00	1.02	0.36	0.76
2	1.00	1.29	0.34	0.97	1.00	1.55	0.45	0.71	1.00	0.84	0.76	1.38
3	1.00	0.84	0.45	0.50	1.00	1.24	0.09	0.01	1.00	0.67	5.28	60.43

Figure 5. **Redox regulation in *Anopheles* mosquitoes and *Ag*Trx-1 expression levels under different oxidative stress conditions (A)Interactions between the Trx and GSH systems in redox homeostasis in *Anopheles* mosquitoes. GR is absent in the GSH system of *Anopheles* mosquitoes and is crossed out to convey this point. Therefore, *Anopheles* mosquitoes and other dipterans recycle glutathione disulphide through a dithiol-disulphide exchange with reduced thioredoxin. Reduced thioredoxin is recycled from its oxidized form by thioredoxin reductase thus maintaining sufficient levels of itself for subsequent glutathione disulphide recycling. GSSG = glutathione disulfide, GSH = glutathione, glutathione reductase = GR, NADPH = reduced nicotinamide dinucleotide phosphate, TrxR = thioredoxin**

reductase, $TrxS_2$ = thioredoxin disulfide, $Trx(SH)_2$ = reduced thioredoxin, Trx-1= thioredoxin-1, Trx-2 = thioredoxin-2, and TPx = thioredoxin peroxidase.

(B) *Ag*Trx-1 protein expression in *An. gambiae* midgut epithelial cells. Immunoblot with α-*Ag*Trx-1 antiserum of female *An. gambiae* midgut lysates obtained by incubation of midguts (5 per sample) under varied concentrations of tBHP in *ex vivo* organ culture media for 15 minutes. Female *An. gambiae* midgut lysates treated with *ex vivo* organ culture media (lanes 1, 5, and 9), 50 µM t-BHP (lanes 2, 6, and 10), 125 µM tBHP (lanes 3, 7, and 11), and 200 µM tBHP (lanes 4, 8, and 12) for the upper panel. Immunoblot with α-*Ag*Trx-1 antiserum of female *An. gambiae* midgut lysates obtained by incubation of midguts (5 per sample) under varied concentrations of tBHP in *ex vivo* organ culture media for 15 minutes. Female *An. gambiae* midgut lysates treated with *ex vivo* organ culture media (lanes 1, 5, and 9), 250 µM tBHP (lanes 2, 6, and 10), 500 µM tBHP (lanes 3, 7, and 11), and 1 mM tBHP (lane 5, 9, and 13) for the lower panel. Lanes 1-4 (biological replicate 1), lanes 5-8 (biological replicate 2), lanes 9-12 (biological replicate 3). *An*APN1 (~135 kDa), as a loading control is shown below each treatment column. *P*-values ($P \leq 0.05$) were calculated by the parametric one-way Analysis of Variance (ANOVA) followed by Bonferroni's correction.

3.3.2 Global Proteomics Profiles

The absence of significant regulation in *Ag*Trx-1 expression level prompted us to expand our investigation into the antioxidant response. To this end, the global proteomic profile in midgut epithelial cells in response to varying tBHP challenges was analysed.

Three experimental groups were generated: (1) untreated *ex vivo* organ culture only (control), and two tBHP-treated groups exposed to (2) 50 μM (low), and (3) 200 μM (high). A total of 1,567 quantifiable proteins were identified using the VectorBase *An. gambiae* protein FASTA sequence database (http://www.vectorbase.org, *Anopheles gambiae* PEST, AgamP4.2) (Giraldo-Calderón et al., 2015) with MASCOT version 2.5 used as the search engine as shown in Appendix7-9. The three experimental groups shared 1,195 of the proteins (76.3%), while 83 proteins (5.3%) were found only in the control group, 49 proteins (3.13%) were found only in the low tBHP group, and 5 proteins (0.32%) were found only in the high tBHP group as shown in Fig. 6A. Furthermore, proteomic profiles of the different experimental groups identified 1,356 proteins (86.5.7%) shared between the control and low tBHP groups as shown in Fig. 6B, 1233 proteins (78.6%) shared between control and high tBHP groups as shown in Fig. 6C, and 1,231 proteins (78.6%) shared between low and high tBHP groups as shown in Fig. 6D.

Antioxidant proteins, which fell into the following groups: heat shock proteins (HSP), cytochromes (CYT), Trx-dependent, and GSH-dependent proteins were identified as shown in Appendix 7-9. However, only 20 antioxidant proteins out of total 1567 proteins (1.27%) were significantly enriched suggesting that the midgut cells were not initiating an antioxidant response. It was then hypothesized that they may be responding through other mechanisms to tBHP generated ROS.

Further analysis of the proteomic profiles of the experimental groups based on their spectral count values ($P \leq 0.05$; Student's t-test) identified additional 89 proteins that were differentially expressed between the groups as shown in Fig. 7A-C and Appendix 7-9. Out of these, we found 10 proteins that were enriched (highly expressed) in the low treatment group as shown in Fig. 7A and Appendix 7; 18 proteins enriched in the high treatment group as shown in Fig. 7B, C and Appendix 8, 9. Evaluation of the respective annotated functions of the enriched proteins revealed

cellular roles in either ribosome biogenesis or in cellular trafficking as part of the lysosomal exocytosis machinery. In this report, a focus on proteins with annotated functions in ribosome biogenesis is assessed due to their close interlink to cellular stress response, including oxidative stress, and the potential application of the ribosome biogenesis machinery as a transmission blocking intervention for Malaria. Details about the other proteins involved in cellular trafficking and lysosomal exocytosis are shown below in Table 5.

Table 5. Proteins enriched with function in either posttranslational modification, cellular transport, or energy metabolism

Protein description	tBHP group	Fold change	*P*-value	Function	Signal peptide (Y/N)
SCPEP1 (AGAP011442) Serine carboxypeptidase 1	Low	5.00	0.000 1	Posttranslational processing(Pshezhetsky & Hinek, 2009).	Y
VHASFD(AGAP009486) V-type transporting ATPase 54 kDa subunit	Low	2.30	0.001 4	Electrogenic pump(Forgac, 2007).	N
MT-ATP6 (AGAP005134) F-type H^+ transporting ATPase	Low	1.40	0.01	Energy metabolism(Yoshida, Muneyuki, & Hisabori, 2001).	Y (mTP)
ISCS (AGAP009094) cysteine desulfurase	Low	3.30	0.011	Biosynthesis of iron-sulfur (Fe-S) clusters(Braymer & Lill, 2017).	Y (mTP)
PMPCB (AGAP005558) peptidase (mitochondrial processing) beta	Low	1.5	0.013	Catalyses the cleavage of nascent/pre-proteins newly imported into the mitochondria(Mossmann, Meisinger, & Vogtle, 2012).	Y (mTP)
NDUFV1 (AGAP010039) NADH dehydrogenase [ubiquinone] flavoprotein1, mitochondrial	Low	3.20	0.014	Energy metabolism (Yagi & Matsuno-Yagi, 2003).	Y (mTP)
GLEANR (AGAP008861) Female reproductive tract protease	Low	2.2	0.018	Posttranslational processing(Pshezhetsky & Hinek, 2009).	Y
EHD1 (AGAP004593) Eps 15 homology domain-containing protein 1	Low	1.5	0.026	Cellular transport of compounds (Kieken, Jovic, Naslavsky, Caplan, & Sorgen, 2007).	N
SEC11 (AGAP003069) Signal peptidase, ER-type	Low	8.2	0.048	Posttranslational processing(Pshezhetsky & Hinek, 2009).	N
CLIC (AGAP000943) Chloride intracellular channel	High	3.1	0.006 1	Chloride ion transport across membranes (Littler *et al.*, 2010).	N
VHASFD (AGAP009486) V-type transporting ATPase 54 kDa subunit	High	2.80	0.006 1	Electrogenic pump (Nishi & Forgac, 2002).	N
SCPEP1 (AGAP011442) Serine carboxypeptidase 1	High	4.70	0.013	Posttranslational processing (Pshezhetsky & Hinek, 2009).	Y

ANPEP (AGAP012745) Alanyl aminopeptidase	High	1.9	0.019	Peptidase activity; posttranslational modification(Bauvois & Dauzonne, 2006; Zhang & Xu, 2008).	N
SRPRA (AGAP010894) Signal recognition particle receptor alpha	High	1.7	0.026	Cellular transport (Gilmore, Blobel, & Walter, 1982; Gilmore, Walter, & Blobel, 1982).	Y
PSMC4 (AGAP003008) 26Sproteosome regulatory subunit T3	High	1.7	0026	Protein homeostasis (Hiller, Finger, Schweiger, & Wolf, 1996).	N
APN3 (AGAP013255) Aminopeptidase N3	High	2.1	0.028	Peptidase activity; posttranslational modification (Luan & Xu, 2007).	Y
SLC22 (AGAP004309) Solute carrier family 22	High	1.8	0.031	Cellular transport (Koepsell, 2013).	N
ATP6V1D (AGAP010298) V-type H^+ transporting ATPase subunit D	High	2.6	0.045	Electrogenic pump (Dow, 1999).	Y (mTP)
NUP210 (AGAP006280) Nuclear pore complex protein glycoprotein 210	High	2.0	0.000 6	Cellular transport.	N

In the first column, the name of the protein is described with its abbreviated form and accession number. The second column indicates the treatment group. The third column shows the fold change in enrichment level for each of the described proteins. The fourth column shows the *P*-value (P ≤0.05) results of Student's t-test comparisons on the fold change in enrichment level. Only P-values that are significant are provided. The fifth column describes the function of the described protein and associated references and the last column denotes whether the protein contains a signal peptide. Y = yes, N = no, and mTP = mitochondrial Target Peptide

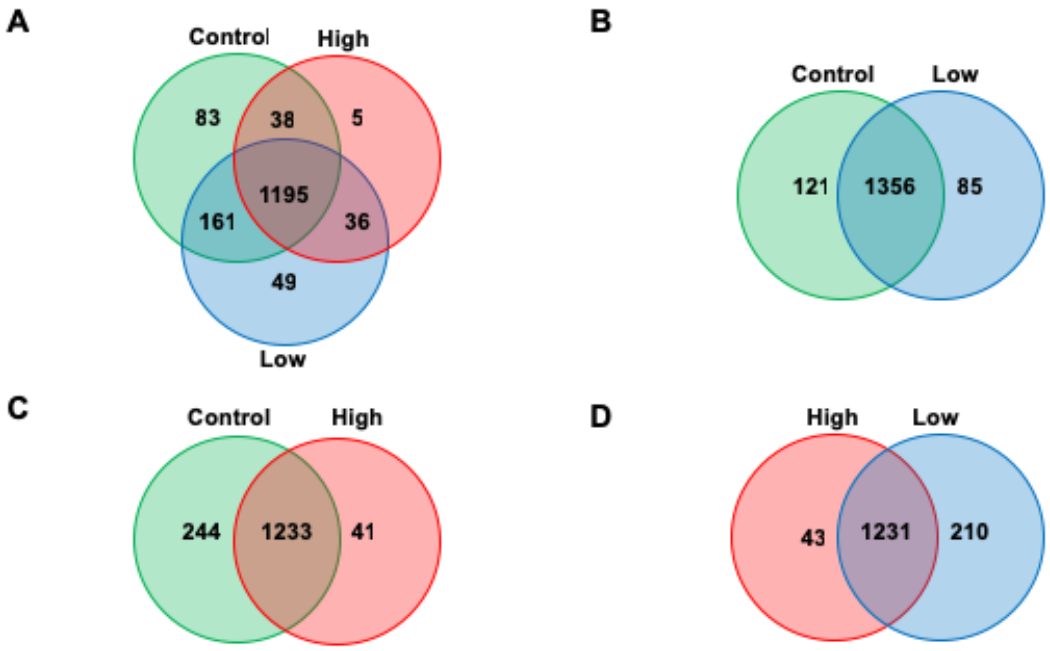


Figure 6. **Protein identification comparisons between treatment groups in *An. gambiae* midguts. Midgut lysates from female *An. gambiae* mosquito midguts treated with varied concentrations of tBHP were subjected to a LC-MS/MS analysis to identify expressed proteins. (A) Proteins identified in all three experimental groups of control (untreated *ex vivo* organ culture media only), low (50 μM tBHP), and high (μM tBHP). (B) Proteins identified in control and low tBHP groups. (C) Proteins identified incontrol and high tBHP groups. (D) Proteins identified in high andlow tBHP groups**

3.3.3 Alteration in Ribosomal Proteins Profile

Differential expression of several RPs was observed in *An. gambiae* midgut epithelial cells that were exposed to different treatments of tBHP as shown in Fig. 7A-C and Table 6-7. Enrichment of 60S ribosomal protein L7 (RpL7) by 1.9-fold was identified in the low tBHP group as shown in Fig. 7(A) and Table 6. In this group, 60S L13 (RpL13) and L22 (RpL22) ribosomal proteins by were underrepresented by 1.5- and 1.33- fold, respectively as shown in Fig. 7A and Table 7.

Enrichment of 60S ribosomal protein L10a (RPL10A), 40S ribosomal protein S15 (RPS15), 40S ribosomal protein S3a (RPS3A), 60S ribosomal protein L19 (RpL19), and a putative RNA binding protein enriched by 3.0-, 5.30-, 1.55-, 2.31-, and 2.72-fold, respectively, was identified in the high tBHP group as shown in Fig. 7B, C and Table 6. In contrast, seven RPs showed reduced expression in the high treatment group: 60S ribosomal protein LP1 (RpLP1), 40S ribosomal protein S26 (RpS26), 60S ribosomal protein L32 (RpL32), 60S ribosomal protein L13a, (RpL13a), 60S ribosomal protein L11 (RpL11), 40S ribosomal protein S14 (RpS14), and 40S ribosomal protein S18 (RpS18) with 2.86-, 3.85-, 2.63-, 1.81-, 16.67, 2.5-, and 3.3-fold, respectively as shown in Fig. 7B, C and Table 7. Taken together these results are an indication that a challenge to mosquito epithelial cells with tBHP induces an altered expression of RPs.

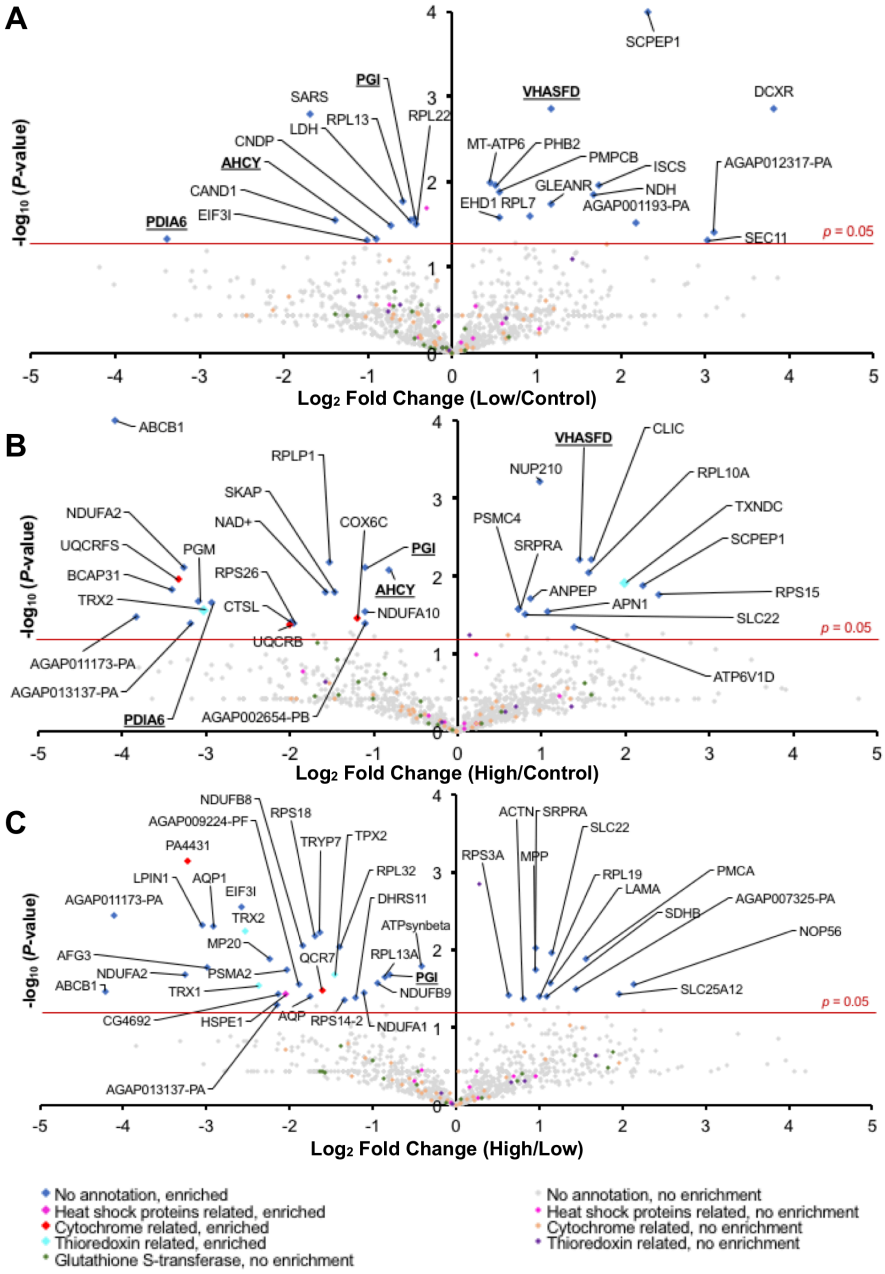


Figure 7. **Female *An. gambiae* mosquito midguts treated with various concentrations of tBHP were subjected to a LC-MS/MS analysis to identify expressed proteins. Volcano plots of quantifiable protein comparisons. (A) Low (50µM tBHP) versus control (untreated *ex vivo* organ culture only) experimental groups (B) High (200µM tBHP) versus control (untreated *ex vivo* organ culture only) experimental groups. (C) High (200µM tBHP) versus low (50µM tBHP) experimental groups. Significant fold change was calculated using Student's t-test with P-value ≤ 0.05. Annotations of significantly enriched proteins are indicated**

Table 6. Overexpressed (enriched) RPs following treatment with low and high tBHP concentrations

Protein description	tBHP group	Fold change	*P*-value	Reference
RpL7 (AGAP008916) 60S ribosomal protein L7	Low	1.9	0.025	Protein translation machinery(Fromont-Racine, Senger, Saveanu, & Fasiolo, 2003).
RpL10A (AGAP011298) 60S ribosomal protein L10a	High	3.0	0.008 8	Protein translation machinery (Koga *et al.*, 2003).
RpS15 (AGAP001274) 40S ribosomal protein S15	High	5.30	0.017	
RpS3A (AGAP003532) 40S ribosomal protein S3a	High	1.55	0.038	Protein translation machinery(Wang, Pakpour, *et al.*, 2015).
RpL19 (AGAP004422) 60S ribosomal protein L19	High	2.31	0.04	Protein translation machinery(Marygold *et al.*, 2007).
AGAP007325 Putative RNA binding protein.	High	2.7	0.032	None

In the first column, the protein is described in its abbreviated form. In the second column, the treatment group where the protein was enriched is mentioned. The third group shows the fold change in enrichment level for each of the described protein. In the fourth column, *P*-value (P≤0.05) results of Student's t-test on the fold change in enrichment level are reported for each of the described protein. Only *P*-values that are significant are given

Table 7. Under-expressed RPs following treatment with low and high tBHP concentrations

Protein description	tBHP group	Fold change	*P*-value	Reference
RpL13 (AGAP001805) 60S ribosomal protein L13	Low	1.50	0.017	Protein translation machinery (Wang, Pakpour*, et al.*, 2015).
RpL22 (AGAP005046**)** 60S ribosomal protein L22	Low	1.33	0.031	Protein translation machinery (Marygold *et al.*, 2007).
RpLP1 (AGAP007740) 60S ribosomal protein LP1	High	2.86	0.006 7	Protein translation machinery (Marygold *et al.*, 2007).
RpS26 (AGAP012100) 40S ribosomal protein S26	High	3.85	0.041	Protein translation machinery (Marygold *et al.*, 2007).
RpL32 (AGAP002122) 60S ribosomal protein L32	High	2.63	0.009 2	Protein translation machinery (Marygold *et al.*, 2007).
RpL13A (AGAP010257) 60S ribosomal protein L13a	High	1.81	0.022	Protein translation machinery (Marygold *et al.*, 2007).
RpL11 (AGAP011173) 60S ribosomal protein L11	High	16.67	0.003 6	Protein translation machinery (Fromont-Racine *et al.*, 2003).
RpS14 (AGAP002346) 40s ribosomal protein S14	High	2.5	0.044	Protein translation machinery (Marygold *et al.*, 2007).
RpS18 (AGAP028693) 40S ribosomal protein S18	High	3.3	0.006 5	Protein translation machinery (Marygold *et al.*, 2007).

In the first column, the protein is described in its abbreviated form. In the second column, the treatment group where the protein was enriched is mentioned. The third group shows the fold change in enrichment level for each of the described protein. In the fourth column, *P*-value (P≤0.05) results of Student's t-test on the fold change in enrichment level are reported for each of the described protein. Only *P*-values that are significant are given

3.4 Discussion

An. gambiae midgut epithelial cells are under frequent oxidative stress either from the digestion of ingestion blood meal or mosquito's innate immunity against the invading *Plasmodium* parasite. In such cases, the epithelial cells need to have their antioxidant defences highly expressed against the oxidative attack (Molina-Cruz *et al.*, 2008; Peterson & Luckhart, 2006). Expression of *Ag*Trx-1, a key player in the cellular redox network, was observed to remain similar under different conditions of oxidative stress resulting from tBHP exposure. A plausible explanation for this is that the *Ag*Trx-1 baseline expression could already be high in midgut epithelial cells most likely due to its other cellular roles in addition to the antioxidant system, so no differential expression was observed (Arner & Holmgren, 2000; Bouvier, Sapin, Bonnard-Gougeon, & Marceau, 2010; Holmgren, 1985; Yin, Xu, & Porter, 2011). Considering this initial observation, the midgut proteomic profiles was further examined to identify oxidative stress proteins that are differentially expressed following tBHP treatment.

Examination of the midgut proteomic profile for redox-related proteins such as HSPs, CYTs, Trx-related, and GSH-related revealed a significantly small proportion of these proteins are enriched following tBHP treatment. This suggests that the midgut epithelial cells are responding to the oxidative stress following tBHP treatment through other non-redox related mechanisms.

A modified expression in several non-redox proteins, most notably an imbalance in the levels of RPs, was observed following treatment with tBHP. In an unstressed cell equimolar amounts of RPs are generated during ribosome biogenesis (James, Wang, Raje, Rosby, & DiMario, 2014). A change in the RP levels due to cellular stresses such as hypoxia, heat shock, ionizing radiation (IR), oxidative stress, and certain drugs could disrupt the balance and thus reduce the number of functional ribosomes impairing protein synthesis (Boisvert, Van Koningsbruggen, Navascues, & Lamond, 2007). In response, the cell induces the ribosomal/nucleolar stress response to mitigate the loss in functional ribosomes (Boisvert *et al.*, 2007). In eukaryotic cells, the most common inducer of ribosomal stress response is the transactivation and accumulation of the tumour suppressor p53 caused by the inhibition of the E3 ubiquitin ligase activity of mouse double minute 2 (MDM2) homolog on p53 (Michael & Oren, 2003). RPs can bind to the MDM2 homolog, inhibiting its E3 ubiquitin ligase activity on p53 which leads to activation of p53 (Daftuar, Zhu,

Jacq, & Prives, 2013). However, certain invertebrates including the dipteran insect *Drosophila* lack a discernible MDM2 homolog (Lane *et al.*, 2010). Not surprisingly, *A. gambiae* also a dipteran, also lacks a discernible MDM2 homolog evident from a thorough BLAST search results of *An. gambiae* genome through the VectorBase (http://www.vectorbase.org, *Anopheles gambiae* PEST, AgamP4.2) database (data not shown), which suggests that induction of ribosomal stress response uses an alternative mechanism (James *et al.*, 2014; Olausson, Nister, & Lindstrom, 2012).

An imbalance in RP levels has been shown to be associated with the "*Minute*" phenotype in *Drosophila* (Marygold *et al.*, 2007). The "*Minute*" phenotype is associated with increased expression of JNK signaling (McNamee & Brodsky, 2009), which has been linked to a wide range of biological processes, including stress response and immunity (Jasper *et al.*, 2001; Kockel, Homsy, & Bohmann, 2001). Interestingly, *Drosophila* homologs of the differentially expressed RPs in our *Anopheles* proteomic data have either been confirmed or predicted to be encoded by a "Minute" locus in the fruit fly (Marygold *et al.*, 2007). Therefore, it is postulate that an overall imbalance in the levels of RPs following tBHP treatment of *An. gambiae* midguts has the same consequence of increasing the expression of JNK signaling as seen in *Drosophila*. Increased expression in JNK signaling increases tolerance to oxidative stress in *Drosophila* as well as in *A. gambiae* (Jaramillo-Gutierrez, Molina-Cruz, Kumar, & Barillas-Mury, 2010). Overexpression of the upstream member JNKK (Hemipterous; Hep) or down regulation of the downstream target *puckered* (puc) in *Drosophila* results in flies that exhibit an increased tolerance to oxidative (Wang, Bohmann, & Jasper, 2003). Interestingly, in *An. gambiae,* JNK signaling regulates the gene oxidation resistance 1 (OXR1), which in turn regulates the expression of antioxidant enzymes such as Catalase and GPx (Jaramillo-Gutierrez *et al.*, 2010).

3.5 Conclusion

The data herein suggest that various inducers of ROS trigger a non-*Ag*Trx-1 pathway, that is likely dependent on the potency of the ROS-inducer. The *Ag*Trx-1 and ribosomal/nucleolar stress response may work in concert to maintain cellular/tissue homeostasis during blood feeding. The induction of ribosomal/nucleolar stress, as the additional response to oxidative stress, could be harnessed as a transmission-blocking strategy. A practical scenario could be the application of druggable small molecules that would induce high ROS activity in the mosquito bloodmeal bolus

in the midgut during digestion (akin to levels induced by tBHP). This would create an environment of selective toxicity wherein the mosquito naturally survives due to its cooperative oxidative stress response pathways, but the parasite would be unable to manage the elevated oxidative stress, resulting in its arrested development and destruction by the mosquito and thus failure to be transmitted to the next human host.

A major limitation to this work is that measurement of ROS/RNS levels in both the control and treated midgut samples was not carried out due to the inherent technical difficulties with this system. This makes it difficult to ascertain if the response observed is entirely due to tBHP treatment. Furthermore, we were not able to carry out the transmission-blocking potential of tBHP *in vivo* against *Plasmodium* due to difficulty in getting the mosquitoes used in our assays to feed on a blood meal containing tBHP owing to its mosquito repellence.

CHAPTER FOUR

4.0 GENERAL DISCUSSION, CONCLUSION, AND RECOMMENDATIONS

4.1 General Discussion

4.1.1 Proteomic and Transcript Profiles Under Different Oxidative Stress Conditions

In chapter 5, enrichment of antioxidant proteins from Trx and GSH pathways was expected following treatment of *An. gambiae* mosquitoes with 1mM of Pqt. Contrary to this, we did not observe any significant enrichment of antioxidant proteins from the Trx and GSH pathways. Furthermore, significant enrichment of any other protein belonging to any class of known cellular antioxidants was not observed.

Other studies looking at the effect of ROS in *Anopheles* mosquitoes used a different route to administer the ROS inducer. It was observed that direct injection (microinjection) of the ROS inducer into *Anopheles* mosquitoes' midgut results into a significant Trx- and GSH-dependent antioxidant response despite much less or similar concentration levels of the ROS inducer used compared to the present study (Jaramillo-Gutierrez *et al.*, 2010; Molina-Cruz *et al.*, 2008). A Trx-dependent antioxidant response was also observed, when ROS inducers were directly added into *Anopheles* mosquitoes cell lines (*in vitro*) similar to the studies by (Peterson *et al.*, 2007). This difference in the results between these studies and our present study could be down to the route of administration of the ROS inducer. In the present study, the ROS inducer (Pqt) was ingested in contrast to direct injection into the midgut in the other studies. The decision to choose "ingestion" over "injection" is with regards of how a a transmission blocking drug will be applied to mosquitoes. Through ingestion, Pqt passes through several *An. gambiae* body organs before reaching the midgut. It's possible that Pqt goes through some detoxification resulting into a slightly diluted concentration of Pqt reaching the midgut. The diluted concentration of Pqt may have not been sufficient to elicit the Trx- and GSH-dependent antioxidant response that was expected.

To our knowledge, the present study is the first in trying to look at the effect of a ROS inducer after ingestion by the mosquito. Comparison of our results to a similar study carried out in *D. melanogaster* shows that the concentration of ROS-inducer and duration of the exposure used in

our study was substantially lower (Hosamani & Muralidhara, 2013). Therefore, it is entirely plausible that the lack of enrichment in Trx and GSH antioxidant proteins was entirely due to this. Furthermore, in the context of a transmission blocking strategy, future studies that identify candidate for this intervention must accurately define a dose would remain effective in the mosquito considering that this dose would get slightly diluted by the time it reaches the midgut.

Invasion of *An. gambiae* midgut cells by *P. berghei* parasite is disruptive and associated with increased oxidative stress. We wanted to observe if similar results would be obtained with invasion of *An. gambiae* midgut cells by *P. falciparum* parasites at 24 hours post blood meal ingestion. *P. falciparum* is known to be less destructive and possibly associated with reduced or absence of oxidative stress. Our results showed that there was no increased expression in Trx and GSH-dependent genes as seen in section 2.3.5 and Appendix 10-11. This suggests that *P. falciparum* invasion of *An. gambiae* midgut is associated with less oxidative stress and settles the debate whether the time bomb theory observed in *An. gambiae*/*An. stephensi-P.berghei* system also happens in the more natural *An. gambiae*/*P. falciparum* system. The extensive co-evolution over many years between the *An. gambiae* and *P. falciparum* could be one of the reasons attributing to this phenomenon. Also, it is worth mentioning that the absence of Trx and GSH-dependent genes overexpression could entirely be due to a throughout increase of an antioxidant response in the midgut post blood meal ingestion. In this regard, the additional presence of *Plasmodium* in the blood meal doesn't make any significant difference. Furthermore, similar results were obtained in a concomitant feeding assay using *An. gambiae* mosquitoes fed on *P. berghei* ANKA 2.34 contrary to our expectation and known literature as shown in Appendix 12, 13. It is not clear why a significant upregulation in Trx- and GSH-dependent genes was not observed but a plausible explanation could be that the read out time of 24 hours is quite late such that reduced or no levels of the oxidative stress associated with the midgut at this timepoint and henceforth an antioxidant response is not necessary.

However, a significant enrichment of proteins that are associated with ER stress pathway in *An. gambiae* midgut cells was observed following Pqt treatment. This observation is not entirely surprising because increased oxidative stress does indeed increase the extent of unfolding and misfolding in proteins. The Pqt concentration used in our study has been shown to be effective against the parasite (Turturice *et al.*, 2013). But it is not directly harmful to the mosquito given the

response observed. Therefore, this creates an environment of selective toxicity in the mosquito midgut where the mosquito is able to handle the oxidative stress, but the parasite succumbs. It should be noted that, this study does not advocate for the use of Pqt as a transmission blocking drug due to its non-specific toxicity in different cell systems. Rather, a suite of Pqt related drugs can be identified and repurposed for this function.

In chapter 3, an *ex vivo* organ culture was used to induce oxidative stress in *An. gambiae* midgut cell. Similar to what was observed in chapter 2, we did not observe an enrichment in antioxidant proteins with this approach as well. Instead we observed an enrichment in proteins associated with the ribosomal/nucleolar stress response. Evidence of upregulation in protein of the ribosomal stress have been observed in another dipteran, *D. melanogaster* (Marygold *et al.*, 2007). The upregulation of ribosomal protein is the mosquito's unique way of dealing with tBHP mediated oxidative stress. This response work in concert with other antioxidant mechanism with the cell in order to bring about redox homeostasis.

4.1.2 *Ag*Trx-1 is not the Cellular Marker for Oxidative Stress in *An. gambiae* Midgut Cells

The absence of GR in *Anopheles* mosquitoes for GSH recycling is substituted by the Trx system, particularly *Ag*Trx-1(Bauer, Kanzok, & Schirmer, 2002; Kanzok *et al.*, 2001). It was expected that an increase in oxidative stress in *An. gambiae* midgut cells will be associated with an increase expression in *Ag*Trx-1. However, in chapter 3, our results showed that there was no significant difference in *Ag*Trx-1 expression across several conditions of oxidative stress. A plausible explanation for this observation is that *Ag*Trx-1 has to remain consistently high to be able to immediately deal with any changes in the cell's redox environment and also to be able to effectively carry out any of its non-redox functions.

4.2 Conclusion

Our study has shown that at fairly low to moderate concentrations of ROS/RNS induction, the *An. gambiae* midgut cell's oxidative response is through a non-Trx or GSH pathways. These responses in concert with the canonical antioxidant response of the Trx and GSH systems work in tandem to bring about redox homeostasis. Furthermore, our data supports the fact that these concentrations

are not directly harmful to the mosquito. Therefore, they create an environment of selective toxicity where they are deleterious to the parasite but not to the mosquito. This would substantially reduce the possibility of development of resistance in the mosquito as there is no fitness cost with the ROS/RNS inducer. However, since there is a fitness cost to the parasite then there is the possibility of resistance development. But an additional antimalaria drug against the parasite will slow due the development of resistance due to an increase number in drug targets that *Plasmodium* genome must mutate to develop resistance against.

4.3 Recommendations

This study is not in support of tBHP or Pqt to be use as transmission blocking drugs. This study provides a proof of principle that oxidative stress in the mosquito midgut could be harnessed as a transmission blocking strategy. The study advocate for future studies that would screen for compounds that are safe and non-toxic to humans with similar mode of action and repurposing them for transmission-blocking.

These future studies should focus on differentiating between the response to oxidative stress due to *Plasmodium* infected blood meal ingestion only and that due to the ingestion of ROS inducing compound(s) only. This would to comprehensive details of the differential expression of protein and transcripts produced under each of these conditions. ROS inducing compound(s) only specific proteins and transcripts could be further evaluated as potential targets for transmission blocking.

The screening efforts for potential compounds could focus on traditional medicines/herbs that have been reported to treat/cure Malaria. Both CQ and Artemisinin are derived from traditional compounds and this underpins the importance of traditional medicines/herb in fighting malaria. Different societies and cultures have different traditional medicines thus priority will have to be given to only those medicines that have shown the most profound effect against Malaria.

Lastly, even though this is years away but once a TBD has been identified and ready for mass consumption the first focus should be in areas of low malaria endemicity. Prevention of malaria transmission in these areas will ensure that the *Plasmodium* prevalence rate drops to a level that will not support transmission of the disease. Subsequent focus should be in areas of high

endemicity where this intervention should be used in addition to existing interventions used both the mosquito vector and parasite as per the WHO's and the respective county's guidelines.

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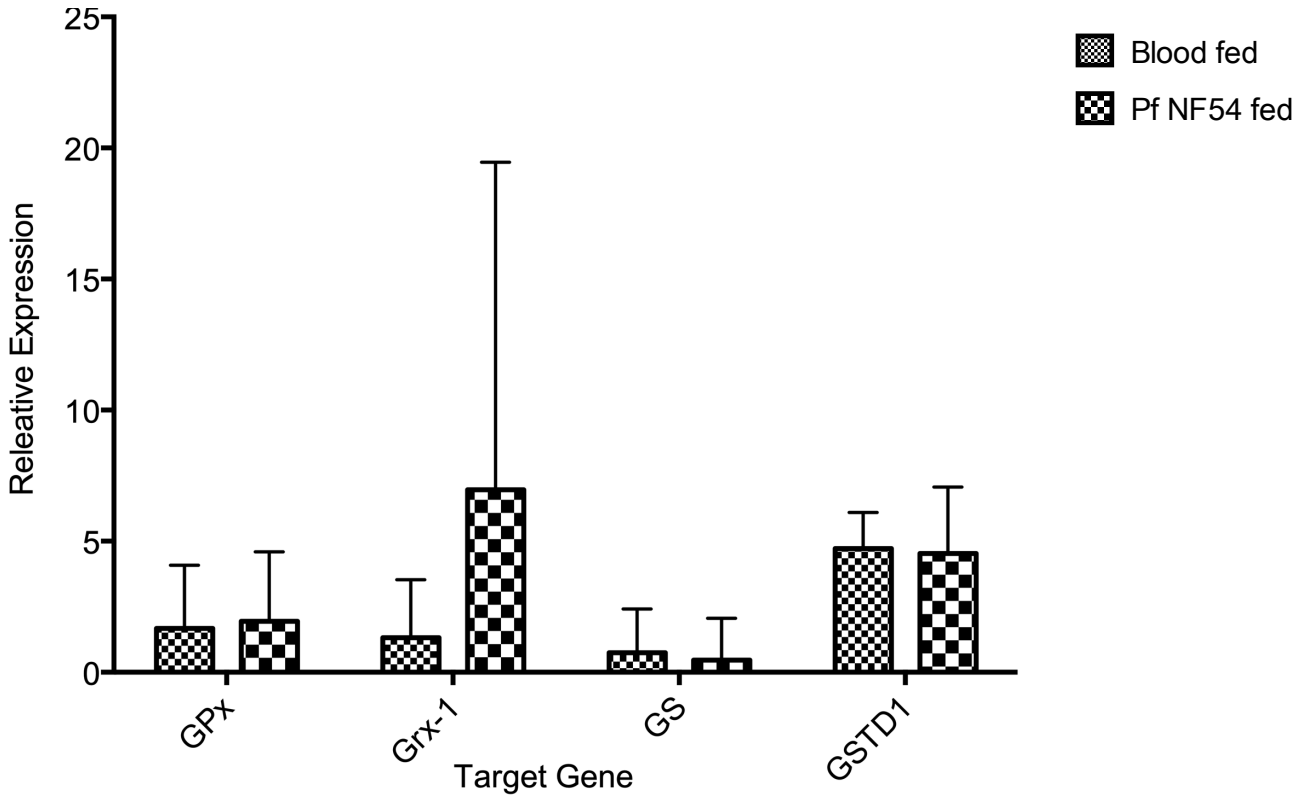
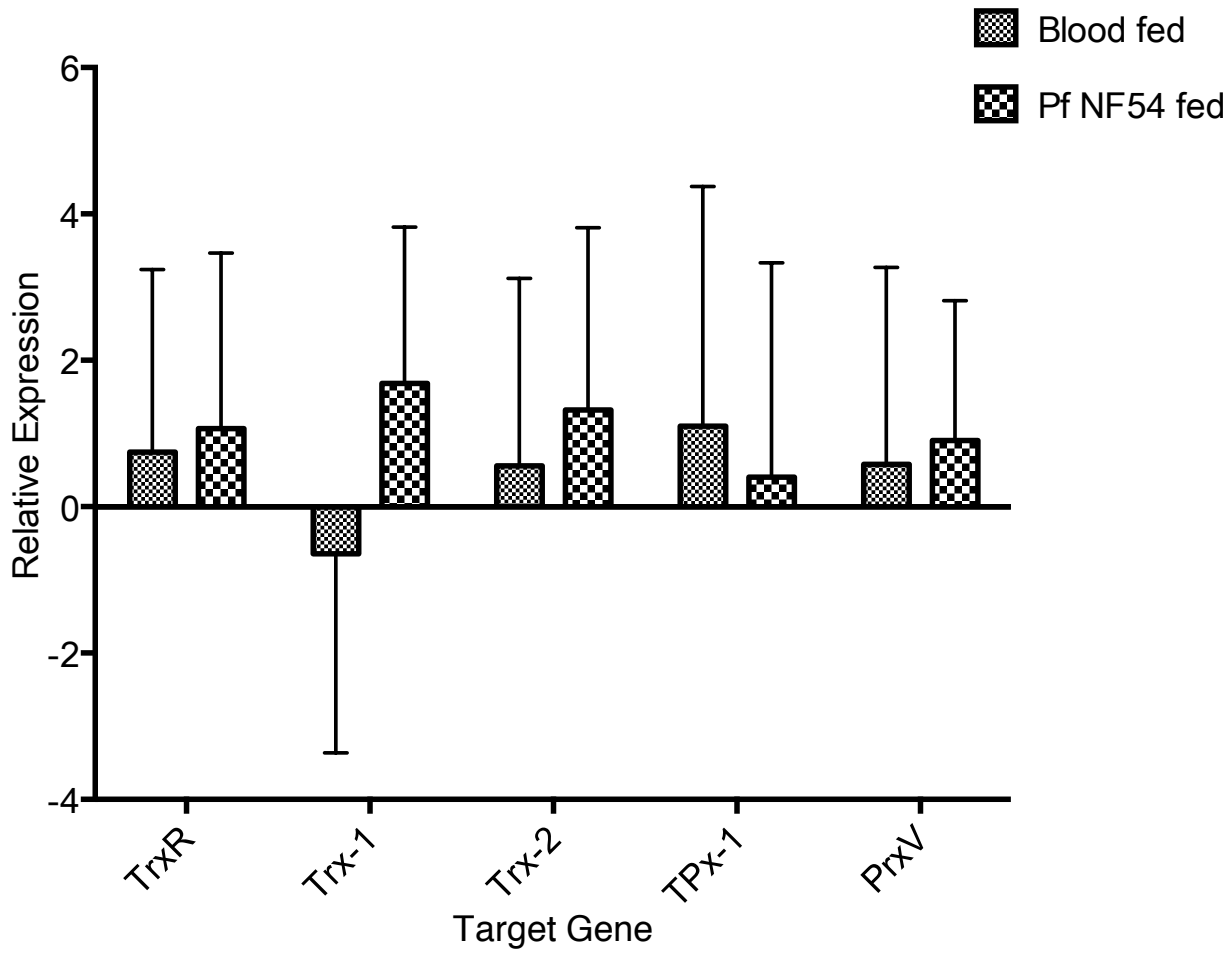
APPENDICES

Appendices 1 – 9: Excel sheets provided as additional files with this document.

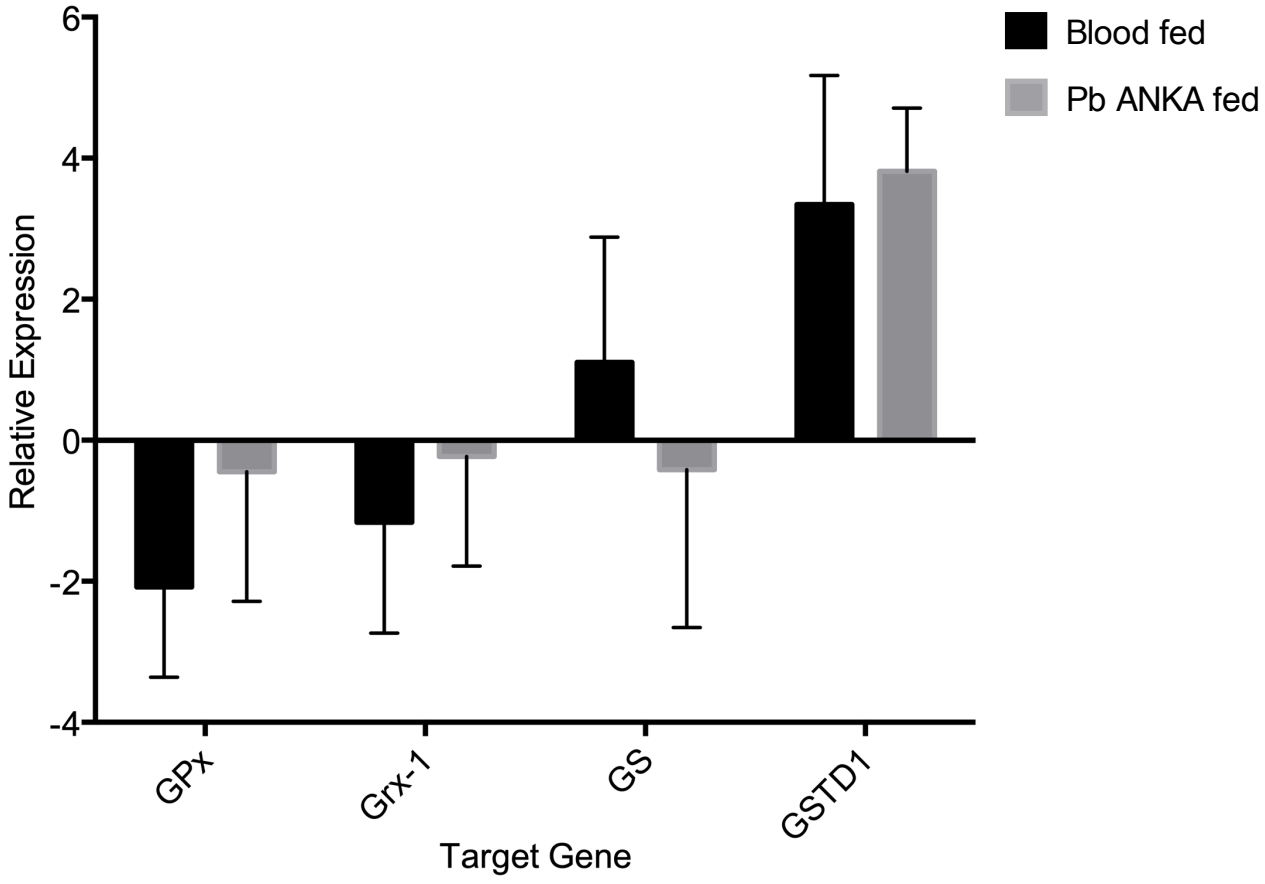
Appendix 10: SMFA with *Pf* NF54 – GSH-dependent Genes.



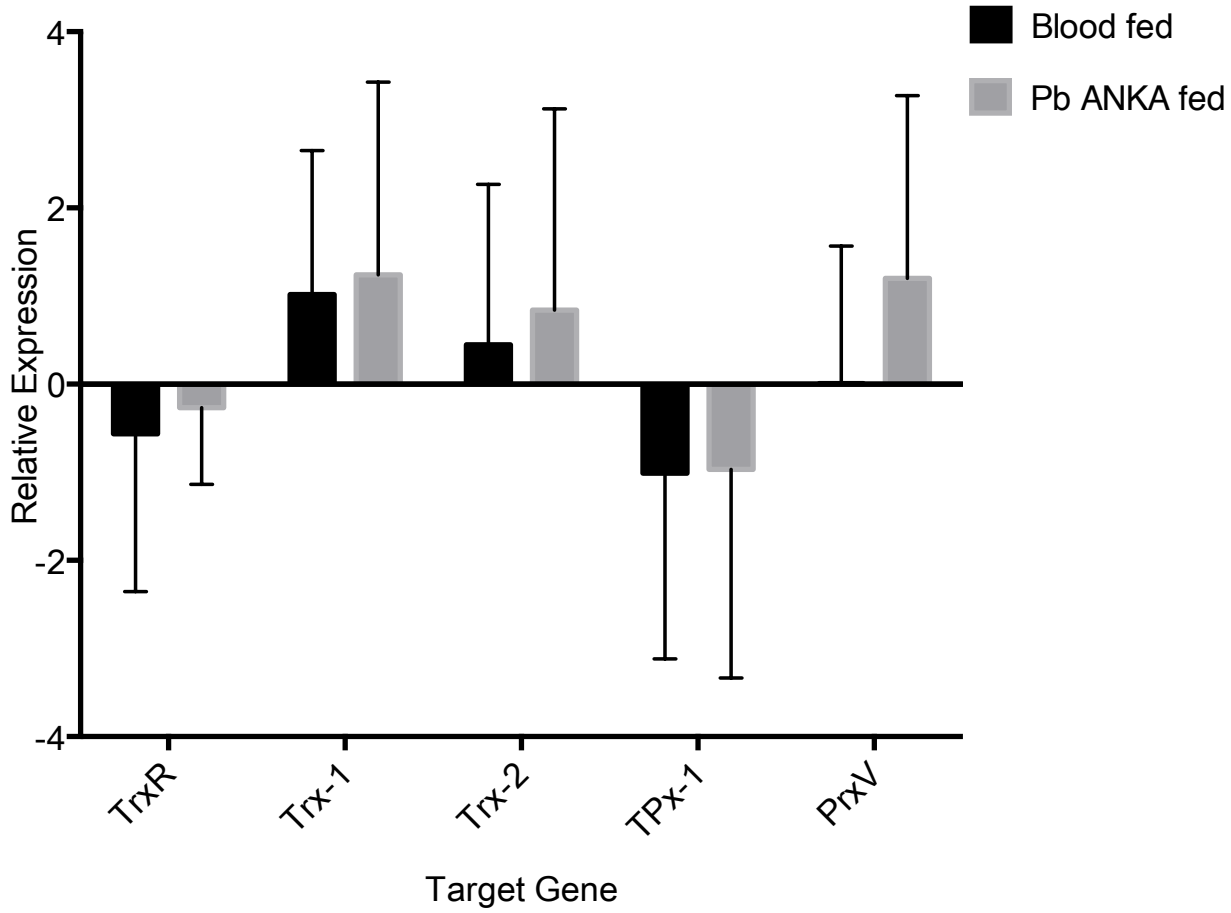
Appendix 11: SMFA with *Pf* NF54 – Trx-dependent Genes.



Appendix 12: DFA with *Pb* ANKA 2.34 – GSH-dependent Genes.



Appendix 13: DFA with *Pb* ANKA 2.34 – Trx-dependent Genes.



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Oxidative Stress Management is Essential for *Anopheles* Mosquito Survival Post *Plasmodium* Infected Blood Meal Ingestion

Brian B. Tarimo[1,2,3], Bernadette A. Hritzo[2], Henry Chun Hin Law[4+], Dingyin Tao[2+], Rebecca Pastrana-Mena[2], Stefan Kanzok[5], Joram J. Buza[1], Rhoel R. Dinglasan[1,2,4]

[1]Department of Health & Biomedical Sciences, Nelson Mandela-African Institution of Science and Technology, P.O. Box 447, Tengeru, Arusha, 23302, Tanzania
[2]W. Harry Feinstone Department of Molecular Microbiology & Immunology & the Malaria Research Institute, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA
[3]Department of Environmental Health and Ecological Sciences, Ifakara Health Institute, Dar es Salaam 14112, Tanzania
[4]Emerging Pathogens Institute, Department of Infectious Diseases & Immunology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA
[5]Department of Biology, Loyola University Chicago, Chicago, Illinois 60660, USA
[+]These authors contributed equally to this work.

Introduction

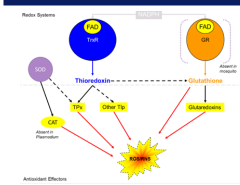


Figure 1. Interplay of redox systems in *Plasmodium* and *Anopheles*. ROS/RNS = reactive oxygen species/reactive nitrogen species, GR = glutathione reductase, FAD = flavin adenosine dinucleotide, NADPH = reduced nicotinamide dinucleotide phosphate, TrxR = thioredoxin reductase, SOD = superoxide dismutase, TPx = thioredoxin peroxidase, CAT = Catalase, and Tlp = thioredoxin-like proteins.

Objectives:

a) To characterize the transcript and protein expression levels of *Ag*Trx-1 in An. gambiae midgut epithelial cells under different oxidative conditions

b) To characterize the proteomic profile of *An. gambiae* midgut epithelial cells under different oxidative conditions

c) To characterize the Trx- and GSH-dependent transcript profile in *An. gambiae* midgut epithelial cells during *P. falciparum* ookinete invasion

Methods

- Oxidative stress induction assays:
  - a) ~ 50 *An. gambiae* (KEELE) female mosquitoes (4-7 days old) were fed an ATP/saline meal supplemented with Paraquat (Pqt) through a glass, water-jacketed membrane feeders and kept for 8 hours (*in vitro* system).
  - b) 50 *An. gambiae* (KEELE) mosquito midguts treated with tert-butyl hydroperoxide (t-BHP) through an organ culture media (*ex vivo* system).
- 5 midguts/sample from ROS/RNS induction assays were used for SDS-PAGE and immunoblot analysis for *Ag*Trx-1 protein expression levels.
- Remaining midguts from ROS induction assays were used for Online 2D LC-MS/MS analysis.
- Standard membrane feeding assay (SMFA) with *An. gambiae* (KEELE) female mosquitoes (4-7 days old) feeding on *P. falciparum* (*Pf*NF54) parasites through and kept for 24 hours.
- Midguts from SMFA were used for qRT-PCR for upregulation of Thioredoxin (Trx)- and Glutathione (GSH)-dependent transcripts. Transcripts analyzed were evidenced from previous studies to be involved in regulation of oxidative stress and detoxification of xenobiotic compounds.

Results

*Ag*Trx-1 expression levels (*ex vivo* system)

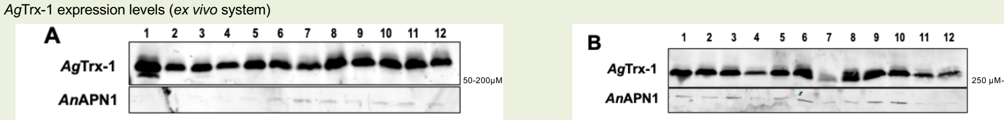


Figure 2. *Ag*Trx-1 protein expression in *An. gambiae* midgut epithelial cells. (A) Female *An. gambiae* midgut lysates treated with *ex vivo* organ culture media lanes 1, 5, and 9), 50 µM t-BHP (lanes 2, 6, and 10), 125 µM tBHP (lanes 3, 7, and 11), and 200 µM tBHP (lanes 4, 8, and 12). **(B)** Female *An. gambiae* midgut lysates treated with *ex vivo* organ culture media (lanes 1, 5, and 9), 250 µM tBHP (lanes 2, 6, and 10), 500 µM tBHP (lanes 3, 7, and 11), and 1 mM tBHP (lane 5, 9, and 13). Lanes 1-4 (biological replicate 1), lanes 5-8 (biological replicate 2), lanes 9-12 (biological replicate 3). *An*APN1 (~135 kDa), as a loading control is shown below each treatment column.

Global Proteomics Response

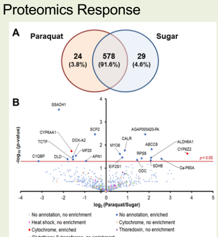


Figure 2. Comparative label-free quantitative proteomic analyses of the *An. gambiae* midgut responses to the ROS-inducer Pqt. (**A**) Global distribution of proteins. (**B**) Volcano plot of quantifiable proteins. Significant fold change was calculated with Student's t-test with *P*-value ≤ 0.05. The red line indicates a *P*-value = 0.05. Annotation of significantly enriched proteins is shown

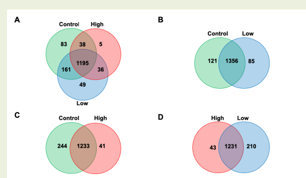


Figure 3. Protein identification comparisons between treatment groups in *An. gambiae* midguts subjected to LC-MS/MS. (**A**) Proteins identified in all three experimental groups of control (untreated *ex vivo* organ culture media only), low (50 µM tBHP), and high (µM tBHP). (**B**) Proteins identified in control and low tBHP groups. (**C**) Proteins identified in control and high tBHP groups. (**D**) Proteins identified in high and low tBHP groups

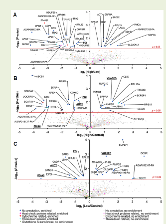


Figure 4. Volcano plots of quantifiable protein comparisons. (**A**) Low (50 µM tBHP) versus control (untreated *ex vivo* organ culture only) experimental groups. (**B**) High (200 µM tBHP) versus control (untreated *ex vivo* organ culture only) experimental groups. (**C**) High (200 µM tBHP) versus low (50 µM tBHP) experimental groups. Significant fold change was calculated using Student's t-test with P-value ≤ 0.05. Annotations of significantly enriched proteins are indicated

Table 1. Proteins enriched (overexpressed) in Pqt-treated midguts directly involved in mitigation of the induced oxidative stress

Protein description	Fold change	*P*-value	Function
ABCC8 (AGAP008437) ATP-binding cassette transporter (ABC transporter) family C member 8	4.37	0.015	Upregulated in bendiocarb resistant *An. gambiae*, a detoxification gene (Antonio-Nkondjio *et al*., 2016)
ALDH6A1 (AGAP002499) Methylmalonate-semialdehyde dehydrogenase (acylating), mitochondrial	4.28	0.035	Classified as environmental and oxidative stress proteins (Sonenshine *et al*., 2011)
Ca-P60A (AGAP006186) Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	5.99	0.039	Function impaired by oxidative stress (Lafleur, Stevens, & Lawrence, 2013; Park, Zhou, Lee, Lee, & Ozcan, 2010; X. Tong, Kono, & Evans-Molina, 2015; XiaoYong Tong, Evangelista, & Cohen, 2010)
CRT (AGAP004212) Calreticulin	1.64	0.017	Ca^{2+} homeostasis (Ihara, Kageyama, & Kondo, 2005; H. Liu *et al*., 1997; Ruddock & Molinari, 2006) and pro-apoptotic protein (Zhang *et al*., 2014)
EIF2S1 (AGAP011190) Eukaryotic translation initiation factor 2 subunit alpha	1.69	0.034	Conserve in eukaryotes, the phosphorylation form of this protein serve as a signal of cell survival by attenuating translation of mRNA (Back *et al*., 2009; Harding, Zhang, & Ron, 1999; Knutsen *et al*., 2015)
ODC (AGAP011806) Ornithine decarboxylase	2.72	0.043	Upregulated after ivermectin-containing blood meals (Seaman *et al*., 2015)
SDHB (AGAP007309) Succinate dehydrogenase (ubiquinone) iron-sulfur subunit	4.23	0.045	Ferredoxin balance system

Table 2. Overexpressed (enriched) RPs following treatment with low and high tBHP concentrations

Protein description	tBHP group	Fold change	*P*-value	Reference
RpL7 (AGAP008916) 60S ribosomal protein L7	Low	1.9	0.025	Protein translation machinery(Fromont-Racine, Senger, Saveanu, & Fasiolo, 2003).
RpL10A (AGAP011298) 60S ribosomal protein L10a	High	3.0	0.008 8	Protein translation machinery (Koga *et al*., 2003).
RpS15 (AGAP001274) 40S ribosomal protein S15	High	5.30	0.017	
RpS3A (AGAP003532) 40S ribosomal protein S3a	High	1.55	0.038	Protein translation machinery(Wang, Pakpour, *et al*., 2015).
RpL19 (AGAP004422) 60S ribosomal protein L19	High	2.31	0.04	Protein translation machinery(Marygold *et al*., 2007).
AGAP007325 Putative RNA binding protein.	High	2.7	0.032	None

Table 3. Under-expressed RPs following treatment with low and high tBHP concentrations

Protein description	tBHP group	Fold change	*P*-value	Reference
RpL13 (AGAP001805) 60S ribosomal protein L13	Low	1.50	0.017	Protein translation machinery (Wang, Pakpour, *et al*., 2015).
RpL22 (AGAP005046) 60S ribosomal protein L22	Low	1.33	0.031	Protein translation machinery (Marygold *et al*., 2007).
RpLP1 (AGAP007740) 60S ribosomal protein LP1	High	2.86	0.006 7	Protein translation machinery (Marygold *et al*., 2007).
RpS26 (AGAP012100) 40S ribosomal protein S26	High	3.85	0.041	Protein translation machinery (Marygold *et al*., 2007).
RpL32 (AGAP002122) 60S ribosomal protein L32	High	2.63	0.009 2	Protein translation machinery (Marygold *et al*., 2007).
RpL13A (AGAP010257) 60S ribosomal protein L13a	High	1.81	0.022	Protein translation machinery (Marygold *et al*., 2007).
RpL11 (AGAP011173) 60S ribosomal protein L11	High	16.67	0.003 6	Protein translation machinery (Fromont-Racine *et al*., 2003).
RpS14 (AGAP002346) 40s ribosomal protein S14	High	2.5	0.044	Protein translation machinery (Marygold *et al*., 2007).
RpS18 (AGAP028693) 40S ribosomal protein S18	High	3.3	0.006 5	Protein translation machinery (Marygold *et al*., 2007).

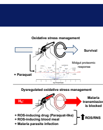
Relative expression of Trx- and GSH-dependent transcripts

Figure 3: qRT-PCR analysis of Trx- and GSH-dependent transcripts.. (**A**) Relative expression levels of Trx-dependent transcripts. (**B**) Relative expression levels of GSH-dependent transcripts. Relative expression levels of targeted transcripts was calculated by Student's t-test followed by Holm-Sïdak score to adjust for multiple scores

Table 4. A list of Trx and GSH-dependent transcripts evaluated following *P. falciparum* infected blood meal ingestion

Transcript/Accession ID	Function/Annotation	Response to *P. falciparum* blood meal ingestion.
Thioredoxin-1 (Trx-1; AGAP009584)	Dithiol-disulfide exchange reaction with GSSG to produce GSH (Kanzok *et al*., 2001)	None (*P*-value = 0.308 8)
Thioredoxin-2 (Trx-2; AGAP007201)	Antioxidative function as electron donor to TPx (Bauer, Kanzok, & Schirmer, 2002)	None (*P*-value = 0.730 9)
Thioredoxin reductase (TrxR; AGAP000565)	Key enzyme of the Trx system responsible for replenishing Trx-1 (Bauer *et al*., 2003)	None (*P*-value = 0.880 6)
Thioredoxin peroxidase-1 (TPx-1; AGAP000396)	Antioxidant enzyme that catalyzes peroxides (Bauer *et al*., 2002)	None (*P*-value = 0.797 6)
Atypical 2-Cys peroxiredoxin (Peroxiredoxin V; PrxV; AGAP001325)	Antioxidant enzyme that protects against ROS/RNS (Peterson & Luckhart, 2006)	None (*P*-value = 0.873 6)
Glutathione synthase (GS; AGAP000534)	Involved in the GSH biosynthesis pathway	None (*P*-value = 0.851 5)
Glutathione peroxidase (GPx; AGAP004247)	Antioxidant enzyme that catalyzes peroxides (Molina-Cruz *et al*., 2008)	None (*P*-value = 0.899 8)
Glutathione S-transferase delta class 1 (GSTD1; AGAP004164)	Implicated in insecticide resistance and detoxifies xenobiotic compounds (Prapanthadara, Hemingway, & Ketterman, 1993; Ranson *et al*., 2001)	None (*P*-value = 0.919 5)
Glutaredoxin-1 (Grx-1; AGAP011107)	Essential component of the GSH system (Giordano, Peluso, Rendina, Digilio, & Furia, 2003; Mercer & Burke, 2016)	None (*P*-value = 0.483 8)

Conclusions



JHMRI
Johns Hopkins Malaria Research Institute
Johns Hopkins Bloomberg School of Public Health