Universidade Nova de Lisboa

Instituto de Higiene e Medicina Tropical

Hemozoin as an immune stimulant of the mosquito *Anopheles gambiae* response against the malaria parasite

Maria Luísa Simões

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE EM BIOLOGIA CELULAR E MOLECULAR

JULHO, 2014

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Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Doutor no Ramo de Ciências Biomédicas, Especialidade em Biologia Celular e Molecular, segundo o Regulamento n.º 474/2012 de 19 de Novembro. Apoio financeiro da Fundação para a Ciência e a Tecnologia, através da Bolsa de Doutoramento SFRH/BD/70110/2010.

Publications

The work presented in this thesis resulted in the following publications:

Simões, ML & Chandrasekar, R. (2014) Immune Pathways in *Anopheles gambiae*. In: Chandrasekar, R, Tyagi, BK, Gui, ZZ & Reeck, G (eds). *Short views on Insect Biochemistry and Molecular Biology*. Kansas State University, KS, Manhattan, USA.

Simões, ML & Silveira, H. (2014) Hemozoin activates the innate immune system and reduces *Plasmodium berghei* infection in *Anopheles gambiae*. In revision.

Resumo

Hemozoína como estimulador da resposta imunitária do mosquito *Anopheles gambiae* contra o parasita da malária

Maria Luísa Simões

Palavras-chave: *Anopheles, Plasmodium,* Imunidade, Hemozoína, Malária.

Hemozoína, um metabolito produzido por *Plasmodium* spp., tem surgido como um potente estimulador, activando o sistema imunitário do hospedeiro e levando à produção de citocinas e quimiocinas em tecidos de mamíferos. Neste estudo, desvendamos o papel deste subproduto do parasita como estimulador da imunidade de *Anopheles gambiae* em resposta à infecção por *Plasmodium berghei*. A malária é uma doença infecciosa de distribuição mundial, causada por parasitas do género *Plasmodium* e transmitida pelas fêmeas de mosquitos do género *Anopheles*. A resposta imunitária do mosquito vector da malária contra o parasita envolve várias vias metabólicas que não se encontram ainda bem caracterizadas. Resultados laboratoriais revelaram que a hemozoína activa a expressão de vários genes da imunidade, incluindo péptidos anti-microbianos e factores anti-*Plasmodium*. Destaca-se a indução, após estimulação com hemozoína, da forma larga (REL2-F) do factor de transcrição REL2, da via *Immune deficiency* (Imd). Estes resultados foram confirmados pela estimulação de tecidos e células de *Anopheles gambiae* com hemozoína sintética e silenciamento do gene que codifica REL2-F e do gene que codifica o seu regulador negativo Caspar. Neste trabalho, mostrou-se pela primeira vez o impacto do tratamento com hemozoína na infecção por *Plasmodium*: a hemozoína reduz eficientemente tanto a taxa como a intensidade da infecção no mosquito. Propomos, assim, que a hemozoína estimula a imunidade inata de *Anopheles*, activando a expressão de genes efectores que tornam o mosquito mais resistente ao *Plasmodium*, e que esta activação é mediada por REL2.

Após identificação de um conjunto de genes associados à imunidade induzidos pela hemozoína, e de acordo com as propriedades da via Imd/REL2 sugeridas pelos resultados obtidos, construímos uma linha de mosquitos *Anopheles gambiae* geneticamente modificados, através da sobreexpressão do gene anti-plasmódico *FBN9 (fibrinogen immunolectin 9)*, sob regulação de *Vitellogenin 1*, um promotor específico do corpo gordo.

Abstract

Hemozoin as an immune stimulant of the mosquito *Anopheles gambiae* response against the malaria parasite

Maria Luísa Simões

Keywords: *Anopheles, Plasmodium*, Immunity, Hemozoin, Malaria.

The *Plasmodium* metabolite hemozoin has emerged as a potent immunostimulator, targeting the host immune system and activating the production of cytokines and chemokines in mammalian tissues. In this study, we disclose the role of this parasite's byproduct as stimulator of *Anopheles gambiae* immunity to *Plasmodium berghei*. Malaria is a worldwide infectious disease caused by *Plasmodium* parasites and transmitted by female *Anopheles* mosquitoes. The malaria vector mosquito *Anopheles* immune response to the parasite involves several pathways which are not yet well characterized. High throughput analyses revealed that hemozoin activates the expression of several immunity genes, including antimicrobial peptides (AMPs) and anti-*Plasmodium* factors. Importantly, we found that the Immune deficiency (Imd) pathway transcription factor REL2, in its full-length form REL2-F, was induced upon hemozoin treatment. These findings were confirmed by stimulation of *Anopheles gambiae* tissues and cells with synthetic hemozoin and silencing of REL2-F and its negative regulator Caspar. Notably, we have for the first time shown the impact of hemozoin treatment on *Plasmodium* infection, effectively reducing both rate and intensity of the infection. We propose that hemozoin boosts the innate immunity in *Anopheles*, activating key effector genes that turn the mosquito more resistant to *Plasmodium*, and this activation is REL2-mediated.

Following identification of a set of key immunity genes induced by hemozoin and encouraged by the properties of the Imd/REL2 pathway suggested by the obtained results, we have successfully engineered a genetically modified *Anopheles gambiae* line, by overexpression of *FBN9* (fibrinogen immunolectin 9) antiparasitic gene under regulation of the fat body-specific Vitellogenin 1 promoter.

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List of Abbreviations

A.- Anopheles

ACT- artemisinin-based combination therapy

A. gambiae s.s.- *Anopheles gambiae* sensu stricto

AMA1- apical membrane antigen 1

AMP- antimicrobial peptide

APL1- *Anopheles Plasmodium*-responsive leucine-rich repeat 1

APL2- *Anopheles Plasmodium*-responsive leucine-rich repeat 2

β2M- beta-2 microglobulin

BC- before Christ

bp- base pair

BSA- bovine serum albumin

ºC- degree Celsius

CDC- Centers for Disease Control and Prevention

cDNA- complementary DNA

CEC- cecropin

CEC1, 3- cecropin 1, 3

CLIPA2, A7, A8, B14, B15, B17- CLIP-domain serine protease A2, A7, A8, B14, B15, B17

CpG ODN- cytosinetriphosphate deoxynucleotide, phosphodiester, guaninetriphosphate deoxynucleotide oligodeoxynucleotide

cRNA- complementary RNA

CSP- circumsporozoite protein

CTL- C-type lectin

CTL4- C-type lectin 4

CTLMA2- CTL mannose binding 2

DDT- dichlorodiphenyltrichloroethane

DEF- defensin

DEF1- defensin 1

DEPC- diethylpyrocarbonate

DGV- Divisão Geral de Veterinária

dH_2O- distilled water

DNA- deoxyribonucleic acid

DsRed- *Discosoma* sp. red fluorescent protein

dsRNA- double-stranded RNA

EBA-175- erythrocyte binding antigen 175

EEC- European Economic Community

e.g.- *exempli gratia* (for example)

Fb- fat body

FBN9- fibrinogen immunolectin 9

$Fe^{III, 3+}$- $iron^{III, 3+}$

FREP- fibrinogen-related protein

g- acceleration of gravity

GAM1- gambicin 1

gDNA- genomic DNA

GFP- green fluorescent protein

GLURP- glutamate-rich protein

GPI- glycosylphosphatidylinositol

Gram +, Gram –- Gram-positive, Gram-negative

hr, h- hour

i.e.- *id est* (in other words)

IGC- Instituto Gulbenkian de Ciência

Imd- immune deficiency

I-PpoI- intron-encoded endonuclease from *Physarum polycephalum*

IRS- indoor residual spraying

ITN- insecticide-treated bednets

IVF- *in vitro* transcription

Jak/Stat- Janus kinase-signal transducer and activator of transcription

L1-L4- larval stages 1 to 4

LB- Luria-Bertani

LLIN- long-lasting insecticide treated net

LPS- lipopolysaccharide

LRIM1- leucine-rich repeat immune protein 1

LRR- leucine-rich repeat protein

LRRD7- leucine-rich repeat-containing protein

M- molar

MeOH- methanol

µg- microgram

mg- milligram

Mg- midgut

µl- microliter

ml- milliliter

µm- micrometre

MSP-1- merozoite surface protein 1

N- total number

NaHCO3- sodium bicarbonate

NaOH- sodium hydroxide

NF-κB- nuclear factor kappa B

nl- nanoliter

NLRP3- NLR family, pyrin domain containing 3

NO- nitric oxide

NOS- nitric oxide synthase

ns- not significant

P.- Plasmodium

PAMP- pathogen associated molecular pattern

Pb- Plasmodium berghei

pbm- post blood meal

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PGN- peptidoglycan

PGRP- peptidoglycan recognition protein

PPO- prophenoloxidase

PRR- pattern recognition receptor

qRT-PCR- quantitative reverse transcription PCR

REL2-F- REL2 full-length form

REL2-S- REL2 short-length form

RHD- Rel-homology domain

RIDL- release of insects carrying a dominant lethal (gene)

RNA- ribonucleic acid

RNAi- RNA interference

ROS- reactive oxygen species

S7- ribosomal protein S7

Sbjct- subject

SD- standard deviation

sHz- synthetic hemozoin

SIT- sterile insect technique

SOC- super optimal broth with catabolite repression

SRPN2- serpin 2

STAT-A, -B- signal transducer and activator of transcription A, B

TAK1- transforming growth factor-beta activated kinase 1

TEP- thioester-containing protein

TEP1, 3, 4- thioester-containing protein 1, 3, 4

TIR- Toll-interleukin-1-receptor

TLR- Toll-like receptor

TLR9- Toll-like receptor 9

TNF- tumor-necrosis factor

USA- United States of America

v/v- volume/volume

w/v- weight/volume

WHO- World Health Organization

1. Introduction

1.1 Malaria: Global Situation

Malaria is one of the world's most significant insect-borne human diseases, caused by *Plasmodium* parasites and transmitted by female *Anopheles* mosquitoes. It is widespread in tropical and subtropical regions, and is presently considered endemic in 104 countries (WHO, 2013). It has been estimated that 207 million cases of malaria and 627 000 deaths due to this infectious disease occurred globally in 2012. Most cases/deaths occurred in Africa and most deaths were in children under five years of age (WHO, 2013). Although in the last decade malaria mortality has dropped by 42% due to increased control interventions (WHO, 2013), the challenge of producing an effective vaccine is still to be met, the host defences are unable to control parasite replication, the parasite has been mounting resistance to antimalarial drugs and the mosquito is growing resistance to insecticides. This observation stresses the importance of intensifying the current malaria control strategies and creating new alternative and effective interventions to fight this devastating parasite infection.

1.2 Malaria: Infection Cycle Overview

Malaria is one of the oldest known diseases, which had already been described in detail in ancient Greece by the 5th century BC. The malarial parasites were first discovered by Charles Laveran (Nobel Prize in Physiology or Medicine, 1907) in 1880, when he observed parasites inside the red blood cells of infected patients. Later, in 1897, Ronald Ross (Nobel Prize in Physiology or Medicine, 1902), observed parasites maturing inside the mosquito, thus discovering how malaria is transmitted and tracing its life cycle.

The success of malaria as a worldwide disease is due in part to the fact that it is transmitted by a mosquito vector. The complex parasite life cycle is shared between two hosts: in the vertebrate host, *Plasmodium* completes the asexual stages of its life cycle (the pre-erythrocytic or exo-erythrocytic stage and the erythrocytic stage) (Figure 1A), while the sexual sporogonic stage takes place inside the female mosquito vector (Figure 1B). In malaria, the mosquito is the definitive host, as well as the carrier of the parasite back to the transient vertebrate host for a new infection.

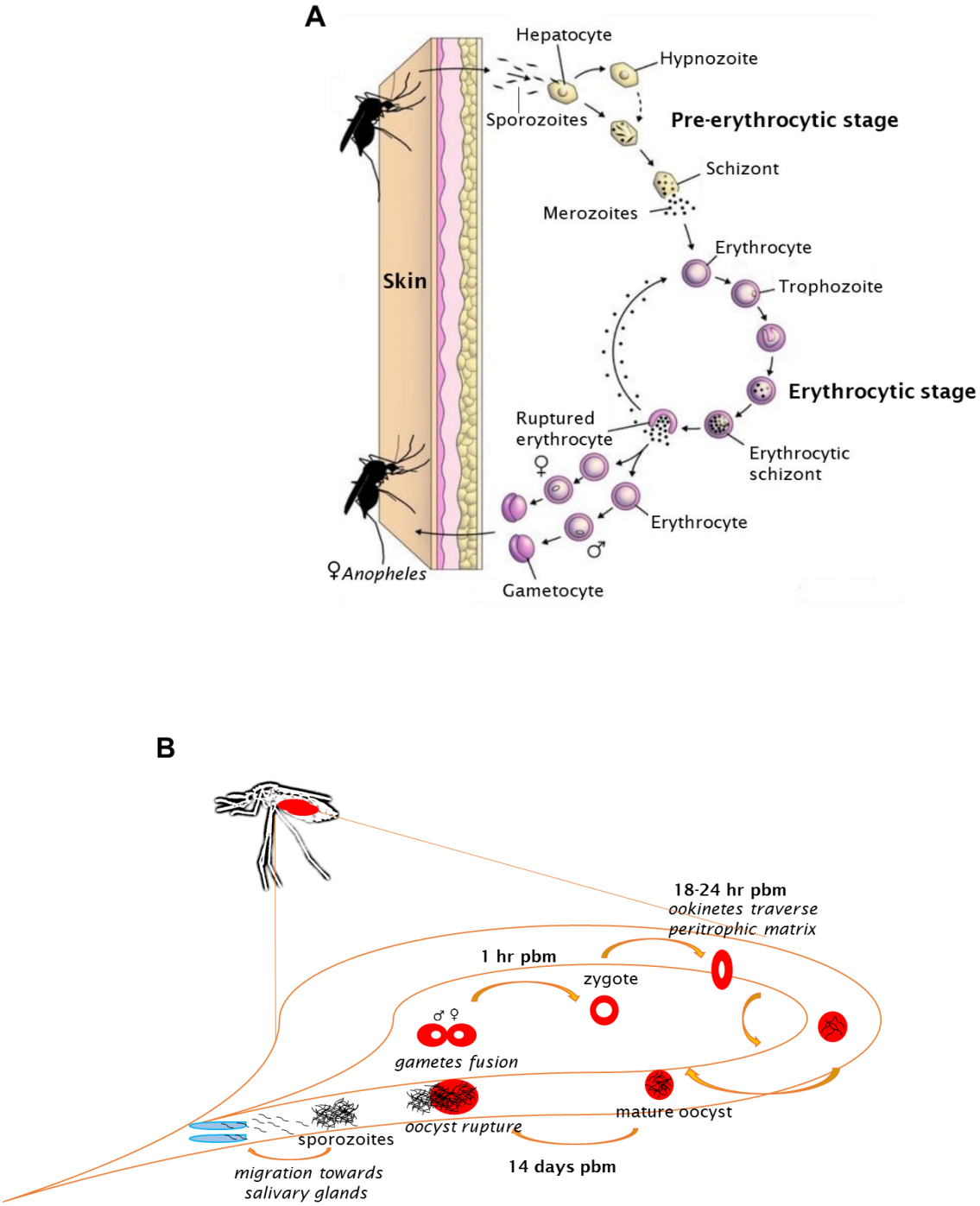


Figure 1. *Plasmodium* life cycle (A) inside the vertebrate host and **(B)** inside the female *Anopheles* vector. **(A)** Adapted from http://www.malwest.gr. **(B)** Adapted from Simões and Chandrasekar, 2014. hr (hour), pbm (post blood meal). Note: figures not drawn to scale.

1.3 *Plasmodium* Parasite

There are five species of protozoan parasites of the genus *Plasmodium* considered responsible for human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi.* Of these, *Plasmodium falciparum* (*P. falciparum*) and *P. vivax* are the most prevalent forms. *P. falciparum* malaria is the most deadly of both and predominates in Africa (Guerra et al., 2008), while *P. vivax* has a wider distribution (Battle et al., 2014), partly due to its higher adaptability and also because it is able to undergo a dormant liver stage (the hypnozoite), enabling its longer survival.

1.3.1 Parasite Life Cycle

Plasmodium parasites suffer several morphological changes along their life cycle:

Pre-erythrocytic stage (or exo-erythrocytic schizogony): When an infected *Anopheles* female mosquito bites the vertebrate host, sporozoites are injected along with saliva into the host skin, until they reach the liver. There they invade cells (hepatocytes) and transform into hepatic schizonts. Schizonts grow and parasites differentiate into invasive motile forms called merozoites, which are released into the blood stream (reviewed by Prudêncio et al., 2006) (Figure 1A).

Erythrocytic stage (or erythrocytic schizogony): Merozoites infect red blood cells (erythrocytes) and develop into immature trophozoites (or ring stages), which then progress to mature trophozoites. Mature trophozoites develop into schizonts, which release merozoites. Merozoites can either invade another erythrocyte and continue this stage, or differentiate into gametocytes (Figure 1A).

Sporogonic cycle: The sexual phase of the *Plasmodium* life cycle starts when the female *Anopheles* mosquito ingests infected blood containing male and female gametocytes. These differentiate into gametes and fertilization occurs within the midgut lumen of the mosquito, resulting in the formation of zygotes. Zygotes develop into motile ookinetes, which penetrate the peritrophic matrix and invade the midgut epithelial cells. Ookinetes settle under the basal lamina surrounding the mosquito midgut, differentiating into oocysts. During oocyst maturation, the parasite undergoes several mitotic divisions to form thousands of sporozoites, which are then released into the hemolymph. Sporozoites

migrate through the hemocoel and invade the salivary glands (Figure 1B), until they are transmitted to the vertebrate host upon another blood meal, thus starting the asexual part of the *Plasmodium* life cycle (reviewed by Cirimotich et al., 2010; reviewed by Yassine and Osta, 2010).

1.3.2 Parasite Control Strategies

1.3.2.1 Antimalarial Drugs

There are different chemotherapeutic agents for the treatment of malaria. Their value depends on the emergence of parasite resistance. Chloroquine was the predominant antimalarial drug in the past; its action interferes with the parasite ability to metabolise hemoglobin, inhibiting its digestion (Foley and Tilley, 1997). However, due to historical overuse, *Plasmodium* parasites resistance to this drug is seriously menacing effective malaria control. Other common antimalarial medications, such as sulfadoxine-pyrimethamine (antifolates which inhibit critical enzymes of the folate pathway, depriving the parasite from essential folate cofactors (Hyde, 2005)) and mefloquine (another aminoquinoline, as chloroquine), are facing the same *Plasmodium*-resistance problem.

Current strategies for malaria treatment (WHO, 2013) recommend the use of artemisinin-based combination therapies (ACTs) for uncomplicated *P. falciparum* malaria. In the case of *P. vivax* infection, in areas where resistance to chloroquine is not yet present, this drug should be used; otherwise, an appropriate ACT must be chosen. In severe malaria cases, treatment should be based on injected artesunate (an artemisinin derivative), followed by a complete course of an effective ACT.

Although ACTs have been adopted as a first-line treatment in malaria-endemic countries, information reporting resistance to artemisinin has recently been disclosed (Dondorp et al., 2009). Parasite resistance to artemisinin has now been detected in four countries of the greater Mekong subregion, in Southeast Asia (WHO, 2013). Nevertheless, many pharmaceutical companies are still marketing artemisinin-based monotherapies in malaria endemic regions, against all advices. The problem is so major, that in April 2013 WHO (World Health Organization) released the *Emergency response to artemisinin resistance in the Greater Mekong subregion: Regional framework for action 2013–2015*, a document that describes priority areas in which action is needed in the coming years to

contain artemisinin resistance. Besides, significant research efforts are now focused as well on the new issue of artemisinin resistance monitoring (Ariey et al., 2014).

1.3.2.2 Vaccines

Vaccines represent one of the most cost-effective tools in the control of malaria. Despite years of intensive research, no licensed vaccine is available yet for malaria; therefore, international efforts from research laboratories, pharmaceutical companies and funding agencies are committed towards its development. However, there exist several candidates being evaluated at present.

Vaccine candidate RTS,S/AS01 is in phase 3 clinical trials. It has been engineered based on a protein expressed by *Plasmodium* sporozoites (circumsporozoite protein, CSP) together with a hepatitis B surface antigen, for targeting of the pre-erythrocytic stage of the disease. First results with African children have shown a 50% reduction in malaria (Agnandji et al., 2011) and more promising results have followed (Otiene, 2013). Other attempts to target the pre-erythrocytic stage of infection for vaccine production consisted in immunizations with irradiated sporozoites (Hoffman et al., 2010) and development of genetically attenuated parasites incapable of progressing beyond the liver stage of infection (Vaughan et al., 2010).

An antimalarial vaccine will undoubtedly be an achievement, as repeated exposure to *Plasmodium* parasites results in some degree of naturally acquired immunity. It is thought that vaccination with parasite antigens can accelerate immunity, hence some *Plasmodium* blood stage antigens (including apical membrane antigen 1 (AMA1), erythrocyte binding antigen 175 (EBA-175), glutamate-rich protein (GLURP) and merozoite surface protein 1 (MSP-1)) have been targeted in vaccine development (Sagara et al., 2009; El Sahly et al., 2010; Hermsen et al., 2007; Hill, 2011).

Another strategy consists in the development of transmission-blocking vaccines that fight the disease at the vector level, thus interrupting its transmission to the human host. Pfs25, Pfs48/45, Pfs230 and Pfs28 are examples of *P. falciparum* surface proteins expressed during the sexual stage development inside the mosquito, that are candidate antigens currently being used as anti-*Plasmodium* transmission blocking targets (Wu et al., 2008; Chowdhury et al., 2009; Williamson, 2003; Hisaeda et al., 2000).

1.4 *Anopheles* Vector

Malaria is spread from one person to another through the bite of the female *Anopheles* mosquito. The *Anopheles* genus comprises more than 400 different species, from which approximately 70 species can transmit human malaria parasites and about 40 species are dominant vectors capable of transmitting malaria at a level of major concern to public health (Hay et al., 2010a; Sinka et al., 2012) (Figure 2). Besides the malaria endemic areas, several other zones of the globe, such as those where malaria has been eliminated but the vector mosquito is present, can be dangerously at risk of re-introduction of the disease (CDC, 2012).

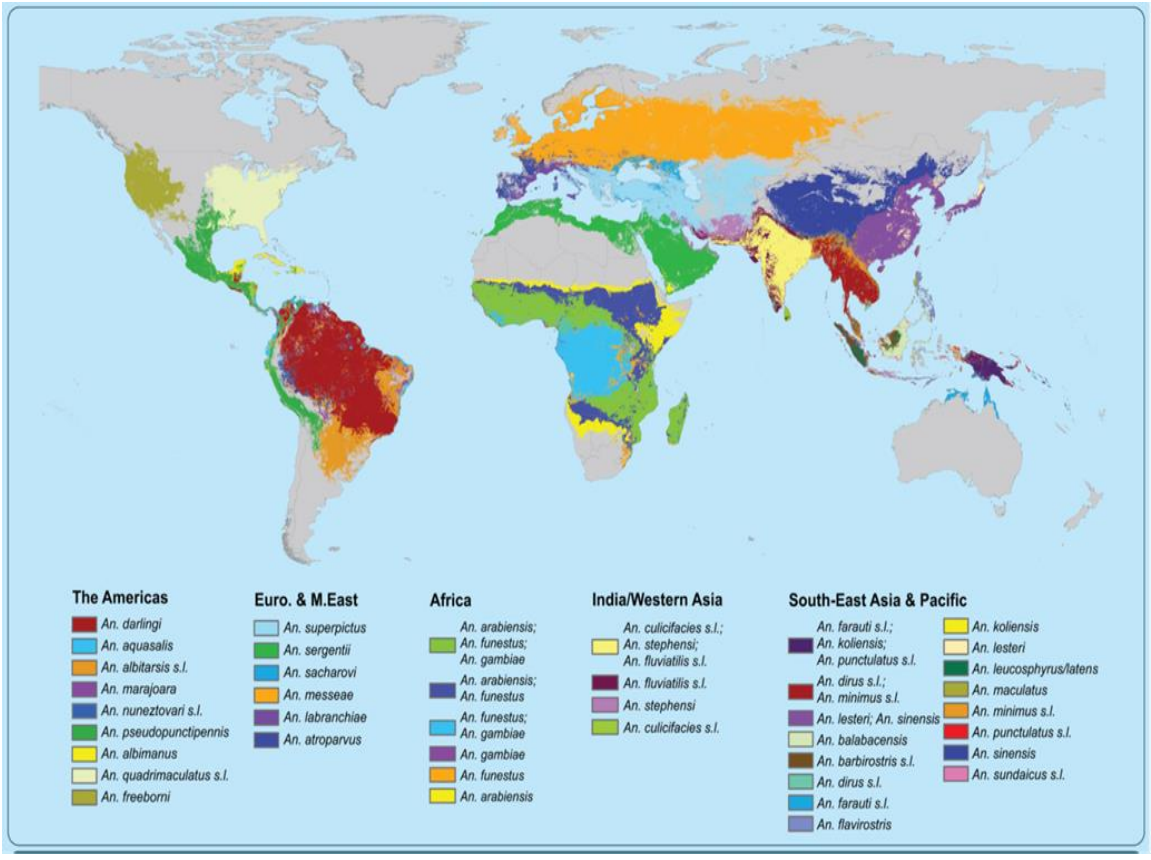


Figure 2. Global distribution of dominant malaria vectors. Adapted from Sinka et al., 2012.

As mentioned above, Africa contains areas with the highest malaria morbidity and mortality in the world, given its highest entomological inoculation rates (Hay et al., 2010b) and prevalence levels globally (Hay et al., 2009). This situation arises partly because here resides the most effective and efficient human malaria vector, *Anopheles gambiae* sensu stricto (*A. gambiae* s.s.) (Coluzzi, 1999), the major vector of the deadliest

human malaria parasite, *P. falciparum*. Although a vast number of *Plasmodium* species and *Anopheles* vectors exist, only a small number of laboratorial models is used. Several studies are mostly based on *A. gambiae* s.s. (hereupon referred as *A. gambiae*), a suitable model for studying the cellular and molecular interactions between vector and parasite, and for which the genome sequencing offers a fundamental tool (Holt et al., 2002).

A. arabiensis, another human malaria vector belonging to the *A. gambiae* complex (which includes at least seven morphologically indistinguishable *Anopheles* species with marked differences in their vectorial capacity (Bass et al., 2007; Sinka et al., 2010)) is also of major importance in sub-Saharan Africa. *A. funestus* is another very antropophilic African vector, which in some cases has a greater impact on human malaria transmission than *A. gambiae* and is considered to be one of the first species to have adapted to the human host (Charlwood et al., 1995; Sinka et al., 2010).

Central and Southeastern Asia is the second area in the world for malaria burden; the Asian-Pacific region has a high diversity of vector species, as shown in the map from Figure 2. In South America, *A. darlingi* is the dominant species, outpaced by *A. albimanus* and *A. pseudopunctipennis* in Central America (Figure 2).

1.4.1 Vector Life Cycle

Anophelines, as any other mosquitoes, undergo four different developmental stages in their life cycle: egg, larva, pupa and adult (Figure 3). The immature phases (before they reach adulthood) are aquatic and can last up to 15 days. Eggs (50-200 eggs per oviposition) are laid on water. Larvae are filter-feeders, feeding on micro-organisms and decaying plant matter, and develop through four stages or instars (L1-L4). Larvae metamorphose into pupae, a stage that doesn't feed but is particularly active. Adults emerge one to three days after pupation; male adults have a shorter life-span than females, which can live up to five weeks in optimal conditions (reviewed by Knell, 1991). Within 24 h (hour) after emerging, the female *Anopheles* is able to mate. Female copulation is almost always a once in a lifetime event and the sperm from this single copulation is stored for all following egg production (Baldini et al., 2013). Indeed, the final act in copulation is the male injection of the mating plug, which blocks the passage of sperm from any subsequent copulations (reviewed by Knell, 1991).

Both females and males feed on plant juices and nectar, but only females feed on blood (hematophagy) (Figure 3). When a female mosquito has mated and ingested its first blood meal in a vertebrate host, it uses the protein of the blood as the basis for the production of a batch of eggs (Dana et al., 2005), which will then be deposited near water for hatching. Therefore, the transmission of the *Plasmodium* parasite by the *Anopheles* vector mosquito is only possible by hematophagy, which is essential for egg production (Dong et al., 2006). The gonotrophic cycle (comprising the host seeking, hematophagy, egg development and oviposition) involves a series of complex biological events, including the formation of the peritrophic matrix, acting as a barrier between the ingested blood and the midgut epithelium of the mosquito (Dana et al., 2005).

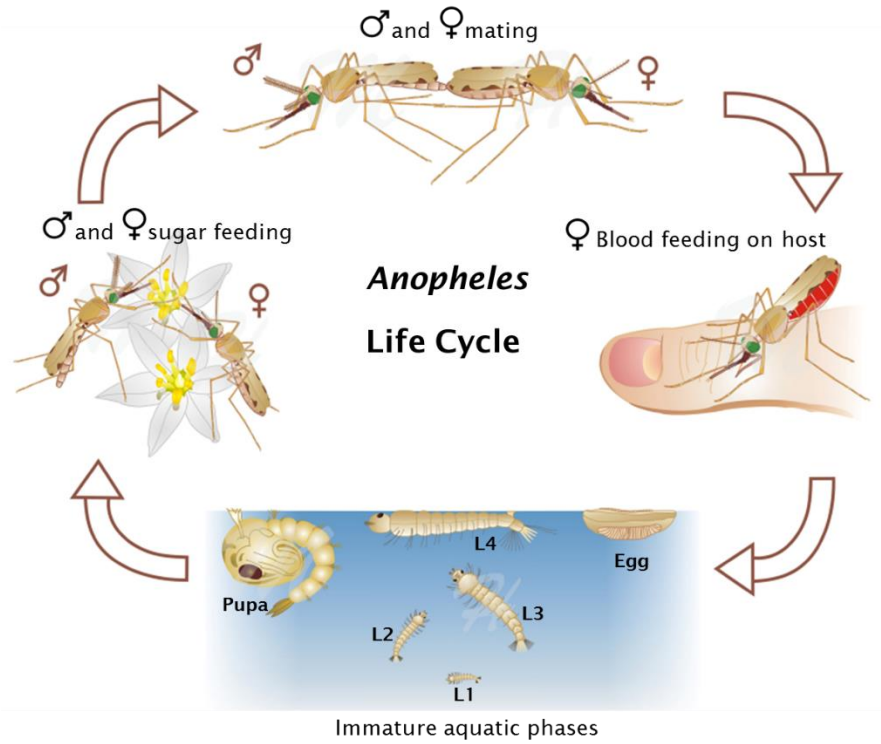


Figure 3. *Anopheles* life cycle. Adapted from http://www.biographix.cz. L1-4- larval stages 1-4.

1.4.2 Classical Vector Control Strategies

Since the discovery of the connection between *Anopheles* vectors and malarial transmission in 1897, vector control strategies have been the most widely used malarial control measures around the world (reviewed by Mabaso et al., 2004). Together with control strategies to reduce *Plasmodium*, the malaria causative agent, policies improvement and creation of new strategies to control the *Anopheles* vector mosquito are being taken, with the final aim of eradicating this devastating disease.

1.4.2.1 Chemical Control by IRS or ITNs

Nearly 70 years ago, the advent of insecticide DDT (dichlorodiphenyltrichloroethane) raised hopes of what was then thought to be the beginning of the worldwide eradication of malaria, through an ambitious programme of systemic control of its vector mosquito. Although indoor residual spraying (IRS) by means of DDT has effectively reduced the mosquito populations in many countries throughout the world (malaria was completely eliminated from Europe, the USA and the former Soviet Union during the 1960s (reviewed by Mabaso et al., 2004)), mosquito resistance to DDT quickly appeared. Together with *Plasmodium* chloroquine resistance and socio-economic-environmental factors, this unfortunate happening compromised the ambitious control programme established by WHO in 1955.

Besides IRS, the most widespread method for the control of adult *Anopheles* is insecticide-treated bednets (ITNs), which provide a protective barrier that prevents vector-host contact. WHO (2013) recommends that, in areas targeted for malaria vector control, all persons at risk should be protected by ITNs or IRS, vector control interventions with demonstrated impact on reducing malaria (reviewed by Lengeler, 2004; reviewed by Pluess et al., 2010); however, this is not the situation yet (Figure 4). New tools, such as long-lasting insecticide treated nets (LLINs, with a typical lifespan of three years) are being implemented, but the cost of such bednets is usually unaffordable to people in developing countries. The only insecticides currently used in most IRS programmes and to treat bednets are pyrethroids; nevertheless, the development of pyrethroid resistance in *A. gambiae* has already been reported (Trape et al., 2011). Recognizing the threat posed by insecticide resistance, WHO released the *Global Plan for Insecticide Resistance Management in Malaria Vectors*, in May 2012.

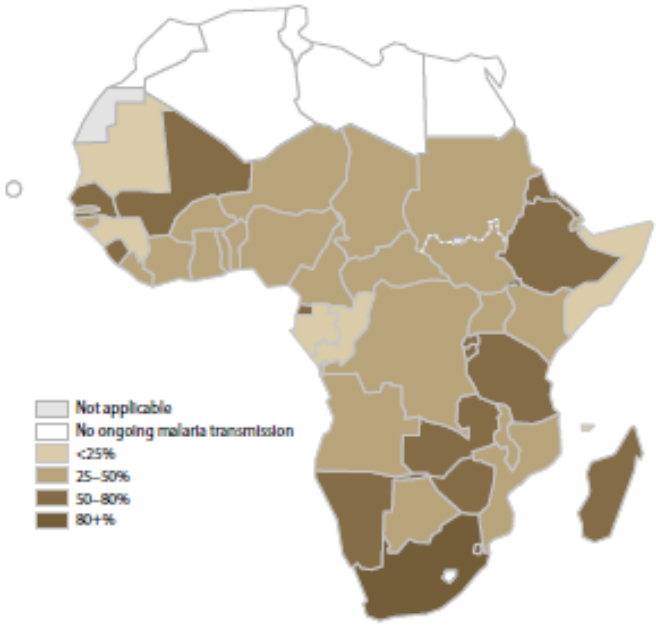


Figure 4. Proportion of population at malaria risk protected by ITNs or IRS, in sub-Saharan Africa in 2012. Adapted from WHO, 2013.

1.4.2.2 Biological Control

Biological control methods are used for control of *Anopheles* vector population at larval and adult stages. Tools for larval control have found some success and include the use of larvivorous fish (that feed on larvae) (Mohamed, 2003; Ghosh et al., 2005), as well as microbial larvicides, formulated to deliver toxins from bacteria such as *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*, that disrupt the cells from the larva gut (Majambere et al., 2007). For the control of adult *Anopheles* vectors, entomopathogenic fungi (which infect insects), such as *Beauvaria bassiana*, have been used with good achievement (Kikankie et al., 2010).

A current trend in vector biology laboratories is the study of the interactions between the mosquito midgut flora and the malaria parasite. In fact, several recent studies revealed that the gut microbiota are able to modulate the vectorial capacity of the *Anopheles* mosquito. More studies are needed to understand the molecular mechanisms that regulate this interaction.

1.4.2.3 SIT

Other biological strategies include the sterile insect technique (SIT), a non-polluting species-specific method of insect control that involves the sterilization of a large number of males, regularly by irradiation, but also by genetic alteration. These sexually active males will mate with wildtype virgin females, resulting in sterile offspring that will thus contribute to population decline. Successful application of this method in *Anopheles* mosquitoes has been achieved in an isolated region of El Salvador, resulting in the eradication of the local *A. albimanus* population (Lofgren et al., 1974). However, SIT has faced several problems which exclude its implementation as universal method for vector control, namely the loss of sterile male fitness following irradiation and the need for laboratorial sex separation to have large numbers of sterile male mosquitoes.

1.4.3 Novel Vector Control Strategies

Genetic manipulation of the mosquito genome has been suggested as another vector control strategy, through either the suppression/eradication of malaria-transmitting *Anopheles* populations or replacement of these populations with mosquitoes refractory to the *Plasmodium* parasite (Marrelli et al., 2006).

1.4.3.1 Population Suppression

1.4.3.1.1 RIDL

Given the several hurdles with SIT implementation mentioned above, a modification to this technique has been proposed, whereby insects are released carrying a repressible dominant lethal gene (RIDL) incorporated into their genome (Thomas et al., 2000). This gene kills the mosquito but can be repressed by an external additive, tetracycline (Alphey, 2002). The more advanced forms of RIDL use a female-specific dominant lethal gene, in which the lethal action of the system is active in females only. This way, the need for a sex separation step as in SIT is avoided, as the tetracycline repressor can be removed from the final stage of mosquito laboratorial rearing, killing all females and leaving males only (Alphey et al., 2002).

Anopheles populations are certainly a possible target for RIDL technology (Nolan et al., 2011a). Homozygous RIDL males could be released into nature, where they would mate with wildtype females; RIDL males would be fitter than SIT males, as they do not suffer

irradiation (Alphey, 2002). Because lethality is female-specific and the lethal gene is dominant, this cross would result in offspring which would only survive if they are males-hence, the female population would be reduced, constituting a very beneficial method of population control, given the central role of *Anopheles* females in malaria transmission. The new males would be heterozygous RIDL and would subsequently pass the lethal gene to a proportion of the following generation, further reducing the target population.

1.4.3.1.2 Homing Endonucleases

Another approach to distort the sex ratio in natural populations is based on the use of engineered mosquitoes expressing a homing endonuclease enzyme targeting the X chromosome. The activity of the X chromosome cutting enzyme intron-encoded endonuclease I-PpoI has been explored to bias the sex ratio in *A. gambiae* mosquitoes (Windbichler et al., 2008; Galizi et al., 2014). This enzyme selectively cleaves a deoxyribonucleic acid (DNA) sequence from the centromeric region of chromosome X, leading to nucleolar fragmentation and cell death. Authors engineered *A. gambiae* male mosquitoes to heterozigously express I-PpoI during spermatogenesis and crossed these males with wildtype females. This resulted in complete early dominant embryo lethality and a strong, negative bias toward X chromosome-carrying spermatozoa (Windbichler et al., 2008; Galizi et al., 2014). This could thereby lead to the reduction or eradication of field populations, through distortion of the sex-ratio in favour of males (Windbichler et al., 2008). The implementation of this strategy to mosquito control in the field is currently under evaluation.

1.4.3.2 Population Replacement

Blocking the transmission of *Plasmodium* using the transgenic manipulation of its mosquito vector, has been achieved in different malaria-transmitting species, such as *A. stephensi* (Ito et al., 2002; Moreira et al., 2002; Yoshida et al., 2007; Dong et al., 2011; Isaacs et al., 2011) and *A. gambiae* (Kim et al., 2004; Meredith et al., 2011). It constitutes a promising malaria control strategy, presently being applied in several vector-studying laboratories. In theory, these *Plasmodium* refractory mosquitoes should be capable of replacing the wild populations and some semi-field studies are currently taking place to validate the implementation of this technology. In such process, a gene's coding region, driven by a tissue- and time-specific promoter, is transformed into the mosquito vector.

In the case of *Anopheles* transgenic manipulation, this could result in the overproduction of an anti-*Plasmodium* factor or silencing of a *Plasmodium* positive regulator, culminating in a decline in the overall number of parasites, therefore interfering with *Plasmodium* cycle through reduction of transmission to the vertebrate host.

Choosing the right promoters to drive the expression of particular effector genes in a stage- and tissue-specific manner is a critical step in engineering transgenic lines for the mosquito. In *Anopheles*, tissue-specific promoters have been widely used to target specific organs, with main focus on the midgut, fat body/hemocoel and salivary glands, the key target organs involved in critical steps of the *Plasmodium* sporogonic cycle. In *Anopheles*, the most commonly used midgut-specific promoter is the *A. gambiae* carboxypeptidase A (Ito et al., 2002; Moreira et al., 2002; Yoshida et al., 2007; Dong et al., 2011; Isaacs et al., 2011; Meredith et al., 2011). The use of another midgut-specific promoter, G12, has been recently validated in *A. stephensi* (Nolan et al., 2011b) as well. Concerning the fat body, the most frequent specific promoters for transgenic studies are the *Anopheles vitellogenin* genes 1 (Dong et al., 2011; Isaacs et al., 2011) and 2 (Chen et al., 2007), which are fat body-specific and blood meal induced. Both the carboxypeptidase and vitellogenin promoters have been extensively used in order to target the malaria parasite at its early stages of development.

1.5 *Anopheles gambiae* Immune System

Given the challenge of finding effective ways to reduce the burden caused by malaria, a wealth of knowledge on the interaction parasite-vector and the mosquito immunity to *Plasmodium* has been building up in the last decade. Several experimental studies have demonstrated that *Anopheles* mosquitoes are able to mount an efficient immune response against *Plasmodium* infection, and that this results in major losses for the parasite during its development inside the vector. The mosquito immune defence involves complex mechanisms of action, which will be described below.

1.5.1 *Plasmodium-Anopheles* Interactions

As previously explained, *Plasmodium* parasites live within three main compartments inside the mosquito vector: the midgut, the hemocoel and the salivary glands. All three of these constitute physical barriers against invasive agents. Moreover, the cells within

these compartments produce immune factors with antimicrobial activity, as we will later discuss in further detail. During the sporogonic cycle (Figure 1B), the parasite experiences three main bottlenecks, when its numbers are largely reduced: 1) the transition between gametocytes and ookinetes, 2) between ookinetes and mature oocysts and 3) between midgut sporozoites and salivary gland sporozoites (Figure 5). Indeed, it is said that *A. gambiae* mosquitoes can kill around 80% of invading *Plasmodium* ookinetes (Lavazec and Bourgouin, 2008).

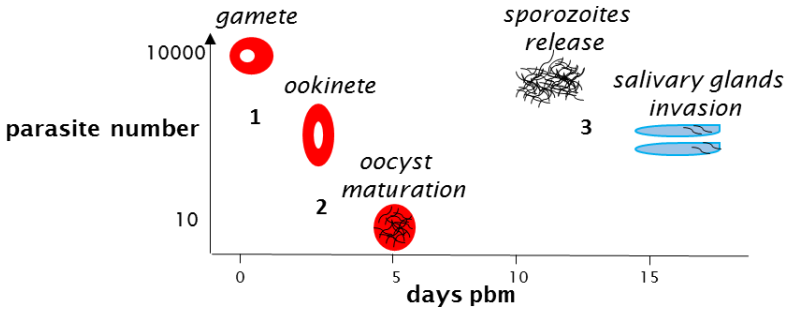


Figure 5. Three main *Plasmodium* bottlenecks during the sporogonic cycle. Adapted from Simões and Chandrasekar, 2014. pbm (post blood meal).

In vertebrates, the fight against invasive pathogens is a result of the interaction between adaptive and innate immunity. Insects, however, lack an adaptive immune system, and thus rely solely on innate immunity for their defence. Insect blood cells (hemocytes), play a prominent role in their immune response, through phagocytosis and encapsulation of foreign invaders (cellular response), as well as their melanization. Hemocytes, together with the fat body, are also able to release immune effectors into the insect circulatory fluid (hemolymph). The production of these antimicrobial effector molecules is regulated by intricate intracellular immune signalling pathways.

1.5.2 Pattern Recognition Receptors (PRRs)

Pathogen elimination in both vertebrates and invertebrates is initiated by pattern recognition receptors (PRRs), which sense, recognize and bind to pathogen associated molecular patterns (PAMPs). Insects fight microbial infections using a limited number of PRRs. In *A. gambiae*, about 150 putative PRRs have been identified (reviewed by Das et al., 2009), which are mostly members of large gene families, such as the thioester-

containing proteins (TEPs), leucine-rich repeat proteins (LRRs), C-type lectins (CTLs) and fibrinogen-related proteins (FREPs or FBNs), the latest being the largest PRR family in this vector (Dong and Dimopoulos, 2009). Among these families are some of the most potent anti-*Plasmodium* immune factors identified to date.

1.5.2.1 TEPs and LRRs

The most studied member of the TEPs family is TEP1 (thioester-containing protein 1), a hemocyte-specific protein first described as being involved in bacterial opsonization (Levashina et al., 2001) and later as mediator of *P. berghei* ookinete destruction in *A. gambiae* (Blandin et al., 2004). TEP1 activation and stabilization is achieved by interaction with LRIM1 and APL1C (leucine-rich repeat immune protein 1 and *Anopheles Plasmodium*-responsive leucine-rich repeat protein 1, PRR proteins of the LRR family) to form a complex prior to binding to ookinetes in the midgut, mediating their lysis (Baxter et al., 2010). The composition of this complex (TEP1, a complement-like protein with structural similarities to vertebrate complement component C3 (Baxter et al., 2007), and two LRR proteins), suggests that mosquitoes have a complement-like system comparable to the one in mammals, which is able to effectively fight *Plasmodium* infection. Independent silencing of these three genes has shown that each one is individually relevant to control the development of *P. berghei* oocysts, besides playing an important role in parasite melanization (Habtewold et al., 2008), an effective immune killing mechanism involving the deposition of melanin on the parasite surface. Another key anti-*Plasmodium* protein of the LRR family is LRRD7 (leucine-rich repeat–containing protein 7, also designated as APL2, *Anopheles Plasmodium*-responsive leucine-rich repeat 2) (Dong et al., 2006).

1.5.2.2 CTLs

Acting in an opposing manner, *A. gambiae* CTL4 and CTLMA2 (C-type lectin 4 and CTL mannose binding 2) seem to be agonists of *Plasmodium* infection in the midgut, where their silencing results in strong ookinete melanization and reduction in the number of oocysts (Osta et al., 2004). Recently, these two CTLs have been identified as essential for mosquito defence against Gram-negative, but not Gram-positive bacteria (Schnitger et al., 2009). The mechanism by which some PRRs fight the parasite in the vector, while other seem to protect it from the mosquito immune responses, is under study.

1.5.2.3 FREPs

Fibrinogen-related proteins (FREPs) constitute a large PRR family in mosquitoes, with 59 genes identified in *A. gambiae* and 37 genes in *Aedes aegypti*. This family seems particularly broad in the mosquito, as only 14 such genes have been reported in *Drosophila melanogaster* (Wang et al., 2005; Waterhouse et al., 2007; Dong and Dimopoulos, 2009). The majority of FREPs in mosquitoes are up-regulated upon immune challenge and several have anti-*Plasmodium* activity. FBN9, an important member of the family mediating *Plasmodium* killing, co-localizes with the ookinete stage of both *P. berghei* and *P. falciparum* parasites in the midgut (Dong and Dimopoulos, 2009). Evidence from observations in vertebrates and co-localization at parasite surface led Garver et al. (2009) to speculate that a mechanism similar to the lectin complement pathway, in which TEP1 and FBN9 cooperate to destroy pathogens, may also exist in mosquitoes.

1.5.2.4 PGRPs

Another important PRR family within the mosquito, the peptidoglycan recognition proteins (PGRPs), with seven genes described in the *A. gambiae* genome (Christophides et al., 2002), is also involved in the activation of various immune reactions in the *Anopheles* mosquito.

1.5.3 Serine Proteases

Serine proteases are proteolytic enzymes present in the hemolymph, where they can rapidly activate immune pathways in response to pathogen detection by PRRs, amplifying the signal and triggering downstream effector responses to eradicate the pathogen invader. The most important components of the serine protease cascade are the CLIP domain serine proteases, 41 of which have been identified in *A. gambiae* so far. Many CLIPs are involved in the melanization of *P. berghei* ookinetes, where they have roles as both positive and negative regulators of this antiparasitic process. CLIPA2 and CLIPA7 are inhibitors of parasite melanization (Osta et al., 2004), hence playing a role in parasite protection, while CLIPA8 and CLIB17 seem to be essential to activate the enzyme prophenoloxidase (PPO), responsible for melanin synthesis (Volz et al., 2006) and thus melanization. Moreover, other CLIPs such as CLIPB14 and CLIPB15, are involved in *P.*

berghei elimination in *A. gambiae*, in a melanization-independent way (Christophides et al., 2002).

Serpins (serine protease inhibitors) are regulators of the serine protease cascade and, similarly to CLIPs, modulate melanization responses. SRPN2 (serpin 2) inhibits melanization of *P. berghei* ookinetes in *A. gambiae* (Michel et al., 2005). It is thought that this protein may interact with CLIPs, CTLs and LRIM1, other molecules known for interfering in the melanization process.

1.5.4 Antimicrobial Peptides (AMPs)

Besides melanization, the humoral responses to pathogen invasion culminate with the production of antimicrobial peptides (AMPs), which are synthesized by the fat body and hemocytes and secreted into the hemolymph upon immune challenge. AMP production constitutes the ultimate step of the defence mechanisms employed by *Anopheles* mosquitoes to fight *Plasmodium*. In *A. gambiae*, four AMP families have been identified to date, comprising four defensins (DEFs), four cecropins (CECs), one attacin and one gambicin (GAM1) (Christophides et al., 2002). Their antimicrobial action comprehends responses against Gram-negative (mainly CEC1 and GAM1) and Gram-positive (mainly DEF1) bacteria. Some are known to have an anti-*Plasmodium* role in *A. gambiae* as well. *CEC1* was one of the first anti-*Plasmodium* factors to be identified (Luckhart et al., 2003). This gene was significantly up-regulated following *Plasmodium* infection (Christophides et al., 2002) and its ectopic overexpression was shown to increase *A. gambiae* resistance to *P. berghei* (Kim et al., 2004). In a more recent study, silencing of *GAM1* resulted in increased *P. berghei* infection (Dong et al., 2006). Defensins are the most extensive insect family of AMPs and play an essential role in the innate immunity of virtually almost all life forms from plants, to insects, amphibians and mammals. Transcriptional regulation of AMPS is controlled by immune signalling pathways.

1.5.5 Immune Signalling Pathways

In both mammals and insects, recognition of PAMPs by PAMP-associated PRRs is followed by signal transduction through immune signalling pathways that activate Nuclear Factor-kappaB (NF-κB)/Rel transcription factors. These factors translocate to the nucleus to initiate the transcription of an array of effector genes, including AMPs. In *Drosophila*, two immune signalling pathways, the Toll and Immune deficiency (Imd)

pathways, have been extensively studied. The Toll pathway is triggered by fungi and Gram-positive bacterial infections, leading to the nuclear translocation of NF-κB transcription factor Dif, as well as Dorsal, which is involved in early developmental processes, whilst the Imd pathway signalling results in nuclear translocation of Relish, following Gram-negative bacterial infection. Both factors induce the transcription of all the AMPs encoded in the *Drosophila* genome (reviewed by Lemaitre and Hoffmann, 2007).

Sequencing the *A. gambiae* genome allowed the comparison of putative immune related genes between *Anopheles* and *Drosophila* (Holt et al., 2002), supporting the conclusion that most intracellular components of these pathways are conserved in the mosquito. A remarkable difference between the two evolutionarily related insects is the lack of Dif in *Anopheles*; therefore, the malaria mosquito genome encodes two NF-κB transcription factors only: REL1 (the orthologue of Dorsal, previously called Gambif1 (Barillas-Mury, 1996)) and REL2, *Drosophila* Relish orthologue.

Both *Anopheles* REL proteins are directly involved in the immune response against invaders. They translocate to the nucleus to initiate the transcription of many effector genes and REL-dependent transcription has been shown to be particularly critical to the mosquito´s ability to manage infection with *P. berghei* and *P. falciparum* (Meister et al., 2005; Frolet et al., 2006; Luna et al., 2006; Hoa and Zheng, 2007; Riehle et al., 2008; Garver et al., 2009, 2012; Chen et al., 2012; Clayton et al., 2013). Therefore, REL1, REL2 and their associated pathways are critical targets for the development of anti-malaria strategies based on vector-parasite interactions (Dong et al., 2011).

1.5.5.1 The Toll Pathway

In humans and mice, respectively ten and twelve Toll-like receptors (TLRs) have been characterized thus far. TLRs play a critical role in the early innate immunity against foreign pathogens. Their name derives from the firstly identified *Drosophila* Toll protein, which is stimulated by the binding of the proteolytically activated extracellular ligand, Spätzle (Weber et al., 2003), following fungal and Gram-positive bacterial infection. Upon activation by Spätzle, the intracytoplasmatic domain of the transmembrane Toll receptor (named TIR, Toll- interleukin-1-receptor), recruits three death-domain proteins, MyD88, Tube and Pelle. Thus forms the Toll receptor complex, which causes the

degradation of Cactus, an ankyrin-repeat protein, hence allowing the nuclear translocation of the above mentioned NF-κB transcription factors Dorsal and Dif.

In *A. gambiae*, the intracellular machinery of the Toll pathway is conserved. Orthologues of MyD88, Tube and Pelle, as well as negative regulator Cactus, have been identified in the malaria mosquito (Christophides et al., 2002). As mentioned, REL1 is the NF-κB factor controlling the transcription of genes regulated by the Toll pathway in *Anopheles*. The importance of REL1-pathway stimulation was made clear when knocking down of *Cactus* (by double-stranded ribonucleic acid, dsRNA, injection) dramatically decreased the parasite load of *P. berghei* in *A. gambiae.* Further, co-silencing of *REL1* and *Cactus* reversed the *dsCactus* phenotype (Frolet et al., 2006). Similar results were obtained by Garver et al. (2009), where a significant reduction in the infection levels of *P. berghei* was observed after Cactus depletion. The same authors have also shown that Cactus silencing altered the expression of several genes from other functional groups besides immunity, suggesting that the Toll pathway is a ubiquitous signalling pathway, with a wide-ranging action, this probably being the reason for the marked reduction in both longevity and fecundity shown in the *dsCactus* mosquitoes in this study (Garver et al., 2009). Importantly, in the above mentioned study (Frolet et al., 2006), it has been said that the mosquito basal immunity level (before the mosquito encounters the parasite) is a key factor for parasite control, i.e., it is significantly more influent in *Plasmodium* development than the induction of immune responses upon parasite infection.

1.5.5.2 The Imd Pathway

Activation of the immune deficiency (Imd) pathway starts when a pathogen is detected by a transmembrane PRR from the PGRPs family, a family of proteins conserved from insects to mammals. This pathway is similar to that of the mammalian tumor-necrosis factor (TNF) (Hoffmann, 2003; Aggarwal and Silverman, 2008). Intracellular signalling begins with the recruitment of IMD, a death domain adaptor located downstream of the receptor and upstream of a series of caspase-like proteins and kinases. This process culminates with caspase-dependent cleavage of Relish (Stoven et al., 2003; Kim et al., 2006), and the consequent release of its Rel-homology domain (RHD) from the inhibitory ankyrin domain. This cascade results in RHD nuclear translocation and the subsequent transcriptional induction of a battery of AMPs (Meister et al., 2005). Caspar has been

identified as a specific negative regulator of the Imd pathway, preventing the nuclear migration of Relish (Kim et al., 2006).

All components of the Imd pathway are conserved in the *Anopheles* mosquito (Christophides et al., 2002). Through alternative splicing, two isoforms of REL2, the Relish orthologue, are present in *Anopheles*: REL2-F (a full-length form) and REL2-S (a short-length form), the latter lacking the inhibitory ankyrin repeats and death domain present in REL2-F. Both transcripts are expressed constitutively throughout *A. gambiae*, as well as in cultured cell lines (Meister et al., 2005; Luna et al., 2006). In the absence of immune stimulation, REL2 exists in the two variants: REL2-S, that is constitutively active, and REL2-F, that is inactive until immune stimulus. Imd pathway activation stimulates cleavage of the inhibitory ankyrin terminal domain of REL2-F, exposing it to nuclear translocation and subsequent transcription initiation (reviewed by Cirimotich et al., 2010). Besides their role against *Plasmodium*, in *A. gambiae* REL2-F was shown to be involved in the defence against Gram-positive *Staphylococcus aureus*, and REL2-S against Gram-negative *Escherichia coli* (Meister et al., 2005), a contrast to the Gram-negative specificity of the Imd pathway in *Drosophila*.

Elucidation of the role of REL2 in fighting *Plasmodium* was obtained through observation that its knockdown increased the number of *P. berghei* oocysts in *A. gambiae* (Meister et al., 2005; Frolet et al., 2006). In a more recent study (Garver et al., 2009), depletion of Caspar reduced the number of *P. berghei* oocysts, in addition to almost abrogating *P. falciparum* infection in three *Anopheles* species: *A. gambiae, A. stephensi* and *A. albimanus*. In contrast to Toll, the Imd pathway is thought to be immunity-specific (Garver et al., 2009). Overall, its properties suggest that this pathway could be used in the development of malaria control approaches, through interference in the parasite sporogonic cycle. Indeed, target expression of REL2 has been recently used to create transgenic lines of *A. stephensi* mosquitoes with particularly reinforced immunity against *Plasmodium* and microbial infection (Dong et al., 2011).

1.5.5.3 The Jak/Stat Pathway

In addition to the Toll and Imd, there is a further mechanism of immune signalling, the Janus kinase-signal transducer and activator of transcription (Jak/Stat) pathway. This pathway has been intensively researched in *Drosophila* but still rather under-explored in

the context of *Anopheles-Plasmodium* interaction. Nevertheless, two Stat transcription factors, Stat-A and Stat-B (signal transducer and activator of transcription-A, B), have been identified in the *A. gambiae* genome (Christophides et al., 2002), with the first playing a role in the mosquito defence against *Plasmodium* (Gupta et al., 2009). In a more recent study, the Jak/Stat pathway was shown to be activated in *A. aquasilis* in response to *P. vivax* challenge, and an increase in *P. vivax* oocysts number was observed after depletion of this pathway (Bahia et al., 2011). The role of the Jak/Stat pathway in the activation of *Anopheles* immunity against the malaria parasite is subject of a growing interest and requires additional investigation. Together with the Imd/REL2 and Toll/REL1 pathways, this immune pathway could be seen as a potential target for development of malaria control strategies.

1.5.5.4 Regulatory Pathways of anti-*Plasmodium* Genes

The process of regulation for the expression of immunity-specific genes is dependent on the instigating PAMP, as well as the selectively activated pathway. PAMPs that are effective elicitors of the mosquito immune response are lipopolysaccharides (LPS), peptidoglycans (PGNs) and β-1, 3-glucans. Current studies are being developed to shed light on *Plasmodium*-specific PAMPs that activate the *Anopheles* immune system. One molecule that has already been identified as an inducer of AMPs production in *A. gambiae* is glycosylphosphatidylinositol (GPI), labelled as the prominent toxin that contributes to malaria pathogenesis in mammals, through the anchoring of parasite proteins on cellular plasma membranes (Arrighi et al., 2009). In our laboratory, we found that the *Plasmodium* metabolite hemozoin acts as a stimulator of *A. gambiae* immune response against *P. berghei*, as will be demonstrated in the present thesis. Both GPI and hemozoin induce the transcription of the enzyme nitric oxide synthase (NOS) (Lim et al., 2005; Akman-Anderson et al., 2007) and the resulting nitric oxide (NO) production prompts the killing of *Plasmodium* ookinetes in the midgut (Luckhart et al., 1998; Peterson et al., 2007). Inhibition of NOS was shown to increase the parasite numbers in *A. stephensi* mosquitoes (Luckhart et al., 1998). Together with reactive nitrogen species, the *Plasmodium* blood meal also activates the production of reactive oxygen species (ROS), to help containing parasite infection (Kumar et al., 2003; Molina-Cruz et al., 2008; Cirimotich et al., 2011; Bian et al., 2013).

In *A. gambiae,* both Toll/REL1 and Imd/REL2 pathways control the expression of important anti-*Plasmodium* factors. Among the most relevant genes described as having anti-malaria activity, are those of the AMPs DEF1, CEC1, CEC3 and GAM1, which appear to be controlled by both pathways/transcription factors. Silencing of the negative regulators of the Toll and Imd pathways (*Cactus* and *Caspar*, respectively) has up-regulated the levels of these four AMPs (Garver et al., 2009). Further, co-silencing of the negative regulator together with the correspondent transcription factor reversed this phenotype, indicating that both immune pathways control the expression of these important anti-*Plasmodium* genes (Garver et al., 2009). These results contradict a previous *A. gambiae* study, where Frolet et al. (2006) had also knocked down the Toll negative regulator *Cactus*, but instead did not observe a distinct up-regulation of any of these four AMPs. Luna and colleagues (2006) have shown *in vitro* (using mosquito cell lines) that both pathways regulate DEF1 and GAM1 expression. Double regulation of AMPs by the Toll and Imd pathways is not a particular *Anopheles* feature, as it has also been described in *Drosophila* (Kim et al., 2006; Tanji et al., 2007). Meister and co-workers (2005) classified *CEC1*, *CEC3* and *GAM1*, among other immune genes, as REL2-regulated, when they assessed gene expression profiles by DNA microarray following REL2 silencing in *A. gambiae* hemocyte-like cells; similarly, Hoa and Zheng (2007) showed a REL2-dependent up-regulation of CEC1 *in vitro*.

Alongside AMPs, some key *Anopheles* anti-malaria effectors belonging to the PRRs group, seem to be regulated by either of the immune pathways. TEP1 has been reported as REL1- and REL2-dependent. In the above mentioned study, Frolet et al. (2006) indicated that before *Plasmodium* invasion, TEP1 basal expression is controlled by both REL factors, but *Plasmodium*-dependent up-regulation of TEP1 is independent of REL1 and REL2. Subsequent studies (Garver et al., 2009; Dong et al., 2011) have shown that TEP1 expression is REL2-dependent. The combined outcomes of these studies suggest that this potent anti-*Plasmodium* factor regulation may not be pathway-specific. APL1C, a TEP1-interacting PRR in antimalarial defence, seems to be Toll pathway-dependent (Riehle et al., 2008), although in Frolet et al. (2006) the authors have not noticed any effect on this gene expression profile upon *REL1*, *REL2* nor *Cactus* silencing, and Garver et al. (2012) considered it to be an Imd pathway member. As for *LRIM1*, another key gene in the *Anopheles* immune response, it was shown to be Imd-regulated in *A. gambiae* cells

(Meister et al., 2005), although a subsequent study (Fraiture et al., 2009) suggested a REL1/Caspar-dependency. Other very important PRRs that act as *Plasmodium*-fighting genes, *FBN9* and *LRRD7*, are thought to be regulated by the Imd-REL2 pathway (Dong et al., 2011; Chen et al., 2012). Moreover, another two immunity factors involved in the *Anopheles* anti-malaria response, *TEP3* and *TEP4*, are likely to be REL2-regulated genes (Luna et al., 2006; Hoa and Zheng, 2007; Garver et al., 2009).

In conclusion, REL1 and REL2 control the *A. gambiae* immune response against *Plasmodium* by regulating the expression of antiparasitic genes. This regulation can be mediated by each transcription factor independently, or, as seen in many cases, both factors can be involved in the transcription of the same gene. The disclosure of the specific regulation for each anti-*Plasmodium* effector is an essential question that awaits further investigation.

1.6 Hemozoin as an Immunity Activator

The *Plasmodium* metabolite hemozoin is a byproduct of the parasite's digestion of host hemoglobin within the erythrocyte. Hemozoin is structurally similar to β-hematin, which is composed of cyclic heme dimers (Fe^{III}-protoporphyrin IX). Heme dimers interact through hydrogen bonds, forming hemozoin crystals (Figure 6).

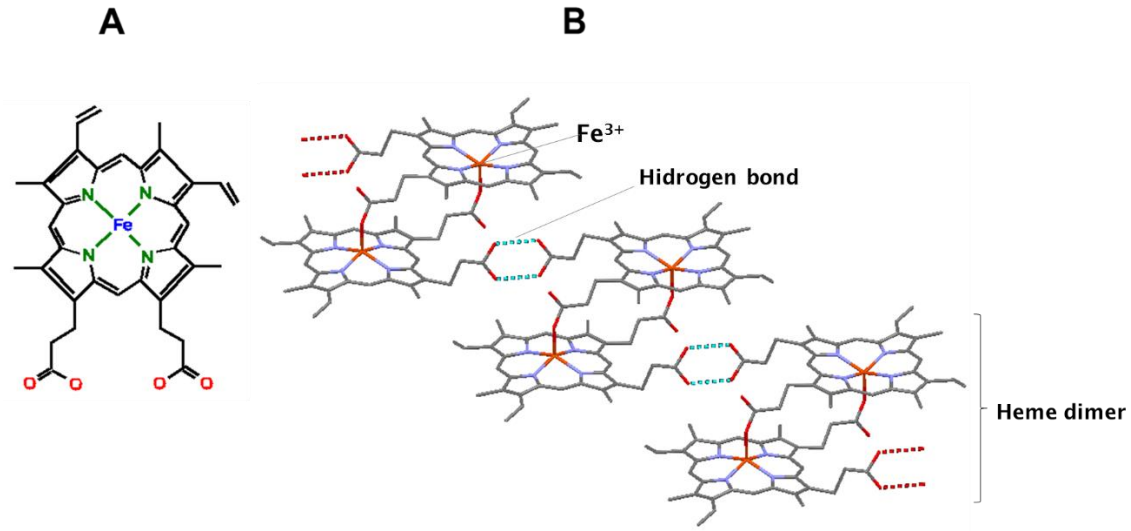


Figure 6. Structure of heme (A) and hemozoin (B). (A) The structure of heme consists of a large ring, porphyrin, with an iron atom in the center (protoporphyrin IX). **(B)** The basic unit of

hemozoin crystals is β-hematin, formed by two heme molecules. Adapted from http://www.tulane.edu.

Hemozoin is released together with merozoites by the rupture of parasitized erythrocytes. It reaches high concentrations in the circulation, and is engulfed by macrophages, monocytes, neutrophils and other immune cells (Arese and Schwarzer, 1997). It has been suggested that free hemozoin leads to the continuous targeting of the host innate immune system in mammals (reviewed by Coban et al., 2010a). In fact during the last decade it has emerged as a potent immunoactivator, both *in vitro* and *in vivo* (Jaramillo et al., 2009). Indeed, hemozoin continuously activates macrophages and dendritic cells to produce pro- and anti-inflammatory cytokines and chemokines in mouse and human cells (reviewed by Coban et al., 2009, 2010a; Jaramillo et al., 2009). Although hemozoin's pro-inflammatory role is generally accepted, the recognition and host response to this molecule, as well as the molecular mechanism(s) by which it activates the mammalian innate immune system, has been a subject of intense debate.

Coban et al. (2005) first identified TLR9 (Toll-like receptor 9, the mammalian receptor for unmethylated CpG (cytosinetriphosphate deoxynucleotide, phosphodiester, guaninetriphosphate deoxynucleotide) motifs in microbial DNA and synthetic oligonucleotides (Hemmi et al., 2003)), as mediator of the host response to hemozoin in a murine model. Parroche et al. (2007) further reported that hemozoin is immunologically inert and that the activation of TLR9 is caused instead by hemozoin-conjugated parasite DNA. Recently, a study by Griffith and colleagues (2009) showed that naïve dendritic cells or macrophages are not stimulated by hemozoin, but it augments the inflammatory response to malarial DNA or TLR ligands, supporting previous results (Parroche et al., 2007). Simultaneously, the work presented by Jaramillo et al. demonstrates that hemozoin possesses immunostimulatory properties by itself and it is unlikely that its immune activity is caused by the ability to bind parasitic DNA (Jaramillo et al., 2009), in contrast with mentioned results (Parroche et al., 2007; Griffith et al., 2009). A more recent study by Wu et al. (2010) affirms that hemozoin is neither stimulatory by itself for activation of mouse and human dendritic cells, nor is it able to confer activity to parasite DNA. Their data also shows that hemozoin is not a TLR9 ligand; indeed they are not the first suggesting that TLR9 has no role to play during malaria infection in both humans and

mice (Togbe et al., 2007; Lepenies et al., 2008). Similar to the hemozoin TLR9 activation of the immune system, the activation through the NLRP3 (NLR family, pyrin domain containing 3) inflammasome is also being extensively debated (Griffith et al., 2009; Shio et al., 2009).

In contrast to numerous studies regarding hemozoin immunity activation in mammals, one single study exploring the effect of this molecule in the *Anopheles* vector has been published so far (Akman-Anderson et al., 2007). In mosquitoes, hemozoin is released from parasitized erythrocytes and leukocytes which enter the midgut with the infected blood meal (Akman-Anderson et al., 2007). *Anopheles gambiae's* peritrophic matrix, acting as a barrier between the ingested blood and the midgut epithelium, is completely formed by 24 h after a blood meal (Dinglasan et al., 2009), which suggests that the midgut epithelium is possibly exposed to hemozoin before the membrane is fully formed (Akman-Anderson et al., 2007). The authors of the mentioned study (Akman-Anderson et al., 2007) demonstrated that both *P. falciparum* and synthetic (sHz) forms of hemozoin contribute to the immune activation in *Anopheles*; in accordance with similar observations in mammalian cells (Jaramillo et al., 2003), hemozoin was found to induce NOS expression in mosquito cells and tissues via multiple signalling pathways.

1.7 Aim

The failure to stop the devastation caused by malaria has stimulated research efforts to understand this disease from the vector perspective, aiming at the development of novel antimalarial strategies. Experimental evidences have revealed the importance of the *Anopheles* immune response against *Plasmodium*; even so, natural immunity is not enough to totally suppress mosquito infection. More effective protective immune responses to the parasite could be tailored by modulation of the mosquito immune system. In our laboratory we are interested in studying the role of immunostimulatory molecules which are potential boosters of the *Anopheles* innate immune system. The interplay between hemozoin and mammalian innate immunity has raised controversy, with some studies showing that hemozoin activates immunity, and others stating that its role as an immunity stimulator is marginal. Moreover, analysis of literature shows that the mosquito

immune response to hemozoin is not yet well characterized and requires further elucidation.

To address these issues, in this study we dissect the activity of this *Plasmodium* metabolite, while unraveling the mechanisms behind its triggering of the immune system of the malaria vector *A. gambiae*. Bearing in mind that *A. gambiae* is not the natural vector for *P. berghei*, this pair was used as a laboratory model, as it is conventionally used for the study of malaria transmission.

Following identification of a set of key immunity genes induced by this parasite's byproduct, as described in the results section of this thesis, our next purpose was to generate transgenic mosquitoes with an enhanced immune response against the parasite. Encouraged by the properties of the Imd/REL2 pathway suggested by the obtained results, and motivated by recent publications (e.g. Dong et al., 2011), in which these properties were explored to construct engineered mosquitoes with an increased robustness against the malarial parasite, we aimed to develop a genetically modified immune enhanced *A. gambiae* line, by overexpression of *FBN9* antiparasitic gene under regulation of the fat body-specific Vitellogenin 1 promoter.

2. Material and Methods

2.1 Ethics Statement

The maintenance and care of experimental animals was carried out in strict accordance with the recommendations in the Europe Directive 86/609/EEC (Louhimies, 2002) and Portuguese law (Decreto-Lei 129/92) for biomedical research involving animals, and was approved by the Divisão Geral de Veterinária (DGV), Portugal. All experiments were performed under anaesthesia, and all efforts were made to minimize animal suffering.

2.2 Synthetic Hemozoin (sHz) Preparation

sHz was prepared from high-purity hemin chloride, using a protocol as described in (Jaramillo et al., 2009; Shio et al., 2009). Briefly, 500 mg (milligram) hemin (≥ 98% pure, Sigma) were dissolved in degassed sodium hydroxide (NaOH, 0.1 molar (M), 100 milliliter (ml)) and pH adjusted with propionic acid. The mixture was heated at 70 ºC for 18 h. After cooling, the solid was separated and washed with three alternate washes of sodium bicarbonate (NaHCO3) and MilliQ water for three hours. MeOH was then alternated with MilliQ water for three final washes. The sample was dried in a vacuum chamber overnight. The pigment was resuspended in endotoxin-free phosphate buffered saline (PBS) at a final concentration of 2.5 mg/ml and kept at -20 ºC until further use.

2.3 Mosquito Treatment and Infection

A. gambiae mosquitoes (Yaoundé strain) were reared at 26 ºC and 80% humidity on a 12/12 h light/dark cycle. Adults were maintained on a 10% (w/v) glucose solution before blood feeding. Three to four day old female mosquitoes were cold-anaesthetized and inoculated intrathoracically with 69 nanoliters (nl) of a 100 or 200 micrograms (µg)/ml solution of sHz or with the same volume of endotoxin-free PBS, using a Nanoject micro-injector (Drummond Scientific). Female CD1 mice (*Mus musculus*) were intraperitoneally inoculated with 10^7 *P. berghei* ANKA parasitized red blood cells/ml and mosquitoes were fed when the parasitaemia reached 10–15% and exflagellation was observed. Fed mosquitoes were kept at 19–21 ºC and 80% humidity for *P. berghei* development. Four

independent biological replicates were performed for each experiment. Between eight and ten days post infection, mosquito midguts were collected to determine infection rate and intensity.

2.4 RNA Isolation

Female mosquitoes were dissected 24 h after sHz inoculation (immediately before feeding). Batches of *circa* 30 fat bodies (abdomen without midgut, ovaries and malpighian tubules, which can also comprise hemocytes) were dissected in cold diethylpyrocarbonate (DEPC) treated PBS and processed for RNA preparation. Total RNA was prepared using NucleoSpin RNA II kit (Macherey-Nagel) and concentration and purity determined by spectrophotometry.

2.5 Microarray Hybridization and Analysis

Microarray Hybridization was performed at Instituto Gulbenkian de Ciência (IGC), in collaboration with the Gene Expression Unit. Each GeneChip experiment was performed with three biological replicates. RNA was processed for use on Affymetrix GeneChip *Plasmodium/Anopheles* Genome Arrays, according to the manufacturer's One-Cycle Target Labelling Assay. Briefly, total RNA was used in a reverse transcription reaction to generate first-strand complementary DNA (cDNA). After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate biotinylated complementary RNA (cRNA). Fragmented cRNA was used in a 300 µl hybridization containing added hybridization controls, on arrays for 16 h at 45 °C. Standard post hybridization wash and double-stain protocols were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000. All quality parameters for the arrays were confirmed to be in the recommended range.

Microarray Analysis was performed at Instituto Gulbenkian de Ciência (IGC), in collaboration with the Gene Expression Unit, according to the protocol described below. Scanned arrays were analysed first with Affymetrix MAS 5.0 software to obtain absent /present calls and for subsequent analysis with dChip 2006. The arrays were normalized to a baseline array with median CEL intensity by applying an Invariant Set Normalization

Method (Li and Wong, 2001). Normalized CEL intensities of the arrays were used to obtain model-based gene expression indices based on a perfect match-only model (Li and Wong, 2001). All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was above 1.2. Li and Wong (2001) have shown that the lower confidence bound is a conservative estimate of the fold change and therefore more reliable as a ranking statistic for changes in gene expression. To validate the microarray data, expression profiles of ten genes differentially expressed by microarray were analysed by quantitative reverse transcription PCR (qRT-PCR) as described below: *AGAP000693*, *AGAP003689*, *AGAP005335*, *AGAP005848*, *AGAP006809*, *AGAP006911*, *AGAP010056*, *AGAP010812*, *AGAP011294* and *AGAP011790*. The sequences of primers used for amplification can be found in Table 1.

2.6 Stimulation of *A. gambiae* Cells

Immortalized *A. gambiae* hemocyte-like cells Sua 5.1* were cultured as in (Akman-Anderson et al., 2007) and incubated at 28 ºC. One to 1.5 x 10^6 cells/well were seeded in 24-well plates and left to grow overnight. Cells were stimulated with different concentrations of sHz or endotoxin-free PBS as a control and incubated at 28 ºC for 24 h or 48 h. Three independent experiments were performed.

2.7 Tissue Collection Before and After Blood Ingestion

Mosquitoes were inoculated with 69 nl of a 200 µg/ml solution of sHz or with the same volume of endotoxin-free PBS, as described above. Batches of *circa* 30 mosquitoes were dissected 24 h after sHz inoculation (immediately before feeding) and fat bodies collected as before. The remaining mosquitoes were fed with either a *P. berghei*-infected or a non-infected (naïve) blood meal and left to rest overnight. 24 h after feeding, fat bodies from different groups were collected. Three independent experiments were performed.

Table 1. Primers used in qRT-PCR and RNAi assays.

Gene name	Gene ID Ensembl	Forward primer (5'-3')	Reverse primer (5'-3')
CEC1	AGAP000693	CAACCCAGAGACCAACCAACCAC	ACTGCCAGCACGACAAAGATGAAG
CLIPC7	AGAP003689	GAGATTGAACACTGCCACGA	GTTCCGACACATTCCACCTT
CTL4	AGAP005335	TTGAATTGGTTTGATGCCGTGTCCTA	GGCGCTCGTTGGTATCCTTTATTGT
FBN50	AGAP005848	ATCACAAGGTTCCGGCTATG	CGTTGGTGTAGGTGAGCAGA
	AGAP006809	CCCTGTTCACCTTCAAGCAG	GGTCAGCACGGCATCATACT
SRPN2	AGAP006911	AGTCTCGAGGGCGCGGTCATTACG	GGGTTTGCCGCGAGTGCCATAGA
	AGAP010056	CGATGATGGTCCTCGTTTTT	TTCCATCGAGGATTTTCACC
TEP4	AGAP010812	AGCACCAGCGCCTTAATCT	CCGCTGATCCAATTTATCGT
DEF1	AGAP011294	GGAGAACTATCGGGCCAAG	GATACAGTGAGCGGCACAAA
CLIPA2	AGAP011790	AGCCCTTCTGCCCTTCTTAACAAC	CGTCGGTGGTGCGTTCTCTTC
S7	AGAP010592	CATTCTGCCCAAACCGATGCGT	CGGGAATACCAGATCCTCCAGG
REL2-F	AGAP006747	ACCGATACGGAAAGTGTGCT	GTATCGTTGCGTCGGATTG
REL2-S	AGAP006747	ACCGATACGGAAAGTGTGCT	CGGTGCTCCTCGTAATGACT
REL1	AGAP009515	GGTCGTGTCGGACATCATCT	TCTCGAAAAAGCGCACCTTA
Cactus	AGAP007938	GACGGCTTATCAGCTTGCAC	AACGCACTCGCTCCGTAGTA
Caspar	AGAP006473	GGTGGGACTTGTCGTTTGAA	CTTCCATCAGCTTGGACAGC
dsCaspar	AGAP006473	TAATACGACTCACTATAGCCGCTTTT CTAAACGCTGTC	TAATACGACTCACTATAGAAACAG GTTGCATGTGTGGA
dsβ2M	NM_009735	TAATACGACTCACTATAGGGAGACA CCCCCACTGAGACTGATACA	TAATACGACTCACTATAGGGAGA AATTAGGCCTCTTTGCTTTACCA
dsREL2-F	AGAP006747	GAATTAATACGACTCACTATAGGGA GAAATCCGACGCAACGATACG	GAATTAATACGACTCACTATAGGG AGAGACCGCAATGTGAAGGATG
FBN9	AGAP011197	CCAAGATGTCGGGCAAGTAT	TTGTGGTACGTCAGCGAGTC
TEP1	AGAP010815	CAGATGGTTCGTTTGGTGTG	GCAATGCCGTCAACACATAC
LRIM1	AGAP006348	AACGGACAGCAGCCTAAAGC	AGATCAAGCTCCTTTACGTTCCA
CLIPB14	AGAP010833	TTCGCGCATTCAACAAGGAG	ATGCCGGCCAAATACCAC
	AGAP003960	TGGTACATACGGGGTTTGGT	GGTACTGCTCGATCCACCTC

2.8 Real-time qRT-PCR Analysis

Total RNA was isolated from mosquito cells and fat bodies using NucleoSpin RNA II kit (Macherey-Nagel), at 24 h and 48 h after sHz treatment. Concentration and purity of RNA were determined by spectrophotometry. cDNA was synthetized using one µg of total RNA reverse transcribed with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Quantitative analysis was performed by quantitative Real-time PCR using SYBR Green Supermix (Bio-Rad), on a final volume of 20 µl, using an iCycler iQ (Bio-Rad). One µl of cDNA was used as template. Cycle conditions were: an initial denaturation step at 95 ºC for 10 minutes, followed by 40 cycles at 95 ºC for 10 seconds and 62-65 ºC for 45 seconds. For all assays, the expression levels of target genes were normalized to the levels of ribosomal protein S7 gene (*AGAP010592*). The sequences of primers used for amplification can be found in Table 1.

2.9 RNAi Gene Silencing

Specific dsRNA was synthesized using the MEGAscript T7 kit (Ambion), according to the manufacturer instructions. Initial PCR products were generated from cDNA using gene specific primers (Table 1) that include a T7 promoter sequence. An exogenous gene, mouse *beta-2 microglobulin (β2M)*, was used to produce control-dsRNA. Each PCR product was purified and dsRNA synthesized following the manufacturer instructions.

Cells: *A. gambiae* Sua 5.1* cells were treated with 22.5 µg/ml *caspar*-targeting or *β2M*-targeting dsRNA (to serve as reference for silencing efficiency and for quantification of gene expression levels). One day following silencing, control-PBS or 12.5 µg/ml sHz was added to cells for 24 h. Total RNA was isolated from cells and cDNA prepared as described above, 48 h after dsRNA treatment (24 h post sHz adding). Efficiency of gene silencing and levels of differentially expressed genes in gene-silenced samples were assessed by qRT-PCR. For all assays, the expression levels of target genes were normalized to the levels of ribosomal protein S7 gene and compared to controls treated with dsRNA against *β2M*, by qRT-PCR using specific primers (Table 1). Three independent experiments were performed.

Mosquitoes: Three day old female mosquitoes were cold-anaesthetized and inoculated intrathoracically with 69 nl of a 3 µg/µl solution of dsRNA (207 ng) for each gene of

interest. A control group was injected with ds*β*2M (to serve as reference for silencing efficiency and for quantification of gene expression levels). All injections were performed using a Nanoject micro-injector (Drummond Scientific). Efficiency of gene silencing was assessed four days after dsRNA injection, by qRT-PCR. Silenced mosquitoes were treated either with control-PBS or 200 µg/ml sHz and the levels of differentially expressed genes in gene-silenced treated samples assessed 24 h later, before *P. berghei*-infected blood feeding. Infection rate and intensity were measured after eight days. Three independent experiments were performed.

2.10 Statistical Analysis

Differences in the infection rate between control/tested groups were compared using the *Fisher's exact test.* The *Student t test* was used for comparison of the levels of expression of genes measured by qRT-PCR. Both tests were used after checking their correspondent assumptions. For data not normally distributed (number of oocysts), the non-parametric *Mann-Whitney's test* was used. GraphPad Prism version 5.00 was used for the statistical analysis.

2.11 DNA Isolation and Amplification

This and the following methods and techniques were performed at the Crisanti laboratory, Imperial College London, UK.

A. gambiae genomic DNA (gDNA) was extracted using a phenol-chloroform method. Briefly, lysis solution was added to the mosquito sample and the sample was homogenised. 8 M potassium acetate was added and the sample was mixed by vortexing and incubated on ice for thirty minutes. After centrifugation at 10 000 g for fifteen minutes, one volume of phenol-chloroform solution (1:1) was added, mixed by vortexing and spun down at 10 000 g for five minutes. Isopropanol was added to the supernatant and the sample was spun again. The obtained pellet containing the DNA was washed with 70% (v/v) ethanol before air drying and resuspended in 20 µl distilled water (dH_2O). cDNA was prepared as described above.

Initial PCR products were generated using *A. gambiae* gDNA or cDNA as template and Phusion High Fidelity PCR Master Mix (New England Biolabs). The PCR cycle included

the following steps: an initial denaturation step at 98 °C for 30 seconds, followed by a denaturation step at 98 °C for 10 seconds, an annealing step for 30 seconds and an extension step at 72 °C, all three latter steps repeated 35 times. The thermocycler program finalized with an extension step at 72 °C for seven minutes, until the reaction was hold at 4 °C. Annealing temperature and extension length were adjusted according to the size of the amplicon. Specific primers were designed for amplification of each particular cloning fragment, namely Vitellogenin 1 promoter (AGAP004203), *FBN9* gene (fibrinogen immunolectin 9, AGAP011197) and Trypsin 1 terminator (AGAP008296) (Table 2).

PCR products were purified using the QIAquick PCR Purification Kit protocol (QIAGEN).

Table 2. Specific primers for amplification of each construct element. Primers include the *BsaI* restriction site (underlined).

Gene name	Gene ID Ensembl	Forward primer (5'-3')	Reverse primer (5'-3')
Vitellogenin 1	AGAP004203	AGGTCTCAATCCGACTTGTGTGCAGGACCTTTCA	AGGTCTCATCGTATGGGTACATGAGGAGGAGTAACTTCGCAATCA
FBN9	AGAP011197	AGGTCTCAACGATGTTCCAGATTACGCTGCCAAACTGGACTACCTAC	AGGTCTCAGAGACTAAACCTCCTTTATCATCATCC
Trypsin 1	AGAP008296	AGGTCTCATCTCTCTCGGCTACAAAACGGTACTG	AGGTCTCAAAGCGAGCTCGAATGGAACTGGCGG

2.12 Restriction Digestion of Plasmid DNA

Purified pDSAR vector plasmid that had been previously grown, was digested with *BSaI* enzyme (Eco31I, Thermo Scientific), in a 40 µl reaction with 10x Fast Digest Green Buffer (Thermo Scientific) and BSA (bovine serum albumin), at 37 ºC for 30 minutes. The plasmid DNA fragments were next run on a 1% (w/v) agarose gel, followed by excision of the band correspondent to the digested plasmid. DNA from the band of interest was purified using the QIAquick Gel Extraction Kit (QIAGEN) and plasmid DNA concentration was determined with a NanoDrop spectrophotometer.

2.13 Cloning

One hundred ng of the linearized vector backbone and equimolar amounts of the other assembly pieces (Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator), were assembled together in a 15 µl reaction containing T4 DNA ligase two million units/ml (New England Biolabs), 10x T4 ligase buffer (New England Biolabs), *BsaI* (Thermo Scientific), BSA (2 mg/ml) and dH_2O. T4 DNA ligase was used for ligation of all three fragments into an entry kanamycin-resistant pDSAR vector containing DsRed (*Discosoma* sp. red fluorescent protein) under the 3xP3 promoter. Restriction-ligation program at the thermocycler was defined with the following steps: incubation for three minutes at 37 °C and four minutes at 16°C, both steps repeated 50 times, followed by five minutes at 80°C (heat inactivation), in a protocol adapted from (Engler et al., 2009). As control, pDSAR backbone without the assembly pieces was used.

2.14 Bacterial Transformation

Five µl of the assembly reaction were added to 100 µl of competent *Escherichia coli* One shot Top10 cells (Invitrogen) for high efficiency transformation. The mix was thawed on ice for 20 minutes, before heat shocked for 30 seconds at 42 °C and kept for two minutes on ice. 250 µl of SOC medium (super optimal broth with catabolite repression) were added to the mix and shaked for 30 minutes at 37 °C. Transformed cells were plated in a kanamycin agar plate (one plate with the complete assembly: pDSAR vector backbone and three fragments; one plate with pDSAR vector alone, for control) and plates were incubated overnight at 37 °C. Kanamycin-resistant grown colonies of cells containing the inserted plasmid were counted in each plate.

2.15 Colony Screening

Several individual colonies were picked from the agar plate with a tip and rubbed against the bottom wall of three PCR tubes, each containing a PCR mix composed by Phusion Master Mix (New England Biolabs), dH_2O and primers for amplification of each of the three construct fragments (Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator). Two colonies from the control plate were amplified as well. The PCR cycle included the following steps: an initial denaturation step at 98 °C for 30 seconds, followed

by a denaturation step at 98 °C for 10 seconds, an annealing step at 60 °C for 30 seconds and an extension step at 72 °C for one minute, all three latter steps repeated 35 times. The thermocycler program finalized with an extension step at 72 °C for seven minutes, until the reaction was hold at 4 °C. PCR products were run on a 1% (w/v) agarose gel and the size of fragments confirmed. Colony-PCR assays were repeated until we found colonies containing the three inserted components expected in our construct (Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator).

2.16 Isolation of Plasmid DNA (Miniprep)

Colonies positive for all three inserted fragments in the colony-PCR assay were grown overnight in a round-bottom polypropylene transparent tube (Fisher Scientific) containing four ml of LB (Luria-Bertani) broth medium with kanamycin, at 37 °C in a shaker incubator. Five hundred µl of culture were added to the same volume of 50% (v/v) sterile glycerol and this glycerol stock was frozen at -80 °C for further use. The overnight cultures were transferred to falcon tubes, which were centrifuged at 6 000 g for three minutes, the supernatant discarded and the pellet saved. Plasmid DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN) and eluted in 50 µl dH_2O.

2.17 Sequencing

Plasmid DNA purified from one of the colonies containing all three inserts was sent for sequencing. To check for percentage of identity between predicted/obtained sequences for the construct, the obtained sequence was BLASTED (http://blast.ncbi.nlm.nih.gov) against the predicted one (Appendix 3).

2.18 Isolation of Plasmid DNA (Midiprep)

Following sequencing of the construct, a polypropylene transparent tube was filled with six ml of LB broth medium. A very small portion of the glycerol stock correspondent to the sequenced construct was added to the medium and the mix was incubated at 37 °C for 10 minutes. Six µl of kanamycin (60 mg/ml) were supplemented, followed by a further incubation lasting three hours at 37 °C. The total culture volume was added to a big glass

jar containing 200 ml of LB broth and 200 µl of kanamycin and this culture was grown overnight at 37 °C.

Large-scale DNA purification, needed for injection of plasmid DNA into the mosquito, was performed with PureLink HiPure Plasmid Filter DNA Purification Kit (Invitrogen), following the Midiprep procedure protocol. The resulting purified plasmid DNA was eluted in 200 µl dH_2O and its concentration measured with a NanoDrop spectrophotometer.

2.19 Embryo Microinjection

Injection solution was prepared by the combination of: 1) a donor plasmid (containing the Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator, cloned into an entry pDSAR vector containing DsRed under the 3xP3 promoter and an *attB* site that, in presence of integrase enzyme, allows the site-specific integration of the gene cassette into the mosquito *attP* site); 2) a helper plasmid, that expresses the φC31 integrase under the germline specific *vasa* promoter; 3) a 10x injection buffer solution, composed by potassium phosphate and sodium phosphate and 4) dH_2O. The donor plasmid was used at a concentration of 200 ng/µl and the helper plasmid at a concentration of 400 ng/µl. All four elements were mixed and filtered through an Ultra Free Centrifuge Filter (Millipore) and spun down at 2 000 g for three minutes.

Newly laid eggs from female *A. gambiae* mosquitoes (G3 strain) of the X1 line (engineered to have an *attP* site on chromosome 2L 21D) were aligned against a glass slide (approximately 20 eggs/slide). The entire embryo was covered with injection buffer, to avoid dryness. Aligned embryos were injected into the posterior pole with a micro-manipulator (Narishige) containing a glass needle with one µl of injection solution (Figure 7) mounted on an inverted microscope (Nikon TE-DH 100W) at 100x magnification. Injected embryos were allowed to hatch in a Petri dish with dH_2O and moved back to the insectary to be kept at the right temperature/humidity.

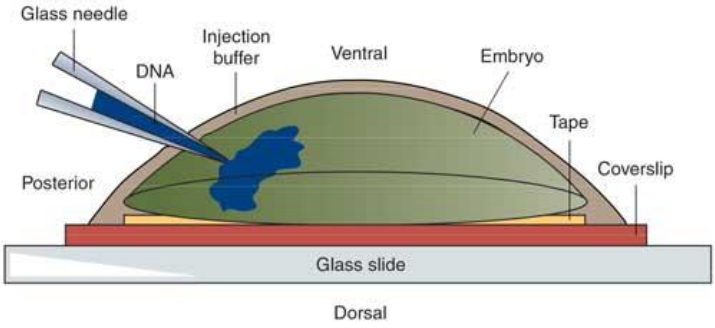


Figure 7. Schematic representation of mosquito embryo injection. Adapted from Lobo et al., 2006.

2.20 Larvae Screening

The hatched larval survivors were screened as early as possible (at L1 larval stage) under epifluorescence on an inverted microscope (Eclipse TE200, Nikon), to look for transient expression of the DsRed marker (red fluorescent protein). Only transient larvae were grown, and pupae were sexed and outcrossed to wildtype *A. gambiae* mosquitoes from the opposite sex in a ratio of at least one female to 10 males. Following blood feeding, the larval progeny of these crosses was analysed for DsRed fluorescence, to search for transgenic mosquitoes. Each transgenic founder was outcrossed to wildtype mosquitoes and blood fed at least three times, thus increasing the chance of finding transgenic offspring from the same founder. To create the transgenic line, at least three generations of transgenic mosquitoes were outcrossed and blood fed and each generation screened for transgenics.

Our engineered *A. gambiae* G3 X1 line overexpressing FBN9 under regulation of the fat body-specific Vitellogenin 1 promoter is currently being kept at the Crisanti insectary at Imperial College London, to be used in future research work. The line is being regularly screened to check for DsRed fluorescence.

3. Results

3.1 Hemozoin Impairs *P. berghei* Infection in *A. gambiae*

To define the impact of hemozoin treatment on the response of *A. gambiae* to *P. berghei* infection, mosquitoes were injected with high purity sHz, comparable to the one used in mice and other mosquito studies. This highly resembles the native *P. falciparum* hemozoin crystals in both size and physicochemical properties, and is devoid of protein and DNA contamination (Pagola et al., 2000; Akman-Anderson et al., 2007; Jaramillo et al., 2009).

Independent experiments were performed using a concentration of 100 µg/ml, with a total number (N) of 221 *A. gambiae* females. Infection rate (number of infected mosquitoes per total number of mosquitoes observed) and intensity (number of oocysts per midgut) were consistently lower in sHz treated mosquitoes when compared to control, sterilized PBS treated ones (Figures 8A and 8B). When sHz concentration was increased to 200 µg/ml, infection rate in sHz treated mosquitoes (44%) was significantly lower (p=0.0025, *Fisher's exact test*) when compared to control (67%) (Figure 8C), in a total of 193 *A. gambiae* females. Oocyst counts from the midguts of both infected and uninfected mosquitoes are shown in Figure 8D, where the difference between sHz and PBS treated mosquitoes is significant (p=0.0007, *Mann-Whitney's test*). Reduction of both parameters was consistently observed in all replicate experiments (Table 3).

Table 3. Infection rate and intensity of *P. berghei* in control/sHz injected *A. gambiae.*

100 µg/ml sHz								
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
N	28		65		91		37	
	PBS	sHz	PBS	sHz	PBS	sHz	PBS	sHz
Infection rate (%)	63.2	44.4	44.7	25.9	72.5	64.7	53.8	41.7
Infection intensity	22.6	16.8	56.1	40.9	63.5	56.7	21.7	20.4

200 µg/ml sHz								
	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
N	36		53		48		56	
	PBS	sHz	PBS	sHz	PBS	sHz	PBS	sHz
Infection rate (%)	81.3	33.3	49.1	31.0	53.0	41.7	84.6	70.0
Infection intensity	68.7	19.3	70.3	21.3	40.1	38.2	98.8	60.6

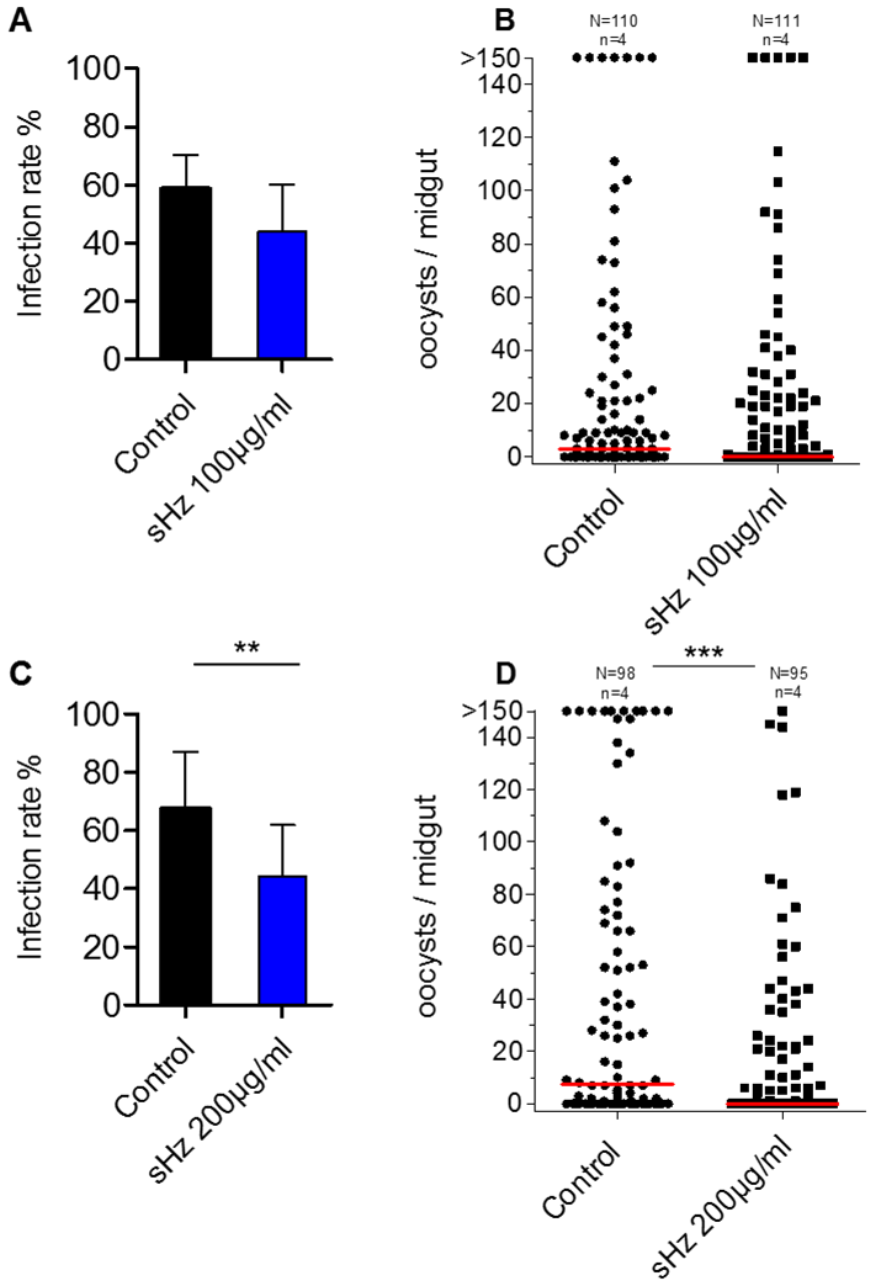


Figure 8. Effect of hemozoin on *A. gambiae* infection by *P. berghei*. Female mosquitoes were treated with sHz or PBS-control and infected with *P. berghei* 24 h later. Oocysts were counted 8-10 days post-infection. **(A, B)** Infection rate and intensity following PBS or 100 µg/ml sHz injection. **(C, D)** Infection rate and intensity following PBS or 200 µg/ml sHz injection. **(A, C)** Values represent the mean and standard deviations of four independent experiments. **(B, D)** Dots indicate the number of parasites in individual midguts following PBS or sHz injection, horizontal red bars represent the median. Differences and *P*-values: ** ($p<0.01$), using the *Fisher's exact test*; *** ($p<0.001$), using the *Mann-Whitney's test*.

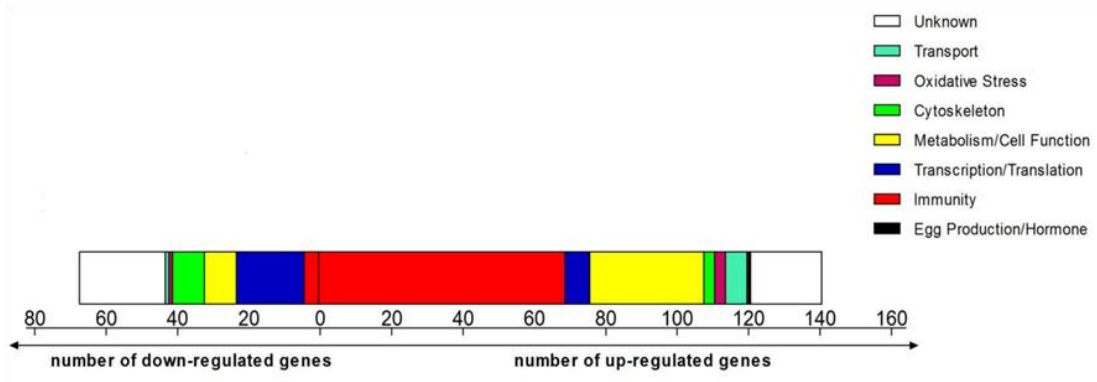
Taken together, these results show that hemozoin increases the mosquito resistance to infection by *Plasmodium*, through an impact on both infection rate and intensity. This suggests that pathways and effector mechanisms triggered by hemozoin are used by *A. gambiae* to control *P. berghei* infection.

3.2 Hemozoin Stimulates the Innate Immune System and Influences anti-*Plasmodium* Effectors

To provide further insight into the mechanism underlying the mosquito decreased susceptibility to *P. berghei* upon treatment with hemozoin, microarray transcription analysis was performed to assess gene expression response of female mosquitoes to injection with 200 µg/ml sHz. Fat body was used, as it would be informative of the response to sHz, being the main source of circulating immune related components. Differential expression associated with treatment was observed in a total of 208 genes, from which 141 were up-regulated and 67 down-regulated (Figure 9A and Appendix 1).

The most represented functional class encoded immune response related genes, comprising 35% of the differentially expressed total genes. From this, 95% were positively induced with sHz treatment compared to control, indicating a robust general activation of the immune system. The majority of down-regulated genes (28%) were involved in transcription and translational functions. Of the up-regulated genes, the most represented class (49%) was immunity, followed by metabolism/cell function (23%) (Figure 9A), which indicates that other processes may be involved in the physiological immune response. Immune up-regulated genes are outlined in Appendix 2.

A



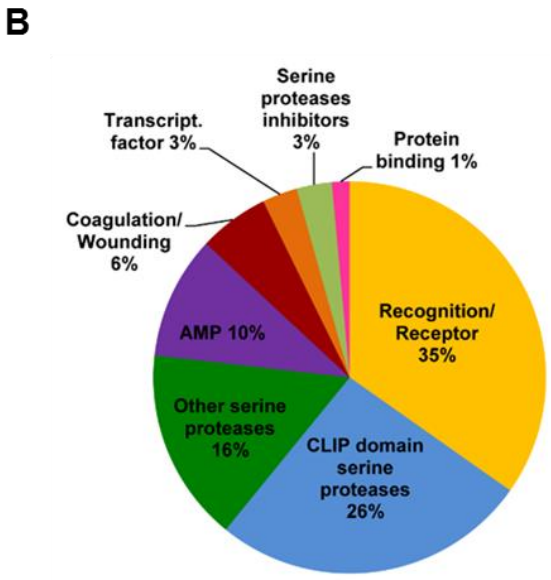


Figure 9. Functional class distribution of genes regulated by hemozoin. (A) Coloured sections correspond to the number of genes either up- or down-regulated in the fat body 24 h after female mosquitoes injection with PBS or 200 µg/ml sHz. **(B)** Pie chart shows sub classes distribution in immunity related up-regulated genes.

In *A. gambiae*, it has been shown that malaria infection induces a large number of immune effector genes, which form an important line of defence against *Plasmodium* parasites, as explained in Chapter 1. In our microarray screen, sHz treatment induced several PRRs which are markers for anti-*Plasmodium* activity (Figure 9B and Appendix 2). Among them are some of the most potent anti-*Plasmodium* immune factors identified to date: TEP1, APL1 and FBN9 (Appendix 2). Our data showed that TEP1/FBN9 and APL1C/LRIM1, PRR pairs of the complement-like pathway (Garver et al., 2009), presented similar expressions in the microarray analysis (Appendix 2). TEP3 and TEP4, another two proteins that interact with the LRIM1/APL1C complex, were up-regulated too after sHz treatment (Appendix 2). The basal expression of TEP1 and LRIM1 is representative of the pre-invasion phase (Frolet et al., 2006), which is in accordance with our results for the induction of these genes in the fat body before parasite invasion. Of note, these receptors are believed to be hemocyte-specific, which shows how fat bodies can also comprise other cellular types besides fat body cells, namely hemocytes that are in part sessile and attached to tissues. Other members of the TEP, FBN and LRIM families were up-regulated as well in our screen.

We have observed a very strong stimulation of CLIP domain and other serine proteases in our microarray results, comprising 42% of the total number of up-regulated immune related genes (Figure 9B). However, as mentioned in section 1.5, some of these genes have been classified as agonists of *P. berghei* infection. CLIPA2, CLIPA7 and SRPN2,

together with receptors CTL4 and CTLMA2, play a role in parasite protection as inhibitors of parasite melanization. This suggests that melanization is not triggered by sHz treatment, and is not the effector mechanism that confers protection against *P. berghei* observed in this study (Figure 8), explaining why we could not observe any melanized oocysts in the sHz-treated mosquitoes. CEC1 and DEF1, two AMPs belonging to the most important AMP families, defensins and cecropins, were induced by sHz in our microarray screen (Appendix 2).

We observed an increased expression of REL2 in our microarray analysis. This observation has drawn our attention to the fact that many induced immune related genes in this screen have been described by different authors as regulated by this nuclear factor kappa B (NF-κB) transcription factor. Indeed *FBN9*, *TEP1*, *TEP4*, *LRIM1*, *CLIPB14* and *AGAP003960* have all been reported to be REL2-regulated/-partly regulated target genes of the Imd pathway (Meister et al., 2005; Luna et al., 2006; Hoa and Zheng, 2007; Cirimotich et al., 2010, 2011; Dong et al., 2011), which prompted us to hypothesize that the up-regulation of fat body immunity genes following sHz treatment may be affected by this transcription factor expression. To validate the robustness of the microarray results, ten genes were analysed by real-time qRT-PCR. The two assays showed a high degree of correlation (Figure 10).

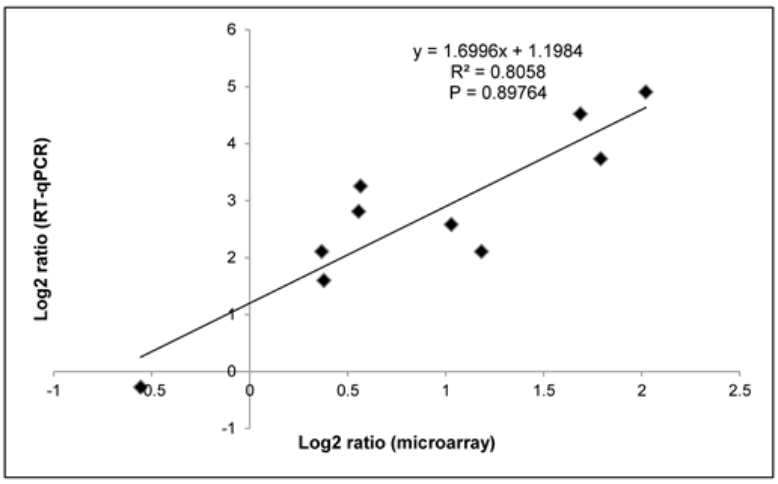


Figure 10. Validation of microarray analysis using qRT-PCR. Gene expression values for ten genes obtained by microarray plotted against the corresponding averages of three qRT-PCR-derived gene expression values from biological replicates. The Pearson correlation coefficient (p=0.8976) and the best-fit linear-regression analysis (R^2=0.8058) demonstrated a high degree of correlation between gene expression magnitudes determined by each assay.

3.3 REL2-F, rather than REL2-S or REL1, is Induced by Hemozoin in *A. gambiae* Cells

Based on previous data that confirmed that sHz could function as a signal in mosquito cells (Akman-Anderson et al., 2007), we used a cell-based approach to explore *in vitro* the suggestion that hemozoin's triggering of immune defence mechanisms is due to the activation of REL2. *A. gambiae* hemocyte-like Sua 5.1* cells were stimulated with a range of sHz concentrations, from 1.25 µg/ml to 200 µg/ml, comparable to the ones used in mosquito cells- and murine macrophages-stimulation assays *in vitro* (Akman-Anderson et al., 2007; Jaramillo et al., 2003). Along with the fat body and epithelial tissues, hemocytes are a source of AMPs production. The levels of REL2-F and REL2-S expression induction were analysed by qRT-PCR 24 h after sHz stimulation, the same time-point used in the microarray experiment, and at 48 h post sHz treatment. At 24 h after treatment, all sHz concentrations used to stimulate Sua 5.1* cells induced REL2-F average expression from 1.91- to 2.67-fold relative to PBS-control, with significant (p=0.0165, p=0.0043) up-regulation shown at 100 µg/ml and 200 µg/ml sHz respectively (Figure 11A). REL2-F induction at 48 h was observed with 12.5 µg/ml and 50 µg/ml sHz, but not significantly and at a much lower level than at 24 h. REL2-S expression was unaltered at both time points and for all concentrations (Figure 11A).

To examine if other factors would respond as well to hemozoin stimulus, we measured the expression of REL1 after sHz treatment. Notwithstanding a subtle induction observed after treatment with the lowest sHz concentration, REL1 expression was mostly unaltered at 24 h and down-regulated at 48 h post sHz treatment (Figure 11A). When the relative induction of REL1's negative regulator Cactus was measured, a general induction was observed, particularly after 48 h and for almost every concentration, which was significant (p=0.0180) when cells were stimulated with 100 µg/ml sHz (Figure 11B). Cactus induction opposed REL1's low expression, thus supporting the idea that REL1 is indeed down-regulated by hemozoin.

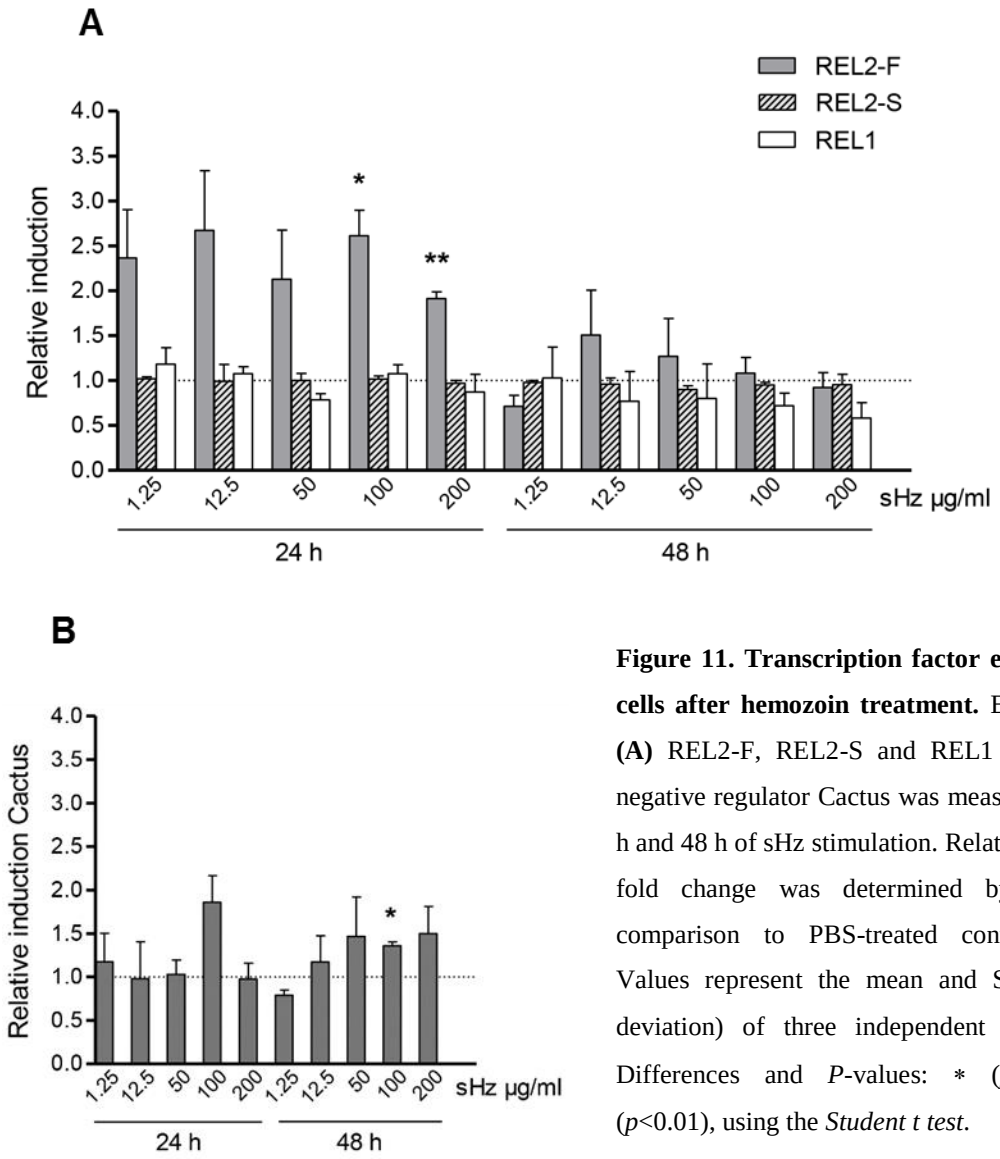


Figure 11. Transcription factor expression in cells after hemozoin treatment. Expression of **(A)** REL2-F, REL2-S and REL1 **(B)** REL1's negative regulator Cactus was measured after 24 h and 48 h of sHz stimulation. Relative induction fold change was determined by qRT-PCR comparison to PBS-treated controls. **(A-B)** Values represent the mean and SD (standard deviation) of three independent experiments. Differences and *P*-values: ∗ (p<0.05), ∗∗ (p<0.01), using the *Student t test*.

These findings seem to confirm that hemozoin induces the expression of REL2 and this induction involves its full-length form, REL2-F (rather than the short form REL2-S), as we had observed in our microarray data (the up-regulated REL2 probeset binds to the ankyrin domain characteristic of REL2-F). Moreover, this induction is maximal at 24 h after sHz stimulation. However, at 48 h post sHz treatment, an increase in REL2-F expression was still detectable, suggesting that REL-F gene expression is sustainable for at least 48 hours. REL1 transcription factor was not positively stimulated by hemozoin, which was confirmed by the consistent activation of its negative regulator Cactus upon sHz treatment.

3.4 Hemozoin Further up-regulates REL2-F Upon *caspar* Silencing

Silencing of target genes in *A. gambiae* and other *Anopheles* species has proved to change the mosquito susceptibility to *Plasmodium* infection. Particularly, knock-down of the negative regulators of the Toll and Imd pathways, *cactus* and *caspar* respectively, has shown to control malaria infection (Frolet et al., 2006; Garver et al., 2009, 2012).

To strengthen our fat body transcriptional profiling and hemocyte-like cell-based observations that suggested hemozoin is an activator of the REL2 pathway, we next investigated, by RNA interference (RNAi) analysis, the effect of *caspar* silencing on REL2-F induction in Sua 5.1* cells treated with hemozoin. A preceding experiment to test which dsRNA concentration would best knockdown *caspar* was performed with this gene silenced from 24 h to 72 h. dsRNA concentrations varied from 5 to 22.5 µg/ml, according to the ones used in other studies for gene silencing by RNAi in *A. gambiae* cells (Luna et al., 2006; Smith and Linser, 2009). The best silencing efficiency was achieved with the highest dsRNA concentration tested and an incubation period of 48 h. Cells were treated with *caspar*-targeting dsRNA or dsRNA targeting *β2M* as control, at the highest tested concentration. One day following silencing, control-PBS or 12.5 µg/ml sHz was added for 24 h, the concentration and time point we observed before as having caused the highest REL2-F induction. The levels of REL2-F were assessed by qRT-PCR 48 h post silencing (24 h post sHz treatment).

The average silencing efficiency obtained was 52.7% (Figure 12A). *caspar* silencing up-regulated REL2-F relative expression and when silenced cells were treated with sHz, REL2-F was further induced up to an average significant (p=0.0247) level of 3.79-fold change compared to control (Figure 12B). Caspar levels of expression were also measured. Following silencing of the gene, Caspar was down-regulated, as expected. Adding sHz has slightly increased this gene relative expression. Although *caspar* had been silenced, it was not completely knocked-down and this slight increase of its expression following sHz treatment might reflect a counterbalanced response to the high levels of REL2-F expression. In parallel, we designed a *cactus*-silencing/REL1 experiment but, as the silencing efficiency obtained was below 50%, results are not shown. However, it is

worth mentioning that although REL1 expression was up-regulated after *cactus* silencing, it didn't change when sHz was added.

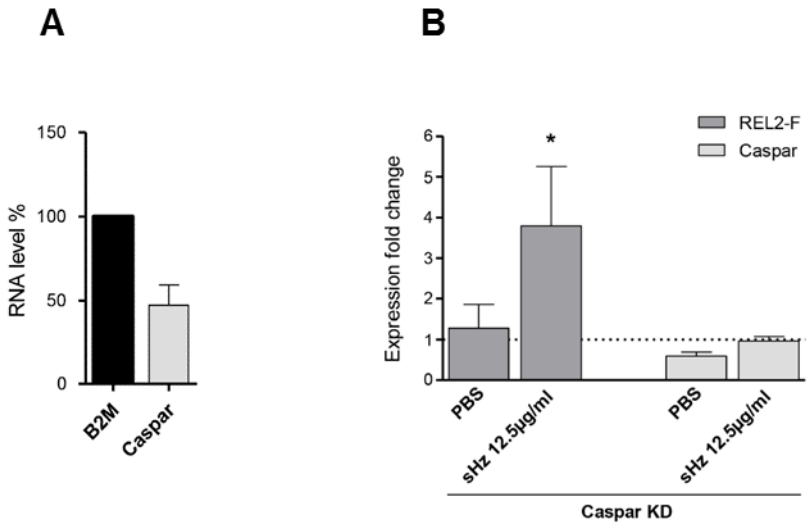


Figure 12. Combined effect of *caspar* silencing and sHz treatment. (A) Cells were silenced using dsRNA specific for *caspar* or control-dsRNA against *β2M*. **(B)** Following silencing, cells were treated with PBS or 12.5 µg/ml sHz. Graph represents expression of REL2-F and Caspar in silenced cells with and without sHz. Relative expressions were measured by qRT-PCR comparison to *β2M*-silenced control. **(A-B)** Values represent the mean and SD of three independent experiments. Differences and *P*-values: ∗ significant (p<0.05).

In conclusion, the results obtained through dsRNA based silencing of its negative regulator, further indicate that REL2-F transcription factor is indeed efficiently induced by hemozoin.

3.5 Hemozoin up-regulates REL2-F and Other Immune Related Genes *in vivo* Before and After *P. berghei* Infection

Having shown that hemozoin's REL2-F activation lasts for 48 h at least, we then analysed *in vivo* the expression of this and other factors at different time points, following stimulation with 200 µg/ml sHz, the same concentration used in the microarray experiment and which incited a very significant REL2-F up-regulation in the cellular assay *in vitro*. The levels of expression induction were measured by qRT-PCR in the fat bodies of female mosquitoes 24 h after sHz stimulation (immediately before blood

ingestion), as well as 48 h after sHz stimulation, 24 h after the mosquitoes were provided with a blood meal. As shown in Figure 13A, at 24 h after treatment, before the blood meal, hemozoin induced REL2-F average expression up to a significant (p=0.0253) high level of 8.71-fold change relative to PBS control. After the blood meal, REL2-F was still markedly up-regulated, but at a lower level than before the blood meal. The expression levels of REL1 and the negative regulators Caspar and Cactus were mostly consistent throughout the assay, fluctuating from unaltered to slightly up-regulated upon sHz stimulation compared to control, with the exception of a higher but not significant Cactus expression level pre-blood meal.

Mosquitoes were fed with either *P. berghei*-infected blood or non-infected (naïve) blood as control. The decrease in REL2-F expression levels after the blood meal is most probably due to the fact that they were measured 48 h after sHz stimulation, rather than *P. berghei* infection altering the expression levels, as the same pattern of REL2-F decreased induction was observed both after the *P. berghei*-infected and the naïve blood meals. These results agreed with the microarray outcome and confirmed what we had observed in cells: REL2-F activation by sHz is maximal at 24 h, however 48 h post sHz stimulation REL2-F is still induced. This shows that REL2-F activation by sHz is a broad mechanism, observed both *in vitro* and *in vivo*.

Similarly, the relative expression levels of six important immune related genes (*FBN9, TEP1, TEP4, LRIM1, CLIPB14* and *AGAP003960*) up-regulated in the microarray analysis, were measured in the female fat body before and after the *P. berghei*-infected blood meal. In agreement with REL2-F's expression, these genes were induced by sHz before the blood meal, as well as after the blood meal though at a lower level for most of them (Figure 13B). Of note, it has been said that the mosquito basal immunity level (before the mosquito encounters the parasite) is a key factor for parasite control (Frolet et al., 2006), which reinforces the importance of these genes' up-regulation in the pre-invasion phase.

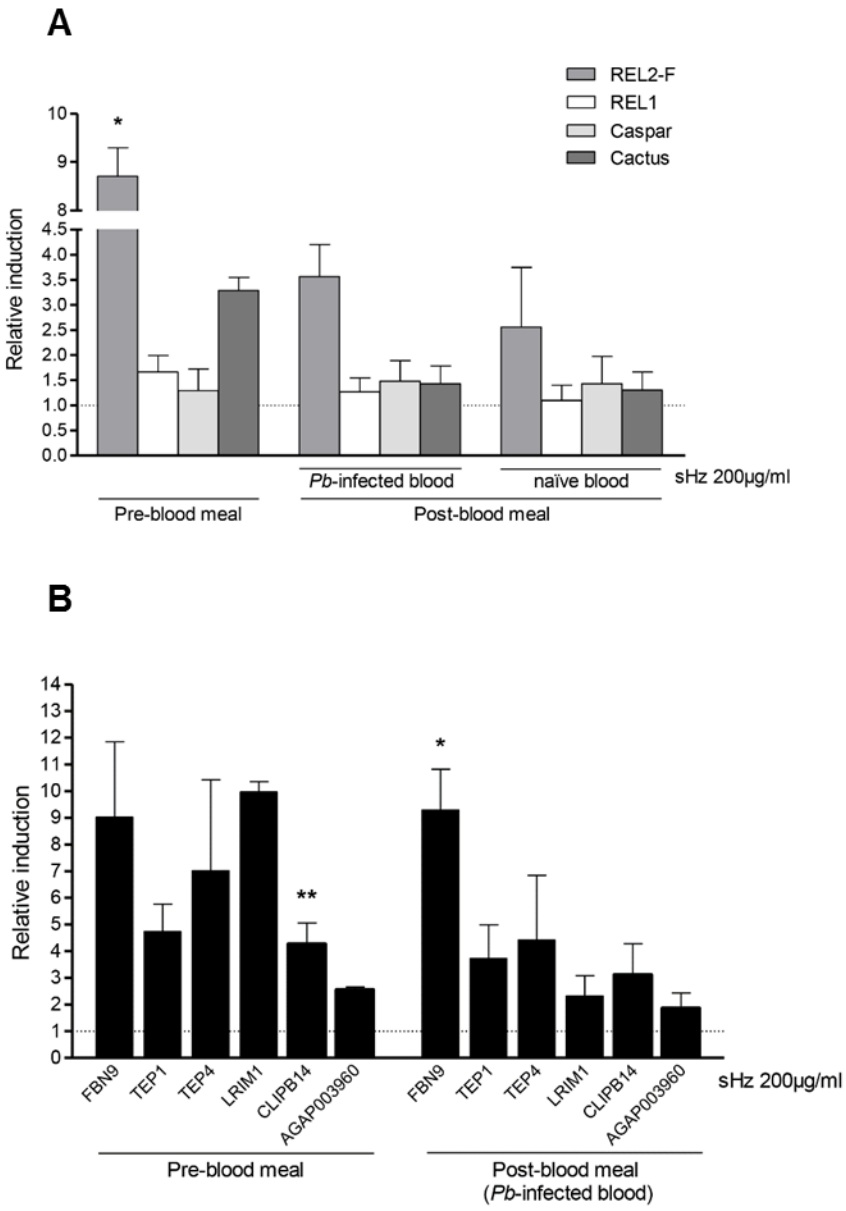


Figure 13. Effect of hemozoin *in vivo* before and after the blood meal. Female *A. gambiae* were treated with 200 µg/ml sHz and fed with *P. berghei*-infected or naïve blood 24 h later. The expression of **(A)** REL2-F, REL1, Caspar, Cactus and **(B)** six immune related genes transcription was measured in the fat body before and after blood ingestion. Relative induction fold change was determined by qRT-PCR comparison to PBS-treated controls. Values represent the mean and SD of three independent experiments. Differences and *P* values: ∗ ($p<0.05$), ∗∗ ($p<0.01$), using the *Student t test*.

3.6 REL2 Mediates the Hemozoin Effect

We have demonstrated above how hemozoin stimulates REL2-F both *in vivo* (Figure 13A, Appendix 1, Appendix 2) and *in vitro* (Figure 11A), and shown this molecule's impact on *Plasmodium* infection, reducing both infection rate and intensity (Figure 8, Table 3).

To explore if REL2 mediates the hemozoin effect, we next investigated whether sHz treatment still had an effect on infection rate and parasite numbers in mosquitoes silenced for REL-F. We used dsRNA as in (Meister et al., 2005), which is specific to target the ankyrin domain of REL2, hence should only affect REL2-F. Silencing efficiency was measured four days after dsRNA injection. REL2-F was efficiently silenced, at both the mosquito fat body (68.1%) and midgut (66.3%) (Figure 14A).

Silenced mosquitoes were treated either with control-PBS or 200 µg/ml sHz and fed with *P. berghei*-infected blood 24 h later, infection rate and intensity being measured after eight days. As shown in Figure 14B, hemozoin did not affect the infection rate in *REL2-F*-silenced mosquitoes, as the percentage of infected mosquitoes is equivalent for both control-PBS treated and sHz-treated mosquitoes, in a total of 190 *REL2-F*-silenced *A. gambiae* females. In contrast, the infection rate in control-ds*β*2M-injected sHz treated mosquitoes (39%) was significantly lower (p=0.0009, *Fisher's exact test)* when compared to *REL2-F*-silenced sHz treated mosquitoes (65%), in a total of 161 *A. gambiae* females. Accordingly, the difference in the oocysts counts between sHz and PBS treated *REL2-F*-silenced midguts was not significant, as represented in Figures 14C and 14D.

Taken together, these results demonstrate that the effect of hemozoin is suspended when REL2-F is knocked-down. The levels of infection and parasite numbers, which are increased with *REL2-F* silencing compared to control-silencing, due to more susceptibility to infection in REL2-F-depleted mosquitoes, as observed by other authors both for *P. berghei* and *P. falciparum* (Meister et al., 2005; Garver et al., 2012), do not show reduction with sHz treatment when compared to control PBS treatment, in contrast with what has been observed in Figure 8 for non-silenced mosquitoes.

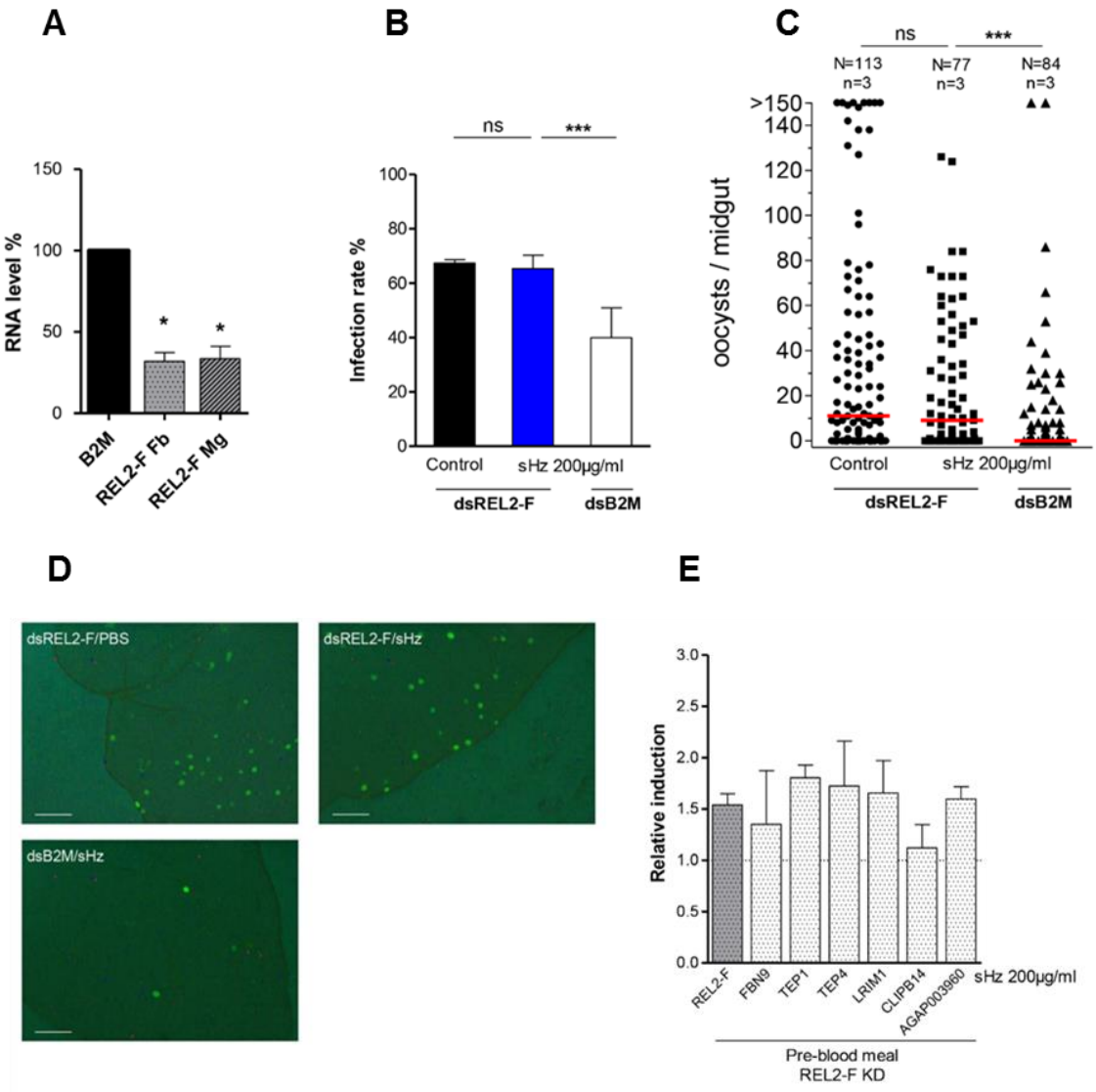


Figure 14. REL2-F silencing suspends the hemozoin effect. Female mosquitoes were silenced using dsRNA specific for *REL2-F* or control-dsRNA against *β2M*. **(A)** Silencing efficiency was measured both at the fat body (Fb) and the midgut (Mg). Differences and *P* values: ∗ ($p<0.05$), using the *Student t test*. Silenced mosquitoes were treated with sHz or PBS-control and infected with *P. berghei*; oocysts were counted 8 days post-infection. **(B, C)** Infection rate and intensity following PBS or 200µg/ml sHz injection in mosquitoes silenced for *REL2-F* or control-*β2M*. Differences and *P*-values: not significant (ns), ∗∗∗ ($p<0.001$), using the *Fisher's exact test* **(B)** and the *Mann-Whitney's test* **(C)**. **(D)** Representative *A. gambiae* midguts showing GFP-tagged *P. berghei* oocysts development in silenced and subsequently treated mosquitoes. Scale bars, 150 µm. **(E)** Relative induction fold change of REL2-F and six other immune related genes measured in the fat body following REL2-F silencing and subsequent sHz treatment, determined by qRT-PCR comparison to PBS-treated controls.

The expression levels of six immune related genes reported by some authors as REL2-regulated which were induced by sHz before the blood meal (Figure 13B), were also analysed, to evaluate these genes response to sHz following REL2-F silencing. As can be seen in Figure 14E, the average relative expression levels of all genes following sHz treatment, was markedly impaired in the REL2-F silenced fat bodies (levels from 1.12- to 1.80-fold change) when compared to the non-silenced ones (levels from 2.54- to 9.96-fold change, Figure 13B), indicating that when REL2-F is silenced, sHz modulation of these genes expression is almost ineffective.

Altogether, the results obtained in Figure 14 suggest that the more efficient antiparasitic response observed upon sHz treatment is mediated by REL2, through activation of the expression of downstream genes, including those involved in parasite elimination.

3.7 *A. gambiae* FBN9, under Regulation of the Vitellogenin 1 Promoter, is Cloned into a Plasmid Vector

The results presented above show that the stimulation of the malaria vector *Anopheles* immune system can decrease the levels of infection by *Plasmodium*. *FBN9*, a target gene of the *Anopheles* Imd pathway, has been described by us and others as playing an active role in anti-*Plasmodium* defence. Therefore, we believe that the development of a genetically modified immune-enhanced *Anopheles* transgenic line, by selective expression of this gene, will result in the generation of vector mosquitoes expressing higher levels of *FBN9*, and hence with an activated antiparasitic immune response. Different degrees of resistance to *Plasmodium* infection will depend on the efficiency of the overexpressed gene of interest and its promoter.

Although most transgenic studies in malaria vectors have been performed in *A. stephensi*, the creation of transgenic *A. gambiae* lines is essential, as this is the major vector of human malaria. Analysis of the literature shows that in *A. gambiae*, our target gene *FBN9* is essentially more expressed in the fat body and carcass than the midgut before the blood meal (Dong et al., 2006; Dong and Dimopoulos, 2009; Baker et al., 2011). Upon naïve blood feeding, *FBN9*-regulation stays higher in the fat body than the midgut (Marinotti et al., 2006). Following *Plasmodium* challenge, this gene shows a generalized increased

expression in the mosquito body (Dong and Dimopoulos, 2009). Given the selective expression of *FBN9* in the fat body, we decided to use the Vitellogenin 1 promoter to drive transgene expression of this gene, and hence target the fat body and hemocytes for an activated immune response.

As explained in section 2.10, DNA from the three construct fragments (Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator) was isolated and amplified, using cDNA or gDNA as template, as shown in Figure 15.

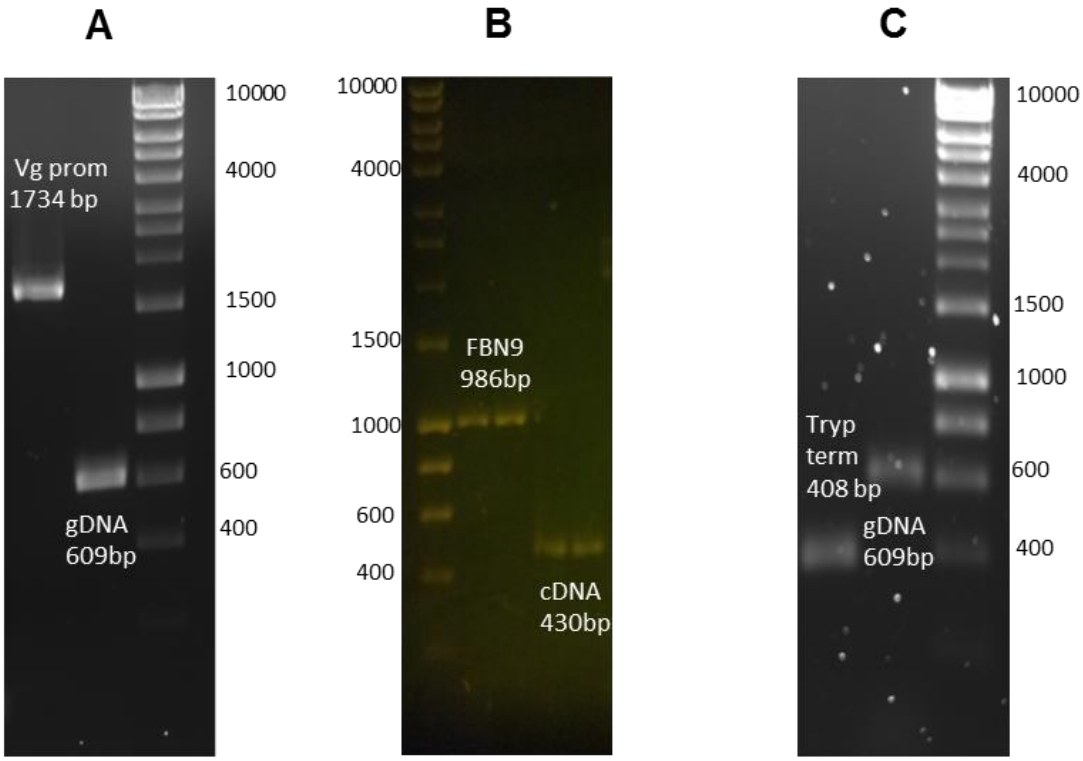


Figure 15. Purified DNA from the three cloning elements. Female *A. gambiae* cDNA/gDNA was amplified using specific primers for each fragment (Table 2). cDNA and gDNA controls were amplified using S7 primers. **(A)** Vitellogenin 1 promoter (1734 base pairs, bp) and gDNA control (609 bp), **(B)** *FBN9* gene (986 bp) and cDNA control (430 bp), **(C)** Trypsin 1 terminator (408 bp) and gDNA control (609 bp). Molecular marker: HyperLadder I (Bioline).

The cloning fragments were ligated into an entry pDSAR vector, thus achieving a final construct 7249 bp long, as summarized in Figure 16.

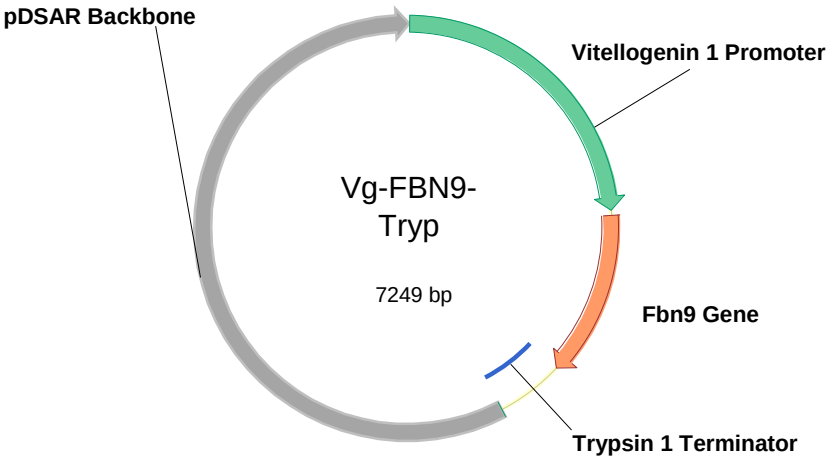


Figure 16. Plasmid map of the Vitellogenin 1-FBN9-Trypsin 1 construct.

3.8 Transformation and Colony Screening

Kanamycin-resistant grown colonies of cells containing the inserted plasmid were counted in each plate. The agar plate with cells containing the complete construct assembly comprised 1040 individual colonies, whilst the control plate with the pDSAR vector backbone alone comprised 35 colonies only (Figure 17). Hence, a 30 times colony difference existed between the two plates. This difference between the target and control plates can be explained by the lack of an insert in the control plate, that did not allow the circularization of the digested plasmid which, this way, could not survive inside the cells.

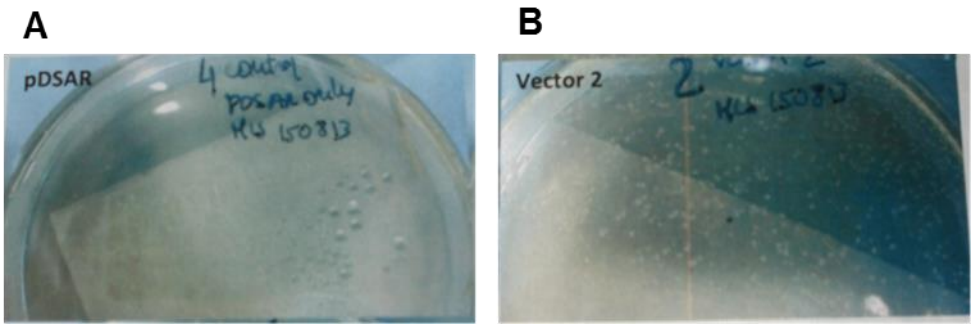


Figure 17. Agar plates with transformed cells grown overnight. (A) pDSAR vector backbone alone, **(B)** pDSAR vector backbone and inserted fragments.

From the plates shown above (Figure 17), several individual colonies were chosen to screen for the inserted fragments. An example of a colony-PCR outcome for *FBN9* gene is presented in Figure 18.

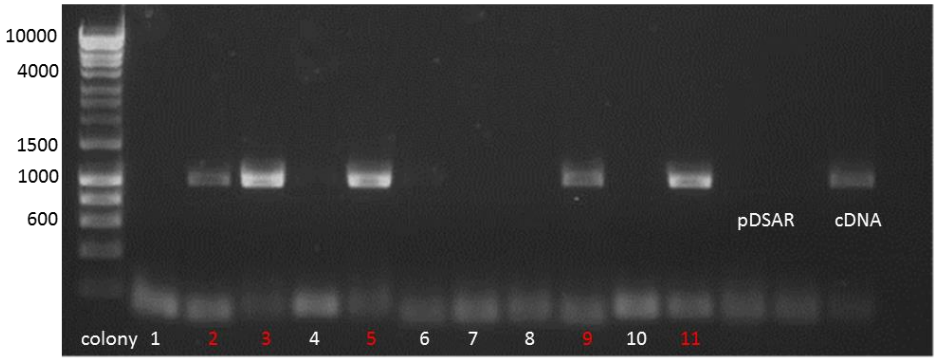


Figure 18. Colony-PCR for FBN9. *FBN9* (986 bp) was amplified by PCR using DNA directly taken from 11 transformed colonies. Five colonies (in red) showed positive results for *FBN9*. DNA from two pDSAR vector alone colonies was used as negative control and *A. gambiae* cDNA was used as positive control. Molecular marker: HyperLadder I (Bioline).

3.9 Sequencing Confirmed Successful Cloning

Sequencing of a purified plasmid DNA sample containing all three inserts, as confirmed by colony-PCR assay, revealed 99% identity (3929 bp in a total of 3988 bp sequenced) between the predicted and the obtained sequences for the construct (Appendix 3). This result confirmed the success of our cloning of the Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator into the pDSAR backbone vector. Purified plasmid DNA from a Midiprep reached a concentration of 967.7 ng/µl, eluted in 200 µl dH_2O, an amount large enough for several embryo microinjections.

3.10 Transgenic *A. gambiae* X1 line overexpressing FBN9 under Regulation of the Vitellogenin 1 Promoter

From the first set of *A. gambiae* embryo injections (*circa* 120 eggs), we obtained seven transient larvae (positive for the DsRed marker) (Figure 19), out of which only three mosquitoes survived to adulthood.

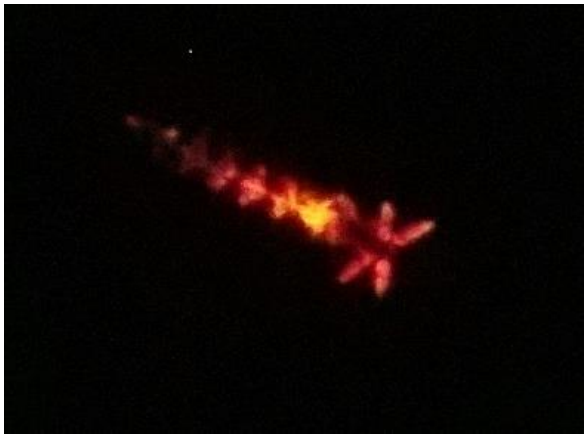


Figure 19. Picture of a transient *A. gambiae* Vitellogenin 1-FBN9-Trypsin 1 larva. Picture shows DsRed marker fluorescence under an inverted microscope (Eclipse TE200, Nikon), using the 10x objective.

All these three transient adult mosquitoes were females, which were subsequently outcrossed to 50 wildtype males before being blood fed and allowed to lay eggs. The larval progeny of these crosses was carefully analysed for DsRed fluorescence. Fifty first generation L1 larvae screened gave rise to one transgenic female individual. This female transgenic was thus the founder of our transgenic line expressing FBN9 under regulation of the fat body-specific Vitellogenin 1 promoter.

The transgenic female was outcrossed to 40 wildtype males and blood fed three times, giving rise to progeny in which 50% of the individuals were transgenic and the other 50% were wildtype (Figure 20). Wildtype mosquitoes were discarded and transgenic mosquitoes were outcrossed with wildtype individuals at least two more times. Each generation was blood fed and offspring screened for transgenics. After at least three outcrosses with the wildtype population, mosquitoes from this constituted transgenic *A. gambiae* X1 line overexpressing FBN9 under regulation of the Vitellogenin promoter, were crossed between themselves and each generation is at present being screened for transgenics. Further sets of embryo injections were performed, in order to give rise to new cages of the same engineered transgenic line, yet originated from different transgenic founders.

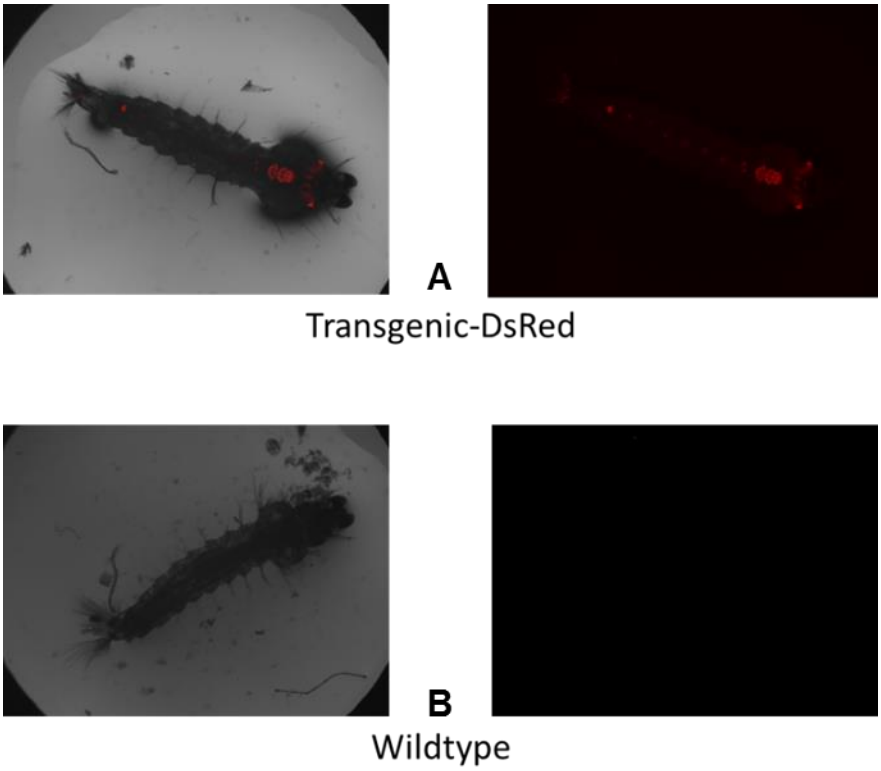


Figure 20. Picture of a transgenic *A. gambiae* Vitellogenin 1-FBN9-Trypsin 1 larva (A) compared to an *A. gambiae* wildtype larva (B). Image taken by EVOS Fluorescence Microscope (VWR), amplified 4x. **(A)** Picture shows DsRed marker fluorescence in the eyes, the gut and the anal gills.

4. Discussion and Conclusions

Several experimental studies have demonstrated that the malaria mosquito is able to mount an efficient immune response against *Plasmodium* infection, which is responsible for big parasite losses during parasite development within the vector. Nevertheless, natural immunity is not enough to totally suppress mosquito infection. In previous works performed in our laboratory, we showed that the *Anopheles* immune system can be modulated by outside factors such as antimalarial drug chloroquine (Abrantes et al., 2005, 2008), and further we observed that synthetic CpG oligodeoxynucleotides (ODNs) can induce protective responses in *Anopheles* against *Plasmodium* (Silveira et al., 2012).

The results of this work firstly demonstrate that the parasite's byproduct hemozoin has a noticeable effect in *Plasmodium* infection, impairing both infection rate and intensity (Figure 8, Table 3). Our transcription analysis based on gene expression responses to sHz, established that hemozoin can act as an immunomodulatory molecule for the mosquito. The most remarkable result obtained from our microarray screen was the differential up-regulation of the NF-κB factor REL2 upon hemozoin stimulation. These observations have prompted us to associate the successful anti-*P. berghei* defence mounted by *A. gambiae* in our infection experiments (Figure 8, Table 3) with the induction of immunity-related genes observed in our microarray data (Figure 9, Appendix 2) and in Figure 13B, and hypothesize that REL2 is regulating the transcription of these genes, following hemozoin stimulation.

Although the model used in this study was the laboratory pair *A. gambiae-P. berghei*, as it is conventionally used for the study of malaria transmission, given the difficulties in manipulating the human malaria parasite *P. falciparum*, hemozoin stimulation triggered the expression of genes described to play a role in the control of *P. falciparum* mosquito infection. In the only study testing hemozoin's effect on *Anopheles* published so far (Akman-Anderson et al., 2007), authors showed that hemozoin activates TAK1 (transforming growth factor-beta activated kinase 1) and proposed it may initiate the response leading to REL2 activation. However, no further work has been reported to elucidate the interaction between the parasite's byproduct and *Anopheles* immunity, until the findings we present here.

Several studies before ours have linked *A. gambiae* Imd pathway/REL2 to the defence against *Plasmodium* and in that way established a role for this pathway in the mosquito antiparasitic response (Meister et al., 2005; Garver et al., 2009, 2012; Dong et al., 2011; Chen et al., 2012; Clayton et al., 2013). After silencing both Imd and Toll pathways' negative regulators, Garver et al. (2009) suggested that Toll is a more ubiquitous signalling pathway, while Imd is more immunity-specific. This is in concordance with our findings, where the activation of the Imd/REL2 pathway by hemozoin led to the transcription of a high number of immunity genes (35% of the total differentially expressed genes) (Figure 9, Appendix 2), including several potent anti-*Plasmodium* factors, which might have contributed to the malaria resistant phenotype observed in this study. Of note, recent publications have stressed the differences in the *A. gambiae* immune responses against *P. berghei* and *P. falciparum*, highlighting the fact that the Imd pathway activation is more effective fighting the human pathogen than its rodent model (Garver et al., 2009, 2012; Dong et al., 2011). This observation emphasizes the need of, as much as possible given all security issues, reinforcing laboratorial studies using the natural pair *A. gambiae-P. falciparum*, thus allowing the establishment of comparisons between parasite species, as well as a more accurate comprehension of the molecular mechanisms regulating vector-parasite interactions in malaria.

The discovery that mosquito immunity to *Plasmodium* is highly modulated by the presence of bacteria in the vector midgut has stimulated a growing interest in this dual interaction. Recent studies have demonstrated that the defence mechanisms mounted by *Anopheles* against bacterial invasion, incite the activation of an anti-*Plasmodium* immune response and the consequent elimination of a large number of parasites (Dong et al., 2009; Meister et al., 2009; Rodrigues et al., 2010; Cirimotich et al., 2011; Bian et al., 2013; Bahia et al., 2014). In *A. gambiae*, REL2 was shown to be involved in the signalling pathway activated by PGRPLC (peptidoglycan recognition protein lc) following bacterial infections (Meister et al., 2009). In a forthcoming study, it will be of great interest to elucidate the interactions between hemozoin stimulation, bacteria initiation and Imd/REL2 pathway activation of the anti-*Plasmodium* response in *Anopheles*.

Previous studies by Akman-Anderson and colleagues (2007), in which *P. falciparum* hemozoin, sHz and hemin chloride were compared both in *Anopheles* cell lines and *in vivo*, showed that the effects of hemozoin stimulation were very distinct from those of

heme (provided as hemin chloride) stimulation. This observation led the authors to conclude that hemozoin's activity is attributable to the β-hematin crystal structure and not to the heme subunit. Further research to explore the specificity of the mosquito immune response to metabolite hemozoin seems, therefore, relevant.

Frolet and co-authors (2006) divided the *Anopheles* anti-parasitic response into pre- and post-invasion phases. Our work hypothesis was that upon stimulation with hemozoin, *Anopheles* basal immunity (corresponding to the pre-invasion phase, before the blood meal) would be activated and hence the mosquito able to face *Plasmodium* subsequent invasion with a stronger immune response. We have indeed demonstrated that the induction of REL2-F and other important immunity genes (reported as REL2- regulated/-partly regulated) by sHz was observable before and after the blood meal (Figure 13), showing that *Anopheles* immunity activated by pre-blood meal injected hemozoin, kept activated during parasite infection.

Our microarray results identified the up-regulated REL2 probeset as specific for the full-length isoform of REL2, REL2-F. The use of primers specific for each isoform, in further experiments, enabled us to confirm that REL2-F was up-regulated when cells and mosquitoes were treated with sHz (Figure 11A, Figure 13A). When Meister and co-workers (2005) silenced REL2 in *A. gambiae* mosquitoes and measured infection by *P. berghei* afterwards, they obtained statistically similar infection results when silencing REL2-F only and both forms of REL2 together concluding that REL2-F, instead of REL2-S, is implicated in this reaction. Additionaly, their data also suggested that processing of REL2-F is important for transcription of anti-*Plasmodium* genes. These findings may well explain our observations and lead us to propose that hemozoin stimulated REL2-F (Figure 13A, Appendix 1, Appendix 2), enabling its translocation to the nucleus, where it further activated several immunity-related genes, including those involved in parasite elimination (Figure 9, Figure 13B, Appendix 1, Appendix 2), this transcription contributing to the *P. berghei*-refractory phenotype in *A. gambiae*, presented in Figure 8. Having shown increased expression of REL2 upon sHz stimulation both in the fat body and in hemocyte-like cells, we wanted to make sure this was the main transcription factor activated by hemozoin. REL1 expression levels revealed that indeed the Toll pathway didn't seem to be meaningfully activated by sHz (Figure 11A, Figure 13A).

Functional analysis by gene silencing using dsRNA has been used in recent works to knock-down specific key factors of central signalling pathways in *Anopheles* anti-*Plasmodium* defence. A few studies focused on the Toll and Imd pathways, either by silencing REL1/REL2 or their negative regulators, both in tissues and in cells (Meister et al., 2005; Frolet et al., 2006; Garver et al., 2009, 2012; Clayton et al., 2013). In our study, by silencing *caspar*, we were able to further confirm hemozoin's REL2-F activation (Figure 12B). Note that only the full-length isoform REL2-F has inhibitory domains that must be cleaved for activation (Garver et al., 2009). Hence, the results we obtained upon *caspar* silencing come in concordance with our suggestion that REL2-F and not REL2-S is the isoform triggered by hemozoin. The role of the recently described *A. gambiae* Imd/REL2 negative regulator *caudal* (Clayton et al., 2013), as well as the role of the Jak/Stat pathway could also be examined in the future in the context of hemozoin's boosting of *Anopheles* immunity.

To explore if REL2 mediates the hemozoin effect, we investigated whether sHz treatment still had an effect on the *P. berghei* infection outcome in *A. gambiae* mosquitoes silenced for REL2-F and found that the action of hemozoin is suspended when REL2-F is knocked-down (Figure 14). Other studies have shown that transient activation of either REL1 or REL2 through gene silencing of their negative regulators *cactus/caspar* impairs the success of *Plasmodium* infection, by activation of an immune response before pathogen challenge (Frolet et al., 2006; Garver et al., 2009). But when the fitness cost of both manipulations was measured, silencing of *cactus* in laboratory conditions affected *A. gambiae* longevity and fecundity, while *caspar* silencing had no apparent fitness cost. Hence authors concluded that the manipulation of the Toll pathway could be unfavourable for the mosquito, probably because, as said, this pathway has a more wide-ranging action, while Imd is more immune specific (Garver et al., 2009). Together with the properties of the Imd/REL2 pathway described in the literature and referred here, the results presented in this thesis suggest that this pathway could be used in the advance of malaria control approaches.

The development of novel interventions against malaria based on vector-parasite interactions, constitutes a promising alternative to support, replace or complement traditional vector control strategies. This can be achieved, for example, by transgene expression of specific immunity genes from immune pathways, using cutting-edge

genetic tools to manipulate the mosquito vector. In a very promising experimental approach, Dong and colleagues (2011) were able to create transgenic lines of *A. stephensi* mosquitoes with increased expression of REL2 transcription factor. The engineered mosquitoes showed higher resistance to *Plasmodium* and bacterial infections, probably due to the action of overexpressed anti-*Plasmodium* effector genes controlled by REL2. Encouraged by this publication and our own results showing the potential of the Imd/REL2 pathway in fighting *Plasmodium* infection in the mosquito, we have successfully engineered a FBN9-enhanced *A. gambiae* line (Figure 20). Generation of this genetically modified mosquito transgenic line was technically challenging, as described in Chapters 2 and 3. Furthermore, it constitutes an accomplishment, given that *A. gambiae* has been overlooked in favor of *A. stephensi* as vector model for transgenic studies, despite the relevance of the former as major human malaria vector.

During the past ten years, hemozoin has emerged as a potent immunoactivator in mammals, influencing adaptive immune responses to malaria infection (Coban et al., 2010a, 2010b). In the work described here, we elucidated for the first time the stimulatory activity of this malarial metabolite in the *A. gambiae* vector, while unravelling effector mechanisms of the mosquito protective immune response.

We have also effectively established a transgenic line by overexpression of an important antiparasitic factor. Future work will be needed to validate its degree of increased robustness against malarial (and bacterial) infection, due to the activation of the *A. gambiae* immune response. Moreover, measurement of the fitness impact of this genetic modification on the mosquito, needs to be addressed.

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6. Appendices

6.1 Appendix 1

Probe set	Gene name	Gene ID Ensembl	Functional class	Sub-class	Baseline mean	Experiment mean	Fold change	Lower bound of Fold change
Ag.2R.37.0_CDS_s_at	CLIPC7	AGAP003689	IMMUNITY	CLIP domain serine proteases	375,41	3245,09	8,64	**4,16**
Ag.UNKN.703.0_CDS_s_at		AGAP010056	METABOLISM-Cell function		320,2	2222,89	6,94	**4,15**
Ag.3R.275.0_CDS_a_at		AGAP010056	METABOLISM-Cell function		212,99	1853,26	8,7	**3,98**
Ag.2L.1635.0_CDS_at	FBN50	AGAP005848	IMMUNITY	Recognition/Receptor	500,2	2605,31	5,21	**3,46**
Ag.2R.37.2_UTR_a_at	CLIPC7	AGAP003689	IMMUNITY	CLIP domain serine proteases	323,21	2635,21	8,15	**3,32**
Ag.2R.1602.0_CDS_at		AGAP003960	IMMUNITY	Other serine proteases	213,78	1354,41	6,34	**3,26**
Ag.3L.414.0_CDS_at	CLIPB20	AGAP012037	IMMUNITY	CLIP domain serine proteases	171,46	772,09	4,5	**3,14**
Ag.3R.44.0_CDS_at	CLIPB15	AGAP009844	IMMUNITY	CLIP domain serine proteases	304,62	1449,62	4,76	**2,97**
Ag.2R.2846.0_CDS_at		AGAP003627	IMMUNITY	Other serine proteases	38,57	259,32	6,72	**2,89**
Ag.2R.118.0_CDS_s_at		Unknown	Unknown gene		261,93	824,97	3,15	**2,55**
Ag.2R.2246.0_CDS_at		AGAP003248	IMMUNITY	Other serine proteases	247,48	1035,79	4,19	**2,47**
Ag.2R.691.0_CDS_at	CLIPB6	AGAP003252	IMMUNITY	CLIP domain serine proteases	85,69	337,91	3,94	**2,41**
Ag.3R.1335.0_UTR_at	Q5XLG8_ANOGA	AGAP009916	IMMUNITY	Recognition/Receptor	34,92	242,62	6,95	**2,37**
Ag.3L.42.0_CDS_at	TEP3	AGAP010816	IMMUNITY	Recognition/Receptor	814,99	3066,76	3,76	**2,34**
Ag.3L.261.0_CDS_at		AGAP010730	IMMUNITY	Other serine proteases	731,55	2675,66	3,66	**2,33**
Ag.2R.815.0_CDS_at		AGAP003627	IMMUNITY	Other serine proteases	245,14	1078,07	4,4	**2,31**
Ag.2L.33.0_CDS_at	CTL4	AGAP005335	IMMUNITY	Recognition/Receptor	694,74	2039,73	2,94	**2,27**
Ag.3L.197.0_CDS_at	CLIPA12	AGAP011781	IMMUNITY	CLIP domain serine proteases	924,51	2760,64	2,99	**2,25**
Ag.3R.270.0_CDS_at		AGAP009110	Unknown function		1225,86	3777,05	3,08	**2,23**
Ag.X.1492.0_at		AGAP000695	Unknown function		62,89	190,47	3,03	**2,22**
Ag.2R.37.2_CDS_a_at	CLIPC7	AGAP003689	IMMUNITY	CLIP domain serine proteases	41,85	432,96	10,34	**2,19**
Ag.2L.34.0_CDS_at	CTLMA2	AGAP005334	IMMUNITY	Recognition/Receptor	954,41	2739,23	2,87	**2,08**
Ag.3L.36.0_CDS_a_at	CLIPA2	AGAP011790	IMMUNITY	CLIP domain serine proteases	736,53	2477,3	3,36	**2,04**
Ag.3L.16.0_CDS_a_at	CLIPA4	AGAP011780	IMMUNITY	CLIP domain serine proteases	929,19	2647,13	2,85	**1,98**
Ag.3L.20.0_CDS_at	CLIPB14	AGAP010833	IMMUNITY	CLIP domain serine proteases	355,62	1191,78	3,35	**1,98**
Ag.3L.191.0_CDS_at	CLIPA7	AGAP011792	IMMUNITY	CLIP domain serine proteases	572,49	1807,76	3,16	**1,97**
Ag.3R.902.0_CDS_at		AGAP009591	METABOLISM-Cell function		430,46	1657,07	3,85	**1,94**
Ag.2L.210.0_CDS_at	LRIM1	AGAP006348	IMMUNITY	Recognition/Receptor	708,16	3137,12	4,43	**1,9**

Ag.3L.6.0_CDS_at	CLIPA1	AGAP011791	IMMUNITY	CLIP domain serine proteases	873,19	2461,81	2,82	**1,9**
Ag.2L.500.0_UTR_at		AGAP007043	IMMUNITY	Other serine proteases	447,36	1277,02	2,85	**1,89**
Ag.X.8.0_CDS_a_at	CEC4	AGAP006722	IMMUNITY	AMP	1740,67	7706,19	4,43	**1,86**
Ag.UNKN.2338.0_s_at		AGAP012851	OXIDATIVE STRESS		65,27	147,91	2,27	**1,86**
Ag.2L.705.0_CDS_a_at	Q7Q5V2_ANOGA	AGAP006193	Unknown function		629,7	1475,29	2,34	**1,83**
Ag.3R.164.0_CDS_at	Q7PVY7	AGAP009146	Unknown function		1640,41	4641,52	2,83	**1,82**
Ag.3L.1790.0_CDS_at		AGAP010934	IMMUNITY	Coagulation/Wounding	85,68	362,76	4,23	**1,81**
Ag.3R.679.0_CDS_at	Q7PVY8	AGAP009145	Unknown function		917,39	2518,35	2,75	**1,8**
Ag.2R.450.1_at		AGAP001509	METABOLISM-Cell function		1966,85	6981,5	3,55	**1,77**
Ag.2R.98.0_CDS_at	GSTD10	AGAP004383	OXIDATIVE STRESS		31,68	174,14	5,5	**1,74**
Ag.3L.364.0_CDS_s_at	CLIPA14	AGAP011788	IMMUNITY	CLIP domain serine proteases	2533,33	5540,24	2,19	**1,73**
Ag.3L.556.0_CDS_at		AGAP010386	METABOLISM-Cell function		649,69	2107,35	3,24	**1,71**
Ag.2L.557.0_CDS_at		AGAP006422	METABOLISM-Cell function		275,3	750,95	2,73	**1,7**
Ag.UNKN.1520.0_CDS_at		AGAP012614	IMMUNITY	Other serine proteases	47,83	132,4	2,77	**1,69**
Ag.3R.324.0_CDS_a_at		AGAP009985	METABOLISM-Cell function		99,52	250,1	2,51	**1,68**
Ag.2L.3944.0_at		AGAP006422	METABOLISM-Cell function		351	708,33	2,02	**1,67**
Ag.3R.314.0_CDS_at		AGAP009859	Unknown function		2016,15	5020,64	2,49	**1,67**
Ag.2L.3062.0_at	FBN50	AGAP005848	IMMUNITY		80,73	194,1	2,4	**1,66**
Ag.2R.102.0_CDS_at	CLIPB17	AGAP001648	IMMUNITY	CLIP domain serine proteases	73,77	367,78	4,99	**1,65**
Ag.X.570.0_CDS_at		AGAP000290	IMMUNITY	Other serine proteases	122,56	256,42	2,09	**1,64**
Ag.2R.25.1_CDS_a_at		AGAP004324	METABOLISM-Cell function		894,09	2368,96	2,65	**1,64**
Ag.2R.119.0_CDS_s_at	CLIPB3	AGAP003249	IMMUNITY	CLIP domain serine proteases	99,46	310,05	3,12	**1,64**
Ag.UNKN.1056.0_CDS_at		AGAP010225	TRANSCRIPTION/TRANSLATION		84,58	213,59	2,53	**1,63**
Ag.2L.528.0_CDS_at	LRIM4 (LRRD5)	AGAP007039	IMMUNITY	Recognition/Receptor	459,15	1318,73	2,87	**1,61**
Ag.3L.18.0_CDS_at	CLIPA6	AGAP011789	IMMUNITY	CLIP domain serine proteases	438,54	1156,16	2,64	**1,6**
Ag.X.264.0_CDS_at	TOLL5A	AGAP000999	IMMUNITY	Recognition/Receptor	607,39	1267,55	2,09	**1,59**
Ag.2L.1068.0_CDS_at		AGAP005612	Unknown function		550,54	2166,99	3,94	**1,59**
Ag.3R.233.0_CDS_at		AGAP009991	METABOLISM-Cell function		37,31	89,44	2,4	**1,57**
Ag.3R.2607.0_CDS_s_at		AGAP010225	TRANSCRIPTION/TRANSLATION		221,31	807,08	3,65	**1,57**
Ag.2R.530.0_CDS_at		AGAP002425	METABOLISM-Cell function		227,38	1114,29	4,9	**1,56**
Ag.3L.31.0_CDS_a_at	CLIPA8	AGAP010731	IMMUNITY	CLIP domain serine proteases	555,09	1767,48	3,18	**1,56**
Ag.3L.13.0_CDS_a_at	CLIPA7	AGAP011792	IMMUNITY	CLIP domain serine proteases	1915,46	4314,01	2,25	**1,55**
Ag.2L.2653.0_CDS_at	REL2	AGAP006747	IMMUNITY	Transcription factors/Regulation	68,4	176,66	2,58	**1,54**

Ag.2L.3005.0_at	APL1C	AGAP007033	IMMUNITY	Recognition/Receptor	2278,68	4956,35	2,18	**1,53**
Ag.3R.16.0_CDS_at	TEP15	AGAP008364	IMMUNITY	Recognition/Receptor	1007,21	2579,99	2,56	**1,52**
Ag.3R.2690.0_a_at		AGAP009620	METABOLISM-Cell function		268,38	530,66	1,98	**1,5**
Ag.X.148.0_CDS_at	Transferrin	AGAP000376	TRANSPORT		1199,29	3681,77	3,07	**1,49**
Ag.UNKN.261.0_CDS_at		Unknown	Unknown gene		71,21	151,52	2,13	**1,49**
Ag.X.8.1_a_at	CECA/CEC1	AGAP000693	IMMUNITY	AMP	3325,39	8932,64	2,69	**1,48**
Ag.2R.177.0_CDS_s_at		AGAP012711	IMMUNITY	Transcription factors/Regulation	142,98	371,08	2,6	**1,48**
Ag.3L.309.0_CDS_at		AGAP010935	METABOLISM-Cell function		231,67	478,27	2,06	**1,48**
Ag.3L.32.0_CDS_a_at	TEP4	AGAP010812	IMMUNITY	Recognition/Receptor	88,15	350,73	3,98	**1,47**
Ag.3L.1878.1_a_at	IAP2	AGAP011326	METABOLISM-Cell function		48,57	200,47	4,13	**1,47**
Ag.2L.2898.0_at		Unknown	Unknown gene		1905,38	4085,36	2,14	**1,46**
Ag.3L.449.0_CDS_at	FBN9	AGAP011197	IMMUNITY	Recognition/Receptor	945,13	2736,07	2,89	**1,46**
Ag.3L.942.0_CDS_at		AGAP011034	IMMUNITY	Coagulation/Wounding	37,69	68,3	1,81	**1,46**
Ag.3L.1022.0_CDS_a_at	Q7PZC7_ANOGA	AGAP011842	TRANSCRIPTION/TRANSLATION		319,04	601,19	1,88	**1,46**
Ag.2L.989.0_CDS_at		AGAP006177	CYTOSKELETON		1288,14	2448,88	1,9	**1,45**
Ag.3R.712.0_CDS_at		AGAP008131	METABOLISM-Cell function		103,04	183,34	1,78	**1,45**
Ag.X.544.0_CDS_at		AGAP000961	METABOLISM-Cell function		31,76	65,98	2,08	**1,44**
Ag.2L.207.0_CDS_a_at	Q9GT44_ANOGA	AGAP005712	METABOLISM-Cell function		2449,04	5482,13	2,24	**1,43**
Ag.2L.2922.0_a_at		AGAP007116	TRANSCRIPTION/TRANSLATION		52,82	101,12	1,91	**1,42**
Ag.3R.2700.0_at		Unknown	Unknown gene		200,23	552,01	2,76	**1,42**
Ag.3L.352.0_CDS_at		AGAP012218	TRANSPORT		91,9	174,25	1,9	**1,42**
Ag.2R.799.2_a_at		AGAP001749	METABOLISM-Cell function		278,17	469,5	1,69	**1,41**
Ag.3R.3441.0_at		Unknown	Unknown gene		30,45	60,07	1,97	**1,41**
Ag.X.302.1_at		AGAP000808	Unknown function		326,39	562,65	1,72	**1,4**
Ag.2R.677.0_CDS_at	IRSP3	AGAP001508	IMMUNITY	AMP	257,18	2682,23	10,43	**1,38**
Ag.3R.208.0_CDS_at		AGAP008761	TRANSCRIPTION/TRANSLATION		294,05	501,8	1,71	**1,38**
Ag.2L.2158.1_a_at		AGAP006962	METABOLISM-Cell function		151,99	250,67	1,65	**1,37**
Ag.3L.539.0_UTR_at		AGAP011519	METABOLISM-Cell function		60,49	100,33	1,66	**1,37**
Ag.3L.686.0_CDS_at		AGAP012388	Unknown function		360,23	711,47	1,98	**1,37**
Ag.3L.1366.0_CDS_s_at	FBN18	AGAP010772	IMMUNITY	Recognition/Receptor	16,94	29,75	1,76	**1,37**
Ag.3R.220.0_CDS_a_at		AGAP008861	IMMUNITY	Other serine proteases	381,77	2072,35	5,43	**1,36**
Ag.3R.2387.0_CDS_at	TGM98	AGAP009098	IMMUNITY	Coagulation/Wounding	78	191,53	2,46	**1,36**
Ag.3L.27.0_CDS_at	TEP1	AGAP010815	IMMUNITY	Recognition/Receptor	140,79	389,8	2,77	**1,36**

Ag.3L.57.0_CDS_s_at	TEP19	AGAP010832	IMMUNITY	Recognition/Receptor	20,3	55,24	2,72	**1,36**
Ag.2L.1137.2_a_at	LYSC4	AGAP007385	IMMUNITY	AMP	2269,86	3605,3	1,59	**1,35**
Ag.2L.1137.0_CDS_a_at	LYSC4	AGAP007385	IMMUNITY	AMP	1047,76	1925,36	1,84	**1,34**
Ag.3L.319.0_CDS_at		AGAP010770	CYTOSKELETON		82,86	163,98	1,98	**1,34**
Ag.2R.537.0_CDS_at		AGAP003627	IMMUNITY	Other serine proteases	680,64	2012,85	2,96	**1,32**
Ag.3R.2409.0_CDS_a_at	SRPN16	AGAP009213	IMMUNITY	Serine protease inhibitors	123,92	289,37	2,34	**1,32**
Ag.3L.390.0_CDS_at		AGAP011806	METABOLISM-Cell function		20,57	93,3	4,54	**1,32**
Ag.2L.2764.1_a_at		AGAP007302	METABOLISM-Cell function		33,13	69,04	2,08	**1,3**
Ag.2L.3089.0_at		AGAP006736	METABOLISM-Cell function		522,3	868,26	1,66	**1,3**
Ag.3R.1217.0_CDS_a_at		AGAP008315	METABOLISM-Cell function		127,43	210,11	1,65	**1,3**
Ag.3L.3.0_CDS_a_at	DEF1	AGAP011294	IMMUNITY	AMP	3937,89	10266,08	2,61	**1,3**
Ag.3L.992.0_CDS_a_at		AGAP011551	METABOLISM-Cell function		99,47	151,14	1,52	**1,3**
Ag.2R.61.0_CDS_at		AGAP001600	TRANSCRIPTION/TRANSLATION		198,31	343,94	1,73	**1,29**
Ag.2R.1200.0_UTR_a_at		AGAP003352	OXIDATIVE STRESS		703,27	1134,88	1,61	**1,29**
Ag.2L.14.0_CDS_at	SRPN2	AGAP006911	IMMUNITY	Serine protease inhibitors	2805,31	4777,54	1,7	**1,29**
Ag.3R.776.0_CDS_at		AGAP008299	METABOLISM-Cell function		581,27	1073,19	1,85	**1,29**
Ag.2R.24.0_CDS_at	CLIPB8	AGAP003057	IMMUNITY	CLIP domain serine proteases	501,22	917,99	1,83	**1,28**
Ag.2R.2279.0_CDS_at		AGAP003626	IMMUNITY	Other serine proteases	68,89	149,7	2,17	**1,28**
Ag.2R.3092.0_CDS_a_at		AGAP001652	METABOLISM-Cell func	FA	271,26	497,28	1,83	**1,28**
Ag.2L.512.0_CDS_a_at	Q7Q9F6 (KTU_ANOGA)	AGAP005250	METABOLISM-Cell function		121,63	195,86	1,61	**1,28**
Ag.3R.748.0_CDS_a_at		AGAP009866	METABOLISM-Cell function		298,57	430,68	1,44	**1,28**
Ag.3R.993.1_at		AGAP009470	TRANSPORT		67,34	103,21	1,53	**1,28**
Ag.2R.825.0_CDS_at		AGAP001447	TRANSPORT		341,25	523,04	1,53	**1,27**
Ag.2L.22.0_CDS_at	CLIPB13	AGAP004855	IMMUNITY	CLIP domain Serine proteases	586,07	1251,87	2,14	**1,27**
Ag.2L.2868.0_a_at		AGAP007366	Unknown function		1032,18	1581,37	1,53	**1,27**
Ag.2L.3544.0_at	LRIM15	AGAP007045	IMMUNITY	Recognition/Receptor	63,48	118,06	1,86	**1,27**
Ag.3L.1215.1_at		AGAP011871	IMMUNITY	Recognition/Receptor	219,64	361,23	1,64	**1,27**
Ag.UNKN.1519.0_CDS_s_at		AGAP012614	IMMUNITY	Other serine proteases	38,7	100,73	2,6	**1,27**
Ag.2R.856.0_CDS_at	CLIPB5	AGAP004148	IMMUNITY	CLIP domain Serine proteases	480,58	912,87	1,9	**1,26**
Ag.2L.19.0_CDS_a_at	SCRASP1	AGAP005625	IMMUNITY	Recognition/Receptor	902,95	1784,93	1,98	**1,26**
Ag.3R.51.0_CDS_at	TEP12	AGAP008654	IMMUNITY	Recognition/Receptor	296,05	819,18	2,77	**1,26**
Ag.3R.619.0_CDS_at		AGAP008267	METABOLISM-Cell function		43,24	73,67	1,7	**1,26**
Ag.2R.379.0_CDS_a_at		Unknown	Unknown gene		1270,33	2157,31	1,7	**1,25**

Ag.2R.1507.0_CDS_at		AGAP001940	Unknown function		266,06	412,2	1,55	**1,25**
Ag.3R.694.0_CDS_at		AGAP009849	IMMUNITY	Other serine proteases	43,85	136,56	3,11	**1,25**
Ag.3L.233.2_CDS_a_at	Spondin fragment	AGAP011765	IMMUNITY	Coagulation/Wounding	532,49	920,18	1,73	**1,25**
Ag.3L.1860.0_CDS_at	FBN10	AGAP011230	IMMUNITY	Recognition/Receptor	66,16	129,09	1,95	**1,25**
Ag.2R.3030.0_CDS_at		AGAP001225	METABOLISM-Cell function		27,76	51,18	1,84	**1,24**
Ag.3R.406.0_CDS_a_at		AGAP009546	IMMUNITY	Protein binding	184,27	279,47	1,52	**1,24**
Ag.3R.1382.0_CDS_at		AGAP007844	TRANSCRIPTION/TRANSLATION		54,97	89,6	1,63	**1,24**
Ag.2R.782.0_CDS_at		AGAP003928	METABOLISM-Cell function		245,15	384,09	1,57	**1,23**
Ag.2R.2142.0_CDS_at		AGAP001983	EGG/PRODUCTION_hormone related		55,46	90,77	1,64	**1,23**
Ag.3R.2631.0_at	Q7Q8S9_ANOGA	AGAP008011	Unknown function		571,7	951,61	1,66	**1,23**
Ag.3L.170.0_CDS_at		Unknown	TRANSPORT		362,65	1031,08	2,84	**1,23**
Ag.UNKN.1005.0_CDS_s_at		AGAP010580	Unknown function		246,57	409,78	1,66	**1,23**
Ag.2R.566.0_CDS_at		AGAP003960	IMMUNITY	Other serine proteases	457,92	920,13	2,01	**1,22**
Ag.2R.903.0_CDS_at		AGAP013252	IMMUNITY	Other serine proteases	44,57	102,97	2,31	**1,22**
Ag.2R.3767.0_at		Unknown	Unknown gene		343,38	482,87	1,41	**1,22**
Ag.2L.29.0_CDS_a_at	LYSC1_ANOGA	AGAP007347	IMMUNITY	AMP	1762,24	4497,49	2,55	**1,22**
Ag.2L.1248.0_CDS_at		AGAP006644	IMMUNITY	Recognition/Receptor	94,72	183,75	1,94	**1,22**
Ag.3R.60.0_CDS_a_at	TEP14	AGAP008368	IMMUNITY	Recognition/Receptor	244,04	669,92	2,75	**1,22**
Ag.2R.410.0_CDS_a_at		AGAP001718	METABOLISM-Cell function		738,34	2176,49	2,95	**1,21**
Ag.2R.3242.0_CDS_at		AGAP002456	CYTOSKELETON		107,09	147,72	1,38	**1,21**
Ag.2L.764.0_UTR_a_at		AGAP006264	METABOLISM-Cell function		277,07	367,24	1,33	**1,21**
Ag.2L.2771.1_CDS_a_at	LYSC8	AGAP007344	IMMUNITY	AMP	14,58	25,94	1,78	**1,21**
Ag.3R.426.0_CDS_at		AGAP009288	TRANSCRIPTION/TRANSLATION		253,34	396,2	1,56	**1,21**
Ag.3L.930.0_CDS_at		AGAP010854	TRANSPORT		49,68	83,69	1,68	**1,21**
Ag.X.64.0_CDS_x_at	CTL1	AGAP000871	IMMUNITY	Recognition/Receptor	64,29	107,53	1,67	**1,2**
Ag.3L.781.0_CDS_at		AGAP010350	IMMUNITY	Recognition/Receptor	18,54	169,84	9,16	**1,2**
Ag.2L.188.0_UTR_a_at		AGAP004877	CYTOSKELETON		2459,65	1718,22	-1,43	**-1,2**
Ag.UNKN.162.0_CDS_at		AGAP012522	Unknown function		112,2	67,91	-1,65	**-1,2**
Ag.2R.1402.0_CDS_at		AGAP002806	TRANSCRIPTION/TRANSLATION		216,67	145,96	-1,48	**-1,21**
Ag.2R.1610.0_CDS_at		AGAP004206	TRANSCRIPTION/TRANSLATION		89,99	46,84	-1,92	**-1,21**
Ag.2L.3753.0_at		AGAP005261	METABOLISM-Cell function		107,63	63,74	-1,69	**-1,21**
Ag.3R.3102.0_at		Unknown	Unknown gene		167,07	54,57	-3,06	**-1,21**
Ag.2L.294.0_CDS_at		AGAP004739	TRANSCRIPTION/TRANSLATION		2461,61	1380,02	-1,78	**-1,22**

Ag.2L.560.0_CDS_at		AGAP006808	TRANSCRIPTION/TRANSLATION		263,09	149,59	-1,76	**-1,22**
Ag.2L.1260.0_CDS_at		AGAP006812	TRANSCRIPTION/TRANSLATION		45,98	26,3	-1,75	**-1,22**
Ag.3L.634.0_CDS_at		AGAP010491	TRANSCRIPTION/TRANSLATION		215,76	142,63	-1,51	**-1,22**
Ag.2R.747.4_CDS_a_at		AGAP001315	CYTOSKELETON		48,1	24,88	-1,93	**-1,23**
Ag.2L.426.1_CDS_a_at		AGAP006254	METABOLISM-Cell function		95,12	64,68	-1,47	**-1,23**
Ag.2L.3021.0_at		Unknown	Unknown gene		185,84	110,91	-1,68	**-1,23**
Ag.3L.1074.0_CDS_at	Q7Q0F5	AGAP012423	Unknown function		60,7	18,33	-3,31	**-1,23**
Ag.2L.2222.0_CDS_at		AGAP007389	METABOLISM-Cell function		51,22	27,72	-1,85	**-1,24**
Ag.3R.2800.1_a_at		Unknown	Unknown gene		351,44	126,98	-2,77	**-1,24**
Ag.3R.3047.0_at		AGAP008137	TRANSPORT		228,91	109,57	-2,09	**-1,24**
Ag.UNKN.211.0_CDS_at		AGAP004684	Unknown function		65,78	40,12	-1,64	**-1,24**
Ag.2R.1042.0_UTR_at		AGAP003047	TRANSCRIPTION/TRANSLATION		1019,25	685,01	-1,49	**-1,25**
Ag.2L.641.0_CDS_a_at		AGAP005549	TRANSCRIPTION/TRANSLATION		79,34	49,15	-1,61	**-1,25**
Ag.2L.744.0_CDS_a_at		AGAP005324	METABOLISM-Cell function		43,04	23,64	-1,82	**-1,25**
Ag.3L.2475.0_at		Unknown	Unkown gene		103,64	70,78	-1,46	**-1,25**
Ag.2R.3737.0_s_at		AGAP003007	CYTOSKELETON		513,82	348,94	-1,47	**-1,26**
Ag.3R.552.0_CDS_at		AGAP008951	TRANSCRIPTION/TRANSLATION		205,06	78,96	-2,6	**-1,26**
Ag.3R.1753.1_at		AGAP008677	METABOLISM-Cell function		328,25	216,86	-1,51	**-1,26**
Ag.3L.2173.0_at		AGAP011718	Metabolism-Cell function	FA	585,19	347,93	-1,68	**-1,26**
Ag.2R.327.0_CDS_a_at		AGAP001730	TRANSCRIPTION/TRANSLATION		192,17	105,39	-1,82	**-1,27**
Ag.3L.2662.0_at		Unknown	Unknown gene		336,41	178,2	-1,89	**-1,27**
Ag.UNKN.114.0_CDS_at		AGAP010315	METABOLISM-Cell function		314,62	191,82	-1,64	**-1,27**
Ag.X.1152.0_s_at		AGAP012956	Unknown function		148,47	51,12	-2,9	**-1,28**
Ag.2L.2177.1_at		AGAP007103	CYTOSKELETON		141,24	87,56	-1,61	**-1,28**
Ag.3R.3130.0_at	Q7PPL0_ANOGA	AGAP008688	Unknown function		4696,61	3035,18	-1,55	**-1,29**
Ag.3L.234.0_CDS_a_at		AGAP011396	CYTOSKELETON		313,43	179,8	-1,74	**-1,29**
Ag.X.556.0_CDS_at		Unknown	Unknown gene		89,56	53	-1,69	**-1,3**
Ag.3L.1517.1_a_at		AGAP011460	IMMUNITY	Serine protease inhibitors	3459,36	1849,56	-1,87	**-1,3**
Ag.UNKN.2335.0_s_at		Unknown	Unknown gene		2754,45	954,04	-2,89	**-1,3**
Ag.2L.3243.0_at		Unknown	Unknown gene		236,97	138,34	-1,71	**-1,31**
Ag.3R.3556.0_at		Unknown	Unknown gene		275,4	163,64	-1,68	**-1,31**
Ag.3L.356.0_CDS_at		AGAP012408	TRANSCRIPTION/TRANSLATION		189,46	104,31	-1,82	**-1,31**
Ag.UNKN.2509.0_at		AGAP012517	Unknown function		53,98	29,96	-1,8	**-1,31**

Ag.3L.124.1_UTR_at		AGAP012844	CYTOSKELETON		683,46	360,76	-1,89	**-1,32**
Ag.3R.365.0_CDS_a_at		AGAP008408	IMMUNITY	Recognition/Receptor	110,65	63,47	-1,74	**-1,33**
Ag.2R.1661.0_CDS_at		AGAP001552	METABOLISM-Cell function		61,61	32,02	-1,92	**-1,36**
Ag.X.1209.0_at		AGAP000088	OXIDATIVE STRESS		169,73	91,79	-1,85	**-1,38**
Ag.UNKN.341.0_CDS_at		AGAP004671	Unknown function		88,33	50,63	-1,74	**-1,38**
Ag.2R.1148.1_a_at		AGAP004556	TRANSCRIPTION/TRANSLATION		376,59	206,02	-1,83	**-1,4**
Ag.2L.212.0_UTR_a_at		AGAP005005	Unknown function		698,58	392,39	-1,78	**-1,4**
Ag.3L.2284.0_at		Unknown	Unknown gene		200,31	101,88	-1,97	**-1,41**
Ag.X.416.1_CDS_a_at		AGAP000086	Unknown function		46,84	26,56	-1,76	**-1,42**
Ag.2L.983.0_UTR_at		AGAP005870	TRANSCRIPTION/TRANSLATION		53,36	30,26	-1,76	**-1,44**
Ag.UNKN.839.0_CDS_s_at		Unknown	Unknown gene		64,34	28,87	-2,23	**-1,44**
Ag.UNKN.2225.0_at		Unknown	Unknown gene		912,43	366,99	-2,49	**-1,44**
Ag.2R.505.0_UTR_at		AGAP003405	TRANSCRIPTION/TRANSLATION		176,51	81,87	-2,16	**-1,45**
Ag.2L.864.0_UTR_a_at		AGAP005075	TRANSCRIPTION/TRANSLATION		169,22	90,15	-1,88	**-1,45**
Ag.2L.713.0_CDS_a_at		AGAP006809	IMMUNITY	Other serine proteases	198,86	96,48	-2,06	**-1,47**
Ag.X.727.0_CDS_at		AGAP000356	IMMUNITY	Serine protease inhibitors	209,26	73,1	-2,86	**-1,5**
Ag.2R.3860.0_at		Unknown	Unknown gene		490,39	242,23	-2,02	**-1,5**
Ag.3R.420.0_CDS_at		AGAP008892	CYTOSKELETON		2258,97	748,46	-3,02	**-1,52**
Ag.X.82.2_UTR_a_at		AGAP001023	CYTOSKELETON		1051,91	469,74	-2,24	**-1,53**
Ag.2R.1238.0_CDS_at		AGAP001261	TRANSCRIPTION/TRANSLATION		204,34	100,45	-2,03	**-1,53**
Ag.3R.683.0_CDS_at		AGAP009310	TRANSCRIPTION/TRANSLATION		279,47	131,33	-2,13	**-1,54**
Ag.3L.635.0_CDS_a_at		AGAP010553	TRANSCRIPTION/TRANSLATION		119,13	62,43	-1,91	**-1,61**
Ag.2R.2275.0_CDS_at			Unknown gene		4407,8	823,97	-5,35	**-1,63**
Ag.2R.3015.0_CDS_at		AGAP004670	METABOLISM-Cell function		77,42	31,82	-2,43	**-1,71**
Ag.3L.1668.0_CDS_at		AGAP012421	Unknown function		184,9	52,84	-3,5	**-1,72**
Ag.UNKN.251.0_CDS_a_at		AGAP010297	CYTOSKELETON		166,77	74,43	-2,24	**-1,99**
Ag.3L.2293.0_at		AGAP012223	TRANSCRIPTION/TRANSLATION		105,84	29,54	-3,58	**-2,03**

Appendix 1. Microarray-derived gene expression in *Anopheles gambiae* fat body following control-PBS or sHz inoculation. The list includes all differentially expressed genes.

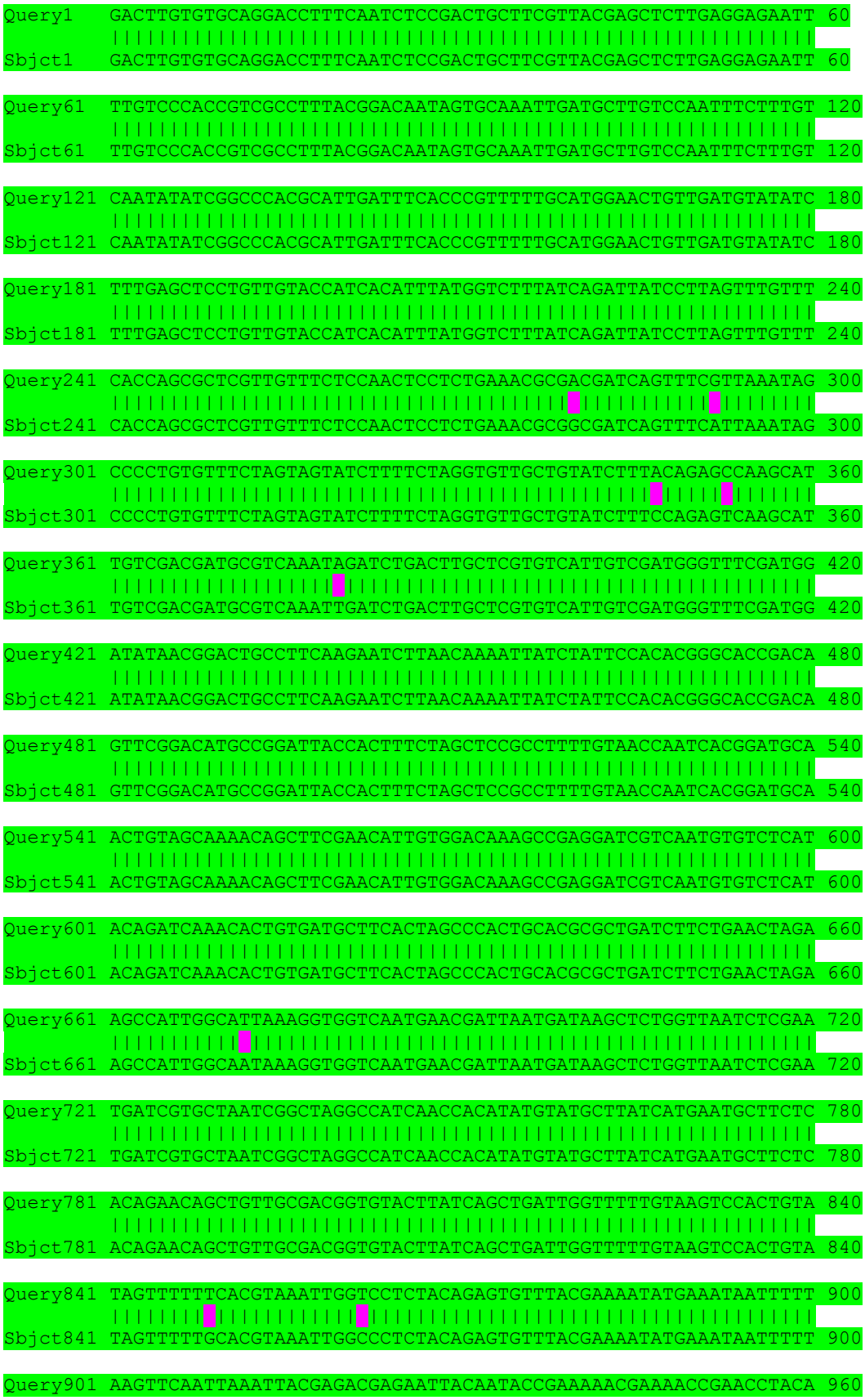
6.2 Appendix 2

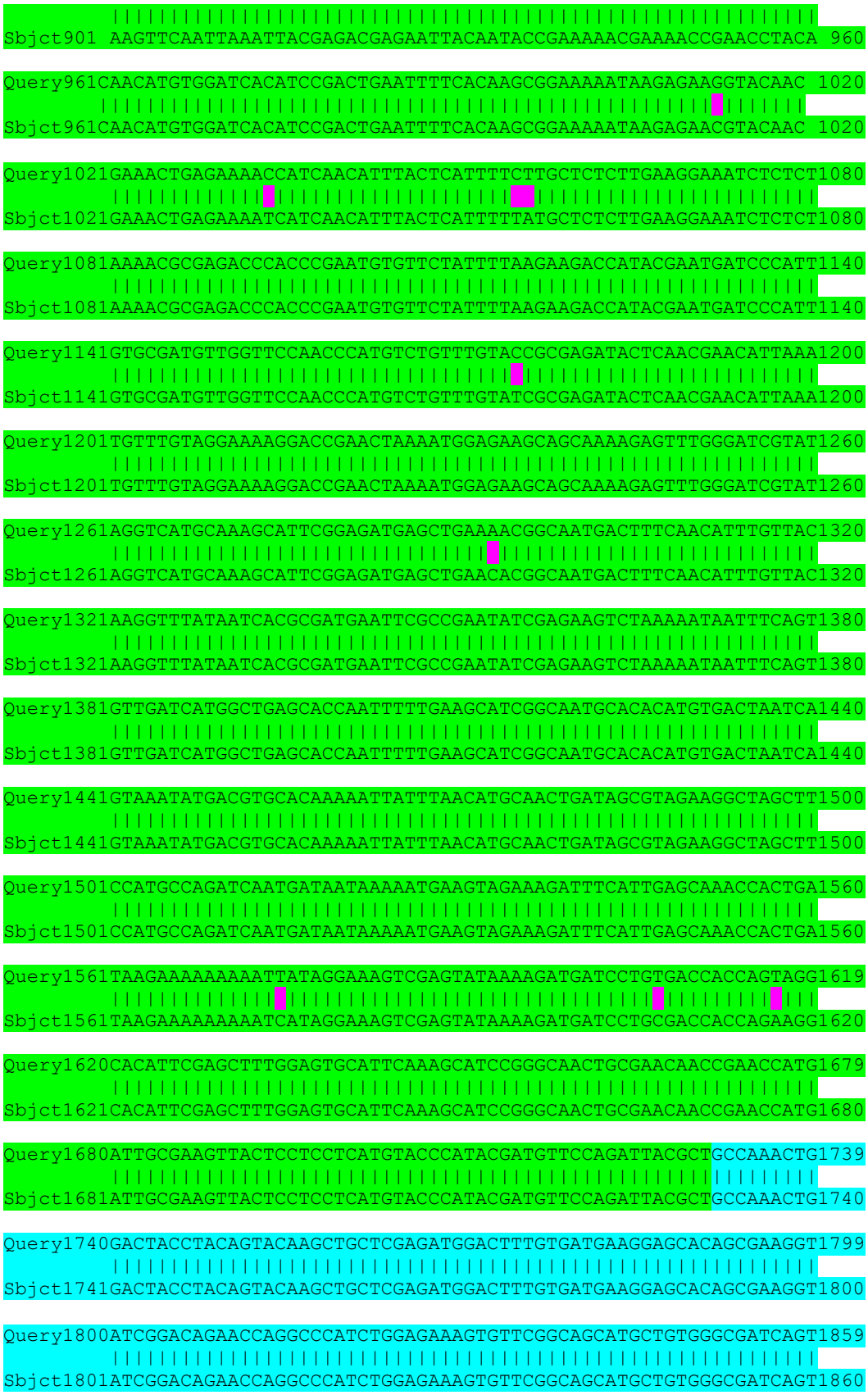
Gene ID	Gene Name	Lower bound
Recognition/Receptor		
AGAP005848	FBN50	3.46
AGAP009916	Q5XLG8_ANOGA	2.37
AGAP010816	TEP3	2.34
AGAP005335	CTL4	2.27
AGAP005334	CTLMA2	2.08
AGAP006348	LRIM1	1.90
AGAP007039	LRIM4	1.61
AGAP000999	TOLL5A	1.59
AGAP007033	APL1C	1.53
AGAP008364	TEP15	1.52
AGAP010812	TEP4	1.47
AGAP011197	FBN9	1.46
AGAP010772	FBN18	1.37
AGAP010815	TEP1	1.36
AGAP010832	TEP19	1.36
AGAP007045	LRIM15	1.27
AGAP011871		1.27
AGAP005625	SCRASP1	1.26
AGAP008654	TEP12	1.26
AGAP011230	FBN10	1.25
AGAP006644		1.22
AGAP008368	TEP14	1.22
AGAP000871	CTL1	1.20
AGAP010350		1.20
CLIP domain serine proteases		
AGAP003689	CLIPC7	4.16
AGAP012037	CLIPB20	3.14
AGAP009844	CLIPB15	2.97
AGAP003252	CLIPB6	2.41
AGAP011781	CLIPA12	2.25
AGAP011790	CLIPA2	2.04
AGAP011780	CLIPA4	1.98
AGAP010833	CLIPB14	1.98
AGAP011792	CLIPA7	1.97
AGAP011791	CLIPA1	1.90
AGAP011788	CLIPA14	1.73
AGAP001648	CLIPB17	1.65
AGAP003249	CLIPB3	1.64
AGAP011789	CLIPA6	1.60
AGAP010731	CLIPA8	1.56
AGAP003057	CLIPB8	1.28
AGAP004855	CLIPB13	1.27
AGAP004148	CLIPB5	1.26

Other serine proteases		
AGAP003960		3.26
AGAP003627		2.89
AGAP003248		2.47
AGAP010730		2.33
AGAP007043		1.89
AGAP012614		1.69
AGAP000290		1.64
AGAP008861		1.36
AGAP003626		1.28
AGAP009849		1.25
AGAP013252		1.22
Antimicrobial peptides (AMP)		
AGAP006722	CEC4	1.86
AGAP000693	CEC1	1.48
AGAP001508	IRSP3	1.38
AGAP007385	LYSC4	1.35
AGAP011294	DEF1	1.30
AGAP007347	LYSC1	1.22
AGAP007344	LYSC8	1.21
Coagulation/Wounding		
AGAP010934		1.81
AGAP011034		1.46
AGAP009098	TGM98	1.36
AGAP011765		1.25
Serine proteases inhibitors		
AGAP009213	SRPN16	1.32
AGAP006911	SRPN2	1.29
Transcription factor		
AGAP006747	REL2	1.54
AGAP012711		1.48
Protein binding		
AGAP009546		1.24

Appendix 2. Immunity related genes differentially induced in the fat body following sHz treatment.

6.3 Appendix 3

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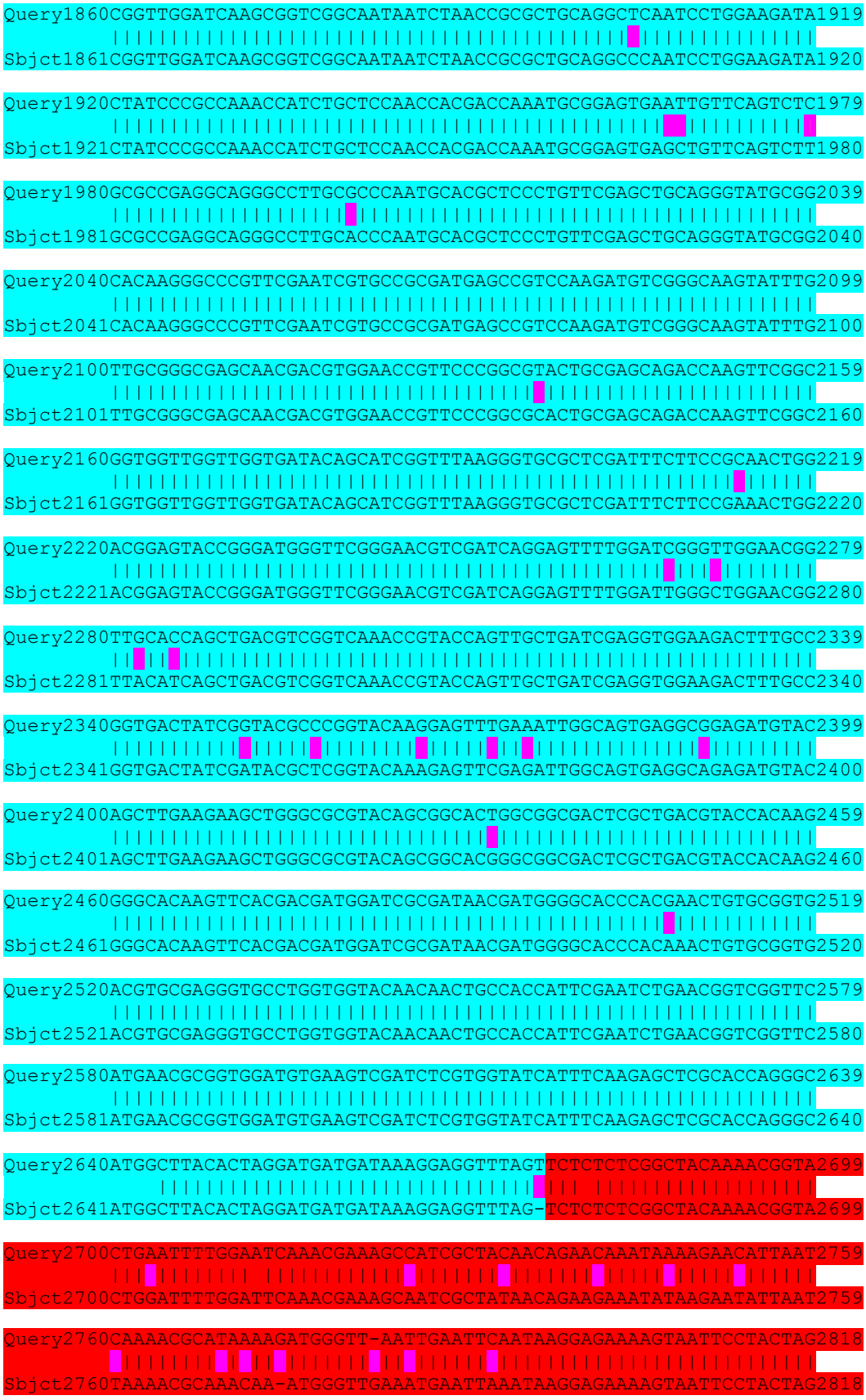
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Appendix 3. Aligned DNA sequences. Query corresponds to the predicted sequence and Subject (Sbjct) corresponds to the obtained sequence. Differences between the two sequences are highlighted in pink. The Vitellogenin 1 promoter, *FBN9* gene and Trypsin 1 terminator sequences are marked in green, blue and red, respectively. The pDSAR backbone was partially sequenced (white background).