Hemolin expression during Cecropia development and its effect on malaria parasites

by

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Abstract

Hemolin is a lepidopteran member of the immunoglobulin superfamily, initially isolated from the giant silkmoth *Hyalophora cecropia*. *Hemolin* is also induced by stimulation with microbial cell wall components and was recently shown to be strongly upregulated by baculovirus and double stranded RNA. An interesting characteristic of the protein is that it is not only highly expressed during infection but also during development.

The work presented in this thesis investigated the expression of hemolin during oogenesis and embryogenesis in *H. cecropia*. Vitellogenic follicles from ovaries were analysed for the presence of the protein by immunohistochemistry in whole-mount preparations and in cryosections. PCR was used to show the presence of Hemolin transcripts throughout vitellogenesis and choriogenesis and in fertilized and unfertilized mature eggs and Western blots showed the protein in unfertilized eggs, yolk cells and embryo.
Injection of the moulting hormone 20-hydroxyecdysone (20E) into hibernating diapausing pupae (low metabolic state), upregulates *Hemolin*. When diapausing pupae were treated with 20E and the protein synthesis inhibitor cycloheximide, its expression stayed low. This shows that the hormone indirectly regulates *Hemolin* by some factor(s) induced by 20E. When both bacteria and 20E were injected into diapausing pupae, an enhanced induction of hemolin gene expression occurred. Despite the seemingly indirect 20E regulation, several putative hormone responsive elements were found in the upstream region of the *Hemolin* (HRE-IR, HRE-M and MRE). When these elements were analysed by gel electrophoresis mobility shift assays (EMSA) to investigate their binding to nuclear factors, all the sites resulted in specific retarded bands. The HRE-IR binding factor was clearly increased by ecdysone. Last but not least we have investigated the effect of Hemolin on development of the malaria parasite *Plasmodium falciparum* in the midgut of the *Anopheles* mosquitoes. Hemolin completely inhibits the development of the parasite into its final transmission stage, the sporozoite. A future goal is to generate para-transgenic mosquitoes, enforced by hemolin, to stop malaria transmission. Importantly, hemolin did not affect the mosquito fecundity when fed to the mosquito. We are currently constructing truncated forms of hemolin to gain insight into which parts are important for its effect on the parasite.

To my beloved family Wubit and Adam

Some said, John, print it: others said, No not so:
Some said, It might do good: others said, No.

John Bunyan 1628-1688
Pilgrim's Progress
Author's Apology

Table of contents

List of papers

This thesis is based on the following publications. In the text the publications are referred by the Roman numerals given below.

I - Bettencourt, R. Assefaw-Redda, Y. and Faye, I. (2000)The insect immune protein hemolin is expressed during oogenesis and embryogenesis. Mechanisms of development 95:301-304.

II - Roxström-Lindquist, K. Assefaw-Redda, Y. Rosinska K. and Faye, I. (2005) 20-hydroxyecdysone indirectly regulates hemolin gene expression in *Hyalophora cecropia.* (Submitted after revision to Insect Molecular Biology).

III - Assefaw-Redda, Y. Hollingdale, M. and Faye, I. *In vivo* development of *Plasmodium falciparum* is inhibited by the moth immune protein hemolin. (Manuscript under revision for resubmission to Nature).

Abbreviations

AMPs	antimicrobial peptides/proteins
C3, C4, C5	complement factors 3, 4 and 5
CD36	cluster of differentiation 36
CIF	Cecropia immunoresponsive factor
CTL4	C-type lectin 4
CTLMA2	mannose binding C-type lectin 2
CTLs	C-type lectins
Dif	*Dorsal-related immune factor*
dSR-CI	*Drosophila* scavenger receptor-C1
dsRNA	double stranded RNA
20E	20-hydroxyecdysone (active form of ecdysone)
EMSA	electrophoresis mobility shift assay
FITC	fluorescein isothiocyanate
GNBP	gram-negative bacteria binding protein
HP	hyperhemocytes
HRE-IR	hormone responsive element–inverted repeat
HRE-M	hormone responsive element-monomer
HREs	hormone responsive elements
Ig-like	immunoglobulin-like
Ig-SF	immunoglobulin-superfamily
Imd	Immune deficiency
IκB	Inhibitor kappa B
IRF-E	interferon regulatory factor-element
κB	kappa B (NF-κB binding sequence)
LPS	lipopolysaccharide
LRIM1	leucine-rich repeat immune protein 1
LTA	lipoteichoic acid

MIM	Multilateral Initiative for Malaria
MRE	monomeric nuclear receptor binding site
NF-κB	nuclear factor-kappa B
NRAMP-1	natural resistance-associated macrophage protein 1
Pen 1	*Plasmodium encapsulation* 1
Pen 2	*Plasmodium encapsulation* 2
Pen 3	*Plasmodium encapsulation* 3
PCR	polymerase chain reaction
PGRP	peptidoglycan recognition protein
PMA	phorbol myristate acetate
PKC	protein kinase C
PKCs	protein kinase C family
ζPKC	zeta protein kinase C
PRR	pathogen recognition receptor
QTLs	quantitative trait loci
RBC	Red blood cell
Rel	reticuloendotheliosis oncogene
RT-PCR	revers-transcriptase polymerase chain reaction
TEP1	thioester containing protein1
TEPs	thioester containing proteins

Introduction

This thesis is a product of three separate studies, with one common denominator, an insect immunoglobulin-related protein named hemolin. Hemolin is a hemolymph protein first isolated from the Giant silk moth, *Hyalophora cecropia*. The gene and/or its product have subsequently been isolated from seven species of Lepidoptera. Its expression is normally at a low level, but it is strongly augmented during infection with bacteria or virus, or when mimicking infection using constituents like Lipopolysaccharide (LPS), Lipoteichoic acid (LTA) and double stranded RNA (dsRNA). Two of the papers (I and II) are based on earlier findings in our laboratory concerning hemolin function and expression, while the paper III is a novel study aiming for a future use in global health emphasis.

Paper I springs from earlier findings that hemolin in *H. cecropia* pupae is expressed in several organs, including the oviposited egg. We investigated when and where hemolin is expressed during oogenesis and embryogenesis. The results in Paper I showed that hemolin is induced, not only during the immune response, but also during development, and we therefore continued with studies on the effect of the moulting hormone ecdysone on *Hemolin* gene expression (Paper II). Encouraged by the discovery of several putative hormone responsive elements in the upstream regulatory sequences of *Hemolin*, we made use of hibernating pupae to clarify the hormone responsiveness of these elements. We injected the pupae with 20-Hydroxyecdysone (20E), a synthetically available active form of the hormone, and tested transcription and the binding of nuclear factors to the above-mentioned hormone response elements.

Finally, Paper III presents results that provide insight into the anti-malarial effect of hemolin. Hemolin is expressed in the midgut of *H. cecropia* pupae, and we tested the effect of hemolin on malaria parasite, *Plasmodium falciparum (Pf), in vivo* by membrane feeding *Anopheles* mosquitoes. Malaria has been under control and was eradicated from many countries 30 years ago.

However, due to drug resistance, development of the parasite and the resistance of mosquitoes to insecticides and other factors, it is now resurging in many parts of the world. It is presently infesting places with no previous history of malaria. One of the reasons suggested is the global warming although that is debated. No vaccine is yet available, despite long-term efforts on vaccine development, underlining the complexity of the parasite.

Our aim is to develop transgenic or para-transgenic mosquitoes enforced with the antiparasitic gene *Hemolin*, to stop malaria parasite development in the gut of the mosquito without affecting the mosquito population.

Background

Eukaryotic organisms need to defend themselves from infections transmitted by agents that surround them. Each organism has solved this problem by different means. The universal system, known as innate immunity, is present from birth and provides the individual with a first line of defence against intruding pathogens. Evolutionarily, this is much older than the adaptive immune system, which first appeared in jawed vertebrates. The vertebrate adaptive system was gained after the lineage separation between the insects and mammals, about 450 million years ago [1]. Human beings combat pathogenic microbes not only with their immune system, but also by inventing antibiotics and other drugs. Although the human intelligence surpasses all or almost all other animals, microorganisms evolve quickly to circumvent the effects of antimicrobial drugs and generate more fit progenies. When the defence against pathogenic organisms is not sufficient or compromised, it can lead to the death of that individual. It can also, more dramatically, lead to the extinction of the entire species, at least locally.

Insects among all phyla in the animal kingdom seem to have

succeeded best in life, accounting for more than one million different species [2]. This is proof that they have developed a potent and efficient immune system. It is noteworthy that despite the hundreds of millions of years between the separation of vertebrates and insects, their innate immune systems still have substantial similarities. Many pathways, effector molecules and mechanisms of action are similar [3]. The main difference between innate and adaptive immunity lies in the ability of the adaptive immunity to remember an antigen that has been encountered. Moreover, the immunological memory of the adaptive immune system offers a faster and enhanced response during succeeding infection with the same or similar pathogens. The adaptive immunity normally gives long-lived protection, as seen in the concept of vaccines. Insects lack the characteristic machinery of adaptive immunity, and it is an accepted belief that they lack the adaptive immune response. Some type of acquired immunity, however, has been proposed in insects [4]. When the copepod, *Macrocyclops albidus* was exposed to the tapeworm, *Shistocephalus solidus,* and three days later challenged with the same or sibling parasite, it showed a less pronounced secondary infection than when infected with an unrelated parasite [5]. This indicates some specificity and memory in the copepod's immunity. Little *et al* also showed specific memory in invertebrates. *Daphnia* clones were infected with different *Pasteuria ramosa* strains and grown for three generations. Offspring then received either homologous (the same) or a heterologous (different) strain of *P. ramosa,* and fecundity was scored. Infection with *P. ramosa* causes fecundity reduction in *Daphnia magna* [6]. The clones that were infected with homologous parasites showed higher fecundity scores, reflecting milder infection due to enhanced resistance. Challenging *P. japonicus* a second time with the same fungal agent gave more protective response than a single challenge, measured by the hemocyte proliferation rate (HPR) [7]. Injection of gram negative bacteria or LPS (a bacterial surface molecule) into the yellow mealworm *T. molitor* leads to protection against fungal infection by

Metarhizium anisopliae [8], suggesting some kind of memory for similar molecules. Vaccine against white spot syndrome in shrimps, *P. monodon*, gave protective immunity and reduced the mortality rate [9, 10]. The number of studies published on innate immunity is growing fast: publications in the innate immunity field have risen from about 60 in 1993 to 950 in 2003 [11]. We hope that this explosive escalation of research into innate immunity will reduce uncertainty concerning adaptive immunity in invertebrates, but first the definition of adaptive immunity must be clarified. If adaptive immunity is described as the presence of B-cell and T-cell clonal selection and antibody production, then adaptive immunity in insects is not demonstrated since such cells and memory derived of such cells has not been described in insects as per today. However, if we describe adaptive immunity and the subsequent "memory" as the ability to recognize molecule(s) that the organism has previously encountered and the accompanying ability to respond faster/better, then some insects show such innate immune memory. Insects have developed a way to fight microbes instantly in an unspecific manner, which may have been of selective advantage since the insect lifespan is generally short. The specific adaptive immune system is slow, and it takes 1-2 weeks to reach an optimal response, a period which is about half of the lifetime of an insect. The large number of insect species, over one million, corresponding to more than half of all animal species, reflects the efficacy and potency of their immune system [2]. The adaptive immunity approach is very energy-costly for the organism, and insects reduce their energy requirement by a factor of about hundred by not practicing adaptive immunity [12]. It is a widespread belief that the short lifespan of an insect makes it unnecessary to have adaptive immunity, but some insects, such as *Formica fusca, Formica rufibarbis, Formica exsecta, Lasius flavus, Leptothorax melas, Atta cephalotes*, have life spans of over ten years [13] and *Lasius niger* even more than 28 years [14]. It would be interesting to study if such long-lived insects posses adaptive immunity.

The insect immune system

Immune systems are thus of two types: innate (natural) immune systems and adaptive (acquired) immune systems. The innate system is the immune system for the species while the adaptive immune system refers to the individual immunity. Although insects are always at high risk of infection by microorganisms, due to their habitat, they are seldom infected. The insect immune system is a biphasic system that relies on humoral and cellular responses [15, 16, 17, 18, 19, 20, 21]. There is no clear dividing line between these two systems since a stimulus can elicit both cellular and cell-free responses at the same time. The cellular response consists of phagocytosis, encapsulation and melanization; while the cell-free or humoral defence refers to the clotting of the hemolymph [22], the prophenoloxidase system [23] and synthesis of antimicrobial peptides [19, 20, 24].

Phagocytosis

Phagocytosis is a process of engulfing and subsequently killing and degrading infiltrating microorganisms or altered own cells. Phagocytosis is carried out by phagocytic hemocytes. It includes four distinct stages, of which the first stage is recognition of the intruder, the recognition being mediated by circulating pathogen recognition receptors (PRRs). These surveillance molecules recognize the “footprints” of the microorganisms by their unique and conserved epitopes, known as pathogen associated molecular patterns (PAMPs) [19, 25]. Recognition leads to the second stage, adhesion, which is followed by the third stage, endocytosis, and finally internalization of the pathogen occurs [26]. Phagocytosis is carried out by hemocytes, the blood cells of insects, and the mechanisms and molecules involved in phagocytosis in vertebrates and invertebrates show homology, which suggests that they have a common origin.

The surface molecules (receptors) on insect hemocytes, such as *Drosophila* scavenger receptor C-1 (dSR-CI) [27], Malvolio [28] and croquemort [29], show similarity to class A macrophage specific scavenger receptors, natural resistance-associated macrophage protein-1 (NRAMP-1) [30] and cluster of differentiation (CD36) respectively [29, 31]. Peroxidasin is another homologous immunity related molecule in insects and mammals. This protein is found on the surface of phagocytic cells and is important during the breakdown of phagosomes [32, 33].

Encapsulation and nodule formation

Encapsulation and nodule formation are important mechanisms of the innate immune response, which takes place in the open circulatory system of an insect. Encapsulation and nodule-formation are similar processes. Nodulation takes place during bacterial aggregation while encapsulation takes place in response to large parasites. Lavine and Strand found that the ultra-structural morphologies of the two processes are very similar [34], which suggests that the same process occurs.

Encapsulation is carried out by the circulating hemocytes. It is normally used when a parasite is too big to be phagocytised by a single hemocyte, or during high number of septic microbial infection. A single hemocyte of the type known as hyperphagocytic hemocytes (HP) can engulf about 500 bacteria: hyperphagocytic hemocytes account for about 1% of the total hemocyte population [35]. Isolating the parasites or the microbes by forming a capsule is an important defence; it limits the infection intensity and hinders the spread of infection to nearby tissues and organs. Hemocytes make a capsule of several layers of cells surrounding the intruder [26]. Encapsulation of a pathogen does not seem to raise a signal for the induction of systemic humoral effector molecules [36]. In wasp egg infected *Drosophila* larvae the

concentrations of antimicrobial peptides are low, but they increased upon ensuing bacterial stimulation to the fully induced level, indicating that the wasp eggs are not recognized in a manner that gives rise to antimicrobial peptide induction [36]. The hemocytes attached to the parasite release sticky substances to recruit more hemocytes that subsequently participate in the agglutination and encapsulation process. The hemocytes in the immediate vicinity of the parasite release enzymes that lead to the prophenoloxidase activation cascade [37, 38, 23] and subsequent melanization.

Different kinds of hemocytes participate in the act of nodulation. In Lepidoptera, the granular cells and plasmatocytes are the active hemocytes, while in Diptera such as *Drosophila* the lamellocytes are involved in encapsulation and nodule formation [39, 40, 34, 20, 41]. Melanization of nodules and encapsulation of parasites do not occur in all insects [42].

Cell-free immunity

Cell-free immunity, also known as the humoral defence, can utilize the molecules that are produced by immunocytes and other organs. In the humoral defence hemocytes are not physically involved but they produce effector molecules and in that way contribute to humoral immunity. Immunocytes are characterized as cells that can distinguish self, altered self and non-self and are able to produce and release effector molecules. The first identification of such effector molecules conferring a potent antibacterial activity was achieved in 1972 in *Drosophila* [43] and later in the Saturniid moths *Samia cynthia* and *H. cecropia* [44]. The first molecules to be isolated were the cecropins from the *H. cecropia* moth [45]. These findings opened a new era of investigations into antimicrobial proteins, not only in insects but also in other eukaryotic organisms. Today about one thousand effector molecules of this kind have been isolated and/or sequenced from vertebrates, invertebrates and plants [46]. These

humoral effector molecules are often referred as antimicrobial peptides/proteins (AMPs). About 50% of these AMPs have been isolated from insects [47].

AMPs function in general by disrupting the integrity of the microbial membranes, thereby causing lysis of the invading microorganisms. Some of these molecules are active against bacteria (Attacin, Diptericin, Drosocin, defensin *etc*), while others (cecropins, Lysozymes and Metchnikowin) are active against both bacteria and fungi. Drosomycin is active only against fungi [19]. Insects are at constant risk of mechanical injuries and are surrounded by infection risk in their daily life. The innate immune system in insects is very efficient – their species diversity and number proves their survival capacity [48]. Injuries that breach the insect's cuticle often lead to septic infection. The insect immune genes may be slightly activated by sterile injury itself, and then is activated strongly in response to microbial invasion. Regulation of the AMP gene is characterized by the initial recognition of foreign entities by receptors and downstream signalling pathways, leading to the induction of a broad array of antimicrobial genes [49]. Immune system genes are triggered during infection to produce large amounts of the antimicrobial peptides [43, 19].

Not much was known about the key regulatory elements and transcription factors in this process until the cloning and sequencing of some insect immune genes [50]. Intensified work revealed further immune genes in *H. cecropia* [51, 52, 53]. Analysing the upstream sequences of AMP genes in *H. cecropia* revealed promoter elements with striking homology to the mammalian κB-motif [54], which is the binding site for the *cis*-acting element. The mammalian factor NF-κB belongs to the Rel-family of transcription factors [53, 55, 56]. Sun and Faye purified the corresponding factor in *H. cecropia* and named it Cecropia immunoresponsive factor (CIF), and demonstrated its similarity to NF-κB in the sense of immunological cross-reactivity, affinity and activation kinetics [57, 58]. CIF is translocated into the nucleus after bacterial infection, as NFκB is. NFκB is retained in the cytosol through the action of an

inhibitor, IκB, which is released upon external stimulation by LPS and other bacterial components. Engström et al. showed that the κB-motif regulates the expression of immune genes by transient expression of reporter gene constructs in *D. melanogaster* mbn 2 cells [59].

The field has grown exponentially by further developments in *Drosophila* genetics and new molecular tools. Two NFκB/Rel transcription factors of importance have been cloned; *Dif* (*Dorsal*-related immune factor) and *Relish,* and these are activated by two main signaling pathways [60] [61]. The *Cactus* gene product is the main inhibitor of the *Drosophila* Rel proteins Dif and Dorsal (IκB-homologue), while Relish has its own inhibitor fused to the Rel part. These two main signaling routes, the Toll and the Imd pathways, are responding to bacteria and fungi through different receptor molecules and activating *Dif*, *Dorsal* or *Relish* [62, 19]. Paper II of this thesis describes hormone regulation as an additional route of immune gene activation. The bacterially mediated induction through CIF seems to synergize with the hormone induction of *Hemolin* transcription.

Malaria

Malaria has been known, at least the symptoms of malaria, since long before the time of Christ. Although we have known about malaria for long time, we have not been successful in eliminating the devastating disease, and no sterile cure is apparent in the near future. The impact of malaria on the endemic and sub-endemic areas of the world is huge. About 40% of the global population, about 2400 million people are at risk of malaria infection. The estimated number of clinical cases, per annum, due to malaria infection lies between 300-500 million (http://rbm.who.int). The estimated number of deaths due to malaria and malaria-related complications is considerably over one

million people per year [63]. This places malaria first among death-causing parasitic diseases in the world. Malaria is a problem not only to the individual infected but also to the nations in the malaria belt, who experience economic loss and the hampering of development due to this disease [64]. The resurgence of malaria in areas from which malaria has been eradicated over three decades ago and its appearance in areas that have previously never experienced malaria are particularly alarming [65]. Moreover, the infection incidence of malaria is worsening in endemic areas [66, 67, 68]. The factors contributing to malaria resurgence may be either direct or indirect. The direct contributory factors are the insecticide-resistance of the vector and the development of multi drug resistance (MDR) in the parasite. Indirect factors that may influence the renaissance of malaria are political instability, global climate changes that favour the spread of malaria to new geographical areas, population dynamics from non-endemic and endemic areas (tourists and refugees) and the dwindling public health system in the malaria endemic areas [65].

The life cycle of the malaria parasite

Malaria is transmitted by the bite of infected mosquitoes. Approximately 3500 mosquito species have been described. About 12% of the mosquitoes belong to the *Anopheles* species, and 1% these of the mosquito species are vectors that can transmit malaria to humans. Only female mosquitoes can transmit malaria, since they need to feed on blood for egg development and production. Malaria has been described in birds, lizards, rodents, primates and humans. Four *Plasmodium* species infect humans: *P. malariae, P. ovale, P. vivax* and *P. falciparum*. The most malignant is *P. falciparum,* which accounts for 90% of malaria deaths. A brief description of the parasite lifecycle is shown in Figure 1 (consult reference [69, 70, 71] for further details).

During a blood meal, an infected mosquito injects sporozoites, which

are resting in the salivary gland, along with anticoagulant into the bloodstream of the host[1]. The sporozoites are transported to the liver by the bloodstream and infect liver cells[2]. They undergo several vegetative replications in the liver cells and generate thousands of merozoites, which are then released to the bloodstream upon lyses of the infected hepatocytes[3]. Once they are present in the bloodstream, the merozoites infect erythrocytes and malaria pathology commences[4]. Merozoites develop in the erythrocytes passing several stages of development that include ring[5], trophozoite[6], and schizont[7]. The schizont ruptures and releases about 15 merozoites that are ready to infect new red blood cells (RBCs). Some of the parasites differentiate to male and female gametes[8]. When the mosquito engorges blood from an infected host, male and female gametes are taken up with the blood bolus and the sexual development in the mosquito gut initiates[9].

The gametocytes escape from the RBC within a few minutes in the mosquito gut and the male gamete exflagellates[10]. As the consequence of exflagellation eight male gametes are released and fuses with a nearby female gametes to form a zygotes[11]. Exflagellation of the male gamete is driven by a fall in temperature, a rise in pH, a fall in CO_2 concentration, the gametocyte-activating factors in mosquitoes, and vector derived xanthurenic acid. The zygote develops into a motile ookinete within 24 hours[12], penetrates the gut epithelium and rests between the epithelial cells and the basal lamina to form an oocyst[13]. Ten to fifteen days later the oocyst matures and ruptures, sporozoites are released into the hemolymph and migrate to invade the salivary gland[14]. The sporozoites reside in the salivary gland to infect a new host during the next mosquito blood meal[15]. The malaria parasite lifecycle proceeds in this manner continuously.

Malaria transition can be stopped by interfering in the lifecycle of the parasite either in vector or in the host. There are several possible ways of doing so. Drugs that kill the parasite in man are one way that has been shown to function fairly well, although the drug resistance development of the parasite is

alarming. Vaccines in man would be a better choice if the vaccines were to give lifelong protection. There are several stage-specific antigens that can be utilized for vaccine development and some of them are mentioned here. From the ring stage of the parasite we can find apical membrane antigen-1 (AMA-1), erythrocyte membrane protein (EMP-1), glutamate rich protein (GLURP), merozoite surface protein (MSP-1), ring-infected erythrocyte surface antigen (RESA) and at the gametocyte stage the Pf25, Pf230, Pfg27, Pfs16, Pfs28 and Pfs45/48 are present [72]. In the sporozoites stage the antigens that are important for vaccine development are circumsporozoite surface protein-1 (CSP-1), thrombospnoding related adhesive protein (TRAP), sporozoite threonine- and asparagines-rich protein (STARP), sporozoite- and liver-stage antigen (SALSA), sporozoite surface protein (SSP-2) and attenuated sporozoites [73]. Vaccination with attenuated sporozoites gives very good protection, however, the production of sporozoite was considered to be impossible to satisfy the need so no one was engaged on producing it. This old idea is revived now and at least one company is aiming to produce attenuated malaria vaccine [74]. Since the development of a vaccine in man has proven to be difficult, transmission blocking is currently receiving more attention than before. A transgenic approach for transmission blocking will be discussed in paper III, when we describe *Hemolin* as a candidate gene to be used in para-transgenics. Para-transgenics means that mosquito midgut symbionts or commensals are modified to express antiparasitic genes.

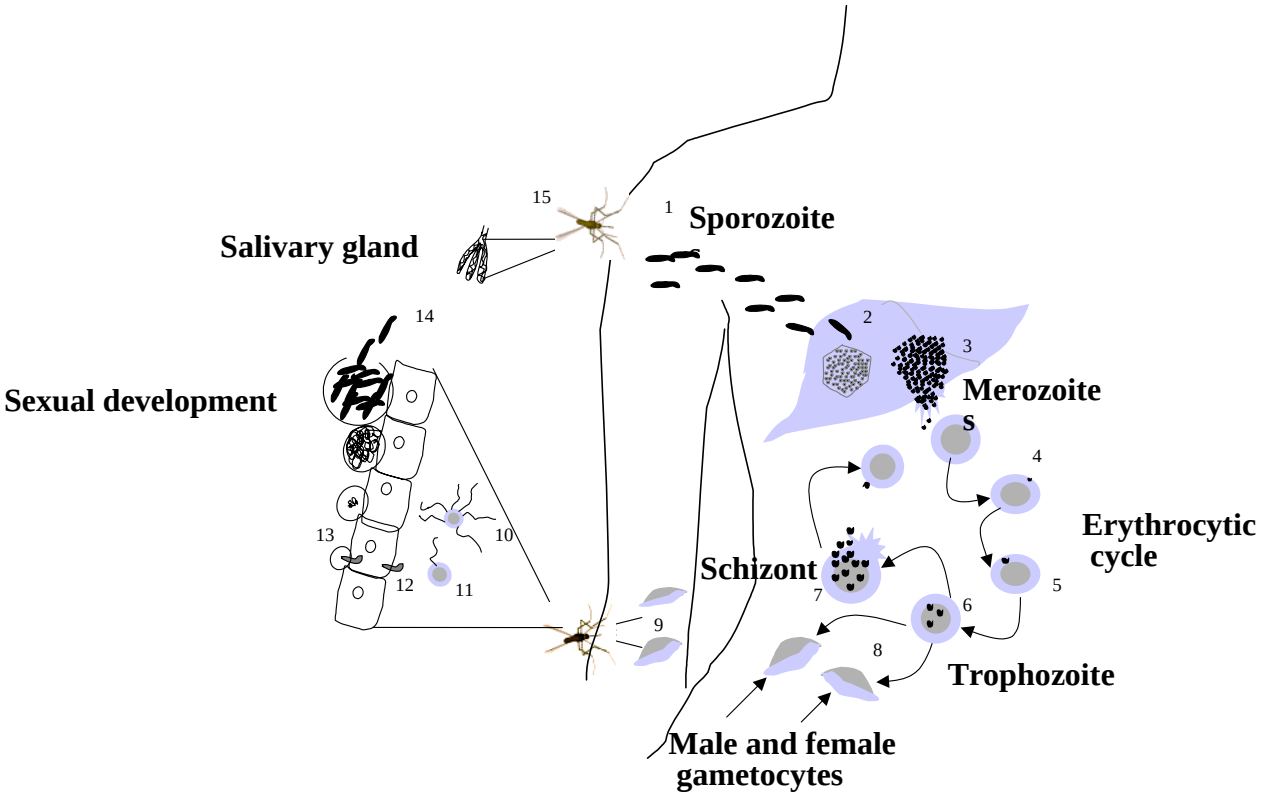

Fig 1. The life cycle of malaria parasite

The immune system in mosquitoes

Mosquitoes, as all insects, run an intense risk of infection during their short lifespan. To overcome pathogenic microbial insults mosquitoes rely on their innate immune system.

The innate immune system has been studied intensively in *Drosophila.* We are gaining knowledge of mosquito innate immunity by drawing parallels with knowledge accumulated from *Drosophila* and other insects. Comparing the genome sequences of the malaria vector *An. gambiae* and *D. melanogaster* is expected to provide much useful information. Moreover, the genome sequence of the human malaria parasite, *Plasmodium falciparum*, together with the

Anopheles genome sequence and the human genome sequence is likely to shed some light on the molecules involved in vector/parasite and parasite/host interaction. The malaria parasite develops for about three weeks in the susceptible vector mosquito (as opposed to refractory mosquitoes that are non-permissive for the parasite development and transmission). In the susceptible mosquitoes, the parasite experiences huge population loss during this mandatory period [75]. In the contrary, a total parasite loss occurs in the refractory mosquito strains [76]. The mosquito gut plays a central role in restraining (refractory/susceptible) or sanctioning (susceptible) the parasite to develop and complete its lifecycle [76, 75]. Prévot et al. compared the *An. gambiae* midgut proteins from sugar-fed males and females and blood-fed females using 2D gel electrophoresis, and found very few differences. About 350 distinct silver-stained proteins were compared in these different groups and only 2.8% of the proteins were specific to blood-fed mosquitoes [77]. Tahar *et al* studied immune responses of *An. gambiae* to malaria parasite *P. falciparum* and found that many mosquito immune genes were affected by parasite infection and the response was different if *P. berghei* were used to infect the mosquitoes [78]. Further studies are needed to be able to exploit the mosquito immune system to interfere with the development of the parasite in the mosquitoes. Such genes are important to identify since they may have promoters that can be used to drive transmission-blocking genes of transgenic mosquitoes. It is also important to find out at what time after the blood meal that these genes are induced. When the trans-gene is to affect the parasite in the gut, which is the most appropriate place, the earlier expressed gene promoter the better, since during blood meal many protolytic enzymes are expressed.

First line barriers

As in other insects, infections activate the innate immune system of the mosquito as well and leads to the production of immune proteins [79, 80, 81]. Insects possess several physical barriers that act as a primary shield against infection. First, the exoskeleton protects the inner organs of the insect from mechanical injuries and from tainted surroundings. Second, the midgut epithelium acts as physical barrier during food intake; in addition, it releases substances that can reduce the consequences of ingested pathogenic microorganisms. The third primary defence in insects is the peritrophic membrane. This may exist permanently or transiently formed, and it participates in limiting the invasion of the gut epithelium by blood born microbes. In some hematophagous insects, such as mosquitoes, the gut flora is augmented because of the nutritious blood, and amplification of gut bacteria by four orders of magnitude has been recorded [82, 83, 84]. The peritrophic membrane is a rigid veil that separates the food bolus from the gut epithelium. In the case of the malaria parasite, it must cross the peritrophic membrane to develop further. It produces chitinase, which locally degrades the chitinous peritrophic membrane [85].

Finally, the chitinous lining of the trachea takes care of the microbes that enter the mosquito respiratory system. Despite all these physical obstructions infection is frequent. Parasites and pathogens that succeed in invading the mosquito soon encounter the local and systemic immune responses.

Pathogen recognition

Pathogen recognition receptors are present in vertebrates and in invertebrates [88, 89, 90, 91, 92]. One *An. gambiae* PRR, the gram-negative bacteria binding protein (GNBP), is upregulated in response to malaria parasite

infection [93].

Another family of pattern recognition molecules that is found in mosquitoes is the family of peptidoglycan recognition proteins (PGRPs) [94, 95, 96]. Seven members of the PGRP family are found in mosquitoes [97]. Several of the PGRP transcripts are up-regulated during bacterial and parasite infections [81, 97, 98]. The C-type lectin families (CTLs) are also important PRRs that are found in mosquitoes.

Melanization

Melanization is a vital mechanism in the immune system of the mosquito for impeding the development of the malaria parasite and it can be cell-mediated or cell-free. Melanization of malaria parasite mostly occurs between the gut epithelium and the basal lamina in cell-free mechanism [76]. The prophenoloxidase cascade is highly controlled by proteolytic enzymes in the serine protease cascade [99]. The zymogen prophenoloxidase circulates in the hemolymph, which is produced and released by the blood cells [99, 100]. Melanization genes and mechanisms have been identified in many mosquitoes [76, 97, 100-102]. Interestingly, *Anopheles* mosquitoes collected in the field do not melanize the parasite, although they are capable of melanizing abiotic beads [103, 104].

Mosquitoes that were treated with dsRNA for CTL4, CTLMA2, or both, produced more ookinetes, showing that the CTL knockout mosquitoes had a greater parasite development promoting character [105]. Osta et al. concluded that CTL4 and CTLMA2 are necessary for the mosquito to perform melanization of the parasite [105]. On the contrary, knocking out the leucine-rich-immune gene (LRIM1) abolished the melanization process, and increased the oocyst load on the mosquitoes [105]. It is interesting that the melanization process by the absence of CTL4 is not functional in the LRIM1/CTL4 double

knockouts if LRIM1 is simultaneously knocked out [105].

Several other gene loci furnish the mosquito defence against *Plasmodium*, in addition to the cloned immune genes. The quantitative trait loci (QTLs) contribute to refractoriness in *An. gambiae*, the predominant *P. falciparum* vector [106, 107, 108]. One of these loci, Pen1, has been studied more intensively and shown to be important in the melanization of ookinetes, while other loci, Pen2 and Pen3, had less pronounced effects [76, 109, 106]. Blandin et al. showed by reverse genetics that TEP1 knockouts of refractory *An. gambiae* strains were devoid of melanization [110, 111] and that susceptible and refractory mosquitoes have different allelic variants of this gene. The thioester-containing protein families in mosquitoes (TEPs) contain important proteins that are produced by hemocytes and secreted to the hemolymph. Interestingly, TEPs are similar to the vertebrate complement factors C3, C4 and C5, that are involved in cell adhesion and lysis, and to the α_2-macroglobulins, which are universal protease inhibitors [112]. These proteins are usually produced in an inactive pro- form. When the inactive form is cleaved, the carboxy-terminal binds to microorganisms through the thioester-site and causes cell lysis [112]. TEPs are also found in a broad variety of species, from *C. elegans* to humans [111].

Immune evasion

Organisms, like *P. falciparum*, that can successfully live as intra-cellular parasites although the host possesses potent innate defence machinery must have evolved mechanisms to evade the immune system of the host. Immune evasion mechanisms are commonly utilized by intracellular parasites. Sporozoites express host-like molecules to evade the assault of the mosquito immune system. *Serratia marcescens* secretes proteases that degrade the effect of cecropins, while *B. thuringiensis* secretes two inhibiting factors that defend it

from the immune system of *H. cecropia* [113]. *Salmonella typhimurium* and *B. cereus* induce hemocytopenia, a reduction of the number of circulating hemocytes in *G. mellonella* in this way [113].

Although the mosquito immune system recognizes *Plasmodium* and responds by synthesising AMPs and other not yet fully understood mechanisms, that reduce the parasite load in a susceptible strain, some of the parasites evade the mosquito immune system and succeed in completing their lifecycle. The parasite can escape the mosquito immune system by evading recognition by the vector immune system and/or by resisting the antiparasitic proteins. It may also actively suppress the innate immune system of the mosquito [114].

The parasite passes several bottlenecks during infection of the mosquito vector before it is transmitted successfully to the vertebrate host. During these transition periods the parasite suffers a huge population loss, the magnitude of which differs from one parasite-vector combination to another [115]. At the later stage of the parasite development, about 1 out of 10 parasites invade the salivary gland, while the rest are eliminated by mechanisms that are not fully understood [69, 70, 71, 115] .

Despite all efforts, malaria remains one of the biggest world health problems. Mosquitoes and the parasites are developing resistance to the available pesticides and drugs respectively. The vaccine development efforts must be intensified and new methods must be tried. One such new method is the generation of transgenic or para-transgenic mosquitoes as is described in Paper III.

Hemolin

Hemolin is a hemolymph immune protein that was isolated first from *H. cecropia* and *M. sexta* [44, 116, 117, 118]. The molecule has subsequently been found in six further *Lepidopteran* species. Hemolin possesses four Ig-like

domains, and is thus structurally similar to members of the Ig-superfamily [52, 117]. A single transcript encodes the gene, which consists of six exons with the introns located within and between the immunoglobulin domains [119]. The crystal structure of hemolin revealed a horse shoe structure, which is unusual for an Ig-molecule, and moreover revealed the interaction of domain 1 with domain 4 and domain 2 with domain 3 [120]. Hemolin is normally present in the hemolymph of *H. cecropia* pupae at a low concentration (0.38 mg/ml). Its transcript is upregulated during immune challenge by a factor of 18. It is also expressed in *M. sexta* larvae at 10 times lower concentration (0.0375mg/ml) and upregulated by a factor of more than 40 times after stimulation by injecting bacteria or bacterial surface components. Although hemolin has no direct antibacterial effect, it binds to bacteria and promotes agglutination of bacteria [88]. Moreover, injection of virus into *H. cecropia* and Chinese oak silk moth *Antheraea pernyi* pupae induces hemolin gene expression (Roxström *et al* unpublished data) [121]. Interestingly, the injection of dsRNA gives a similar pattern of hemolin gene expression as virus infection [121].

Hemolin in recognition

Recognition and discrimination of self from non-self is a principal step in the immune response. Non-self recognition leads to opsonization, phagocytosis, activation of the pro-inflammatory signalling pathways and the complement and coagulation systems. It can also lead to apoptosis in higher organisms [25]. The binding of hemolin to non-self stimulates phagocytosis of the foreign entity, acting as an opsonin [122, 123].

The innate immune system is a genetically imprinted defence mechanism, which identifies conserved molecular patterns that are absent or not expressed in animals. In insects the PRRs include peptidoglycan recognition molecules, beta-1,3-glucan recognition molecules, C-type lectins and these are

crucial for self and non-self determination [88, 24] . Hemolin is induced upon infection and binds to the lipid A part of the LPS [124]. Upon identification and binding to microbial surface molecules, the pattern recognition molecules should pass a signal that stimulates both the cellular immune system and the humoral immune system of the insect. In this sense hemolin may also be seen as a recognition molecule (see below).

The broad spectrum of binding specificity of hemolin (G^+- and G^--bacteria, their surface molecules, virus etc.), and the magnitude of its transcription upon stimulation confirm the overall biological function of hemolin as a surveillance molecule [123, 125]. It binds to and agglutinates microorganisms such as bacteria and yeast in a manner that does not depend on the concentrations of bivalent cations [88]. Hemolin is an Ig-like molecule with the character of PRRs rather than clonal selection molecules like immunoglobulins, which are often specific for particular pathogens.

Hemolin affects phagocytosis

Lanz-Mendoza et al showed that addition of hemolin, LPS, or a mixture of hemolin and LPS to mbn-2 cells or *H. cecropia* hemocytes enhances phagocytic [126]. They incubated yeast cells with hemolin before introducing it to mbn-2 cells lines or hemocytes, and found that the degree of phagocytosis of yeast cells was not affected. In the contrast, pre-incubation of mbn-2 cells or hemocytes with hemolin or LPS enhanced the phagocytic activity**.** The effect was greatest when LPS and hemolin were added simultaneously to the cells**.** They also showed that soluble hemolin prevents the aggregation of hemocytes (by 85%) when phorbol myristate acetate (PMA) was added to induce cell aggregation. The inhibition of cell aggregation may be a means of providing greater capacity of binding of opsonized intruders. Hemocytes can spontaneously adhere to Sephadex beads, form aggregates and proceeding to

melanization. In the presence of hemolin none of these effects takes place. The presence of soluble hemolin inhibits hemocytes aggregate formation**.**

The interaction between hemocytes and foreign molecules can lead to many immune events, which in turn eliminate the intruding foreign molecule. The results described above suggest that hemolin exerts its action after binding to specific membrane molecules on the hemocytes.

Hemolin in intracellular signalling

Sterile wounding or the injection of Lepidopteran Ringer, a physiological salt solution for Lepidoptera, into diapausing pupae of *H. cecropia* induces hemolin gene expression. It is not clear what starts the *de novo* synthesis of hemolin. One speculative model to explain these phenomena may be that physical wounding signals danger, leading to soluble hemolin being recruited to hemocytes. The subsequent fall in the concentration of free hemolin promotes the *de novo* synthesis of hemolin. When the level of free hemolin reaches equilibrium, its synthesis is down regulated in an auto-regulation mechanism, until the concentration reaches the minimum necessary concentration in the hemolymph.

The danger signal that leads to the induction of hemolin expression when the wound is sterile is not yet known. The protein kinase C family (PKCs) are important in many cellular events, and play key roles in regulating biological activities. There are more than 12 members of the PKCs family. Addition of hemolin to hemocytes alters the phosphorylation pattern in the PKC pathway, indicating a signal transduction event mediated by hemolin [127, 126]. Earlier studies have shown that ζKPC is required for the activation of NF-κB. Phosphorylation of the inhibitor IκB by ζKPC primes the inhibitor protein for proteolytic degradation [128]. The κB motif is present in the upstream

regulatory sequence of hemolin, and the activation of hemolin transcription may also take place by this mechanism [119].

We can conclude that hemolin plays a role in the insect's immune defence by recognizing the intruder and possibly signalling to the immune system during infection and wounding by mechanisms not fully understood.

Hemolin in development

Hemolin not only plays a role in the immunity but also in development. As is shown in Paper I, evidence is accumulating that supports its role during development. Hemolin is expressed during oogenesis and embryogenesis in follicles and in the epidermal and neural tissues of developing embryos[129, 130]. Since immunity and development seem to go hand in hand the role of hemolin in development was investigated by RNAi technique in our lab. Interestingly this study revealed that interfering with hemolin RNA in the parents was lethal to the progeny [130]. Together with our work in Paper I, these findings show that hemolin is important not only during infection but also during development. It also suggests that maternally inherited hemolin may play a vital role in the development of the embryos. Hemolin might be a shuttling protein, and depriving the embryos of such a molecule by RNAi during this vulnerable developmental stage, lead to the death of the embryo [131]. Moreover, injection of LPS into fertilized oviposited eggs upregulated the expression of *Hemolin* transcript (Assefaw-Redda et al unpublished data). Thus, it is possible that the embryo is not dependent only on the maternally produced hemolin during infection.

In the gypsy moth, *Lymantria dispar* hemolin is induced during pupation and the titre remains at high level during the mandatory diapause [125]. The titre falls as the chilling period is due, suggesting that the gene is subject to developmental regulation [125]. The role of the high hemolin level in the diapausing pupae

could be and indirect protection from infection since hemolin does not possess antimicrobial activity. Hemolin protein is found in high amounts in the meconium of *H. cecropia* (Bettencourt, unpublished data). This suggests that hemolin could have also a function in clearing waste products during metamorphosis.

Paper I: The insect immune protein hemolin is expressed during oogenesis and embryogenesis

Analysing the kinetics and expression of a molecule both in time and space (the organs in which it is expressed) gives important information that helps to understand its function. At the time when this study was initiated not much was know about the location of expression of the hemolin gene in *H. cecropia*, apart from its expression in hemocytes and fat body organs known to be involved in immunity. The expression of hemolin in other organs such us the eye discs, and in oviposited eggs [129] in neural tissues of developing pupae [129, 132], wing tissue and testis [132]was described. We decided to investigate in more detail the presence of hemolin in oviposited eggs, and the timing of hemolin expression during oogenesis. One of the objectives of the study was to confirm whether the properties of hemolin are consistent with the accumulating facts on the pleiotropic character of hemolin that it is involved both in immunity and developmental events. The work described in Paper I extends the studies on hemolin spatial expression during oogenesis and embryogenesis in *H. cecropia*. Several techniques were used to study the expression of hemolin both at the protein and mRNA levels during oogenesis.

An ovariole consists of a structure known as the germarium at its distal part and one known as the vitellarium at its proximal part. Oocytes are formed from oogonia in the germarium, while yolk is deposited in oocytes in the

vitellarium. The germarium is regulated by the oocyte genes and contains species-specific information of the germ line, while maternal genes, outside of the oocyte, mainly regulate the vitellarium. Many proteins, carbohydrates and lipids are present in the yolk; they originate from outside of the oocyte and are deposited in enclosed vesicles.

In order to investigate the location of hemolin during oogenesis we collected both fertilized and unfertilized eggs from female *H. cecropia* moths and processed them in singlets before proceeding with the immunoblot. Materials from unfertilized eggs, yolk cells and embryos from fertilized eggs were prepared separately and subjected to SDS-PAGE electrophoresis and by Western blots hemolin bands were identified. A single band of hemolin was present in extracts from unfertilized eggs (Figure 3, lane 3) and from yolk cells (Figure 3, lane 4). The size of the hemolin in yolk cells and in unfertilized eggs corresponds to the size of native and recombinant hemolin, shown in lane 1 and 2, respectively, in Figure 3. The size of the embryonic hemolin, on the other hand, was different, (Figure 3, lane 5). This size difference may be due to post-translational modification in the embryos. The size difference was also seen by Bettencourt *et al* in the follow up of this study [131] where they show the importance of hemolin during embryogenesis by dsRNA injection of parent pupae. This size difference was not observed in *M. sexta* whole embryos homogenate [133]. Yu *et al* analysed several time points and the last embryo in their study was 4 days old [133], while we used five days old embryos in our assays, so the concentration and timing difference might play a role in the size discrepancy.

Furthermore, the presence of hemolin in the developing follicle was shown by immunohistology. Hemolin was present in sections of vitellogenic follicles in follicle cells, nurse cells, and in the ooplasm (egg cytoplasm) (Figure 1, F and G). Whole-mount staining with FITC-conjugated secondary antibody showed pronounced staining for hemolin in the yolk and in nurse cells (Figure

1A).

Finally, *in situ* hybridization of the whole-mount follicles showed striking staining in the follicle cells (Figure 1, D and E) demonstrating the presence of hemolin RNA transcripts. The control follicles were not stained (Figure 1, C and D, bottom follicle).

Cryostat sections of five-day embryos were analysed by immunohistochemistry to study the distribution of hemolin. Hemolin was present in the proctodaeum, ectoderm and neural tissues (Figure 2, G and H, pr, ect and nt). Figure 2F shows a control embryo section with almost no staining. Furthermore, yolk cells were immunostained with FITC-conjugated secondary antibody. Hemolin was clearly present in the follicle cells (Figure 2C), while the control cells showed only weak auto-florescence of the yolk cells (Figure 2E). We also investigated the presence of hemolin transcript in the embryo, yolk cells, unfertilized but oviposited eggs, follicles with chorion (dissected) and vitellogenic follicles. We prepared total RNA from each of these, and generated cDNA by RT-PCR. The PCR products gave the predicted fragment size (Figure 4) and the sequencing confirmed that the TA-cloned PCR products contained the hemolin cDNA.

In conclusion we can say that hemolin is present throughout the oogeneseis and embryogenesis and supported by the hemolin RNAi studies we suggest its involvement in development [130,131]. Further studies are needed to get a full understanding of hemolin function during these stages.

Paper II: 20-Hydroxyecdysone indirectly regulates hemolin gene expression in *H. cecropia*

As stated earlier, the expression of *hemolin* is substantially induced during development and immunity. Microbial surface molecules, such as LPS,

LTA, peptidoglycan and β-13-glucans, also induce hemolin gene transcription in a similar way as the live microorganisms [123]. Bacterial surface molecules or the signatures of live bacteria are recognised by PRRs and these molecules influence the level of hemolin gene transcription. Most insect immune genes contain at least one κB-motifs. Indeed, four such κB-motifs are found in the hemolin gene of *H. cecropia*. Hemolin transcription during infection is mediated by the nuclear translocation and specific binding to the regulatory κB-motifs CIF, similar to the mammalian transcription factor NF-κB [57].

Ecdysone hormone regulates development, and works together with juvenile hormone, in hemimetabolous and holometabolous insects. There are four conserved promoter sites in the upstream regions of the hemolin gene in *H. cecropia* and *M. sexta;* three of which are believed to be hormone responsive elements (HREs). These have been given the names HRE-IR (where IR means indirect repeat), HRE-M (M means monomer), and MRE (monomeric nuclear receptor binding site). MRE is found only in *H. cecropia,* while the others are found in both *H. cecropia* and *M. sexta*. A fourth region is found in the upstream region of hemolin gene in both moths. This region is similar to the interferon regulatory factor element (IRF-E) [119]. The experiments described in Paper II investigate the functional relevance of the putative HRE-sites in response to 20E treatment. The first experiment determined whether the expression of hemolin is regulated by 20E. To do this, we injected *H. cecropia* pupae with 20E, the active form of the ecdysone hormone, and determined hemolin expression pattern. Hemolin gene cDNA was used as a probe in Northern blot analysis, which showed that the expression of hemolin was, indeed, stimulated by ecdysone. We then analyzed whether this reflects a primary response to 20E or not. In a primary response the hormone binds to a nuclear transcription factor that acts directly by binding on HRE sites to induce hemolin transcription. A secondary response means that another factor or factors are synthesized after the injection of 20E, and that these factors induce *Hemolin* transcription. If the regulation

were of primary nature we would expect that the hemolin gene transcript to be induced even if a protein synthesis inhibitor is injected. However, if the regulation were secondary, we would expect no hemolin to be produced in the presence of the inhibitor. The Northern blot result showed that minor amounts of *Hemolin* transcript was expressed when the protein inhibitor cycloheximide was injected, indicating that hemolin expression needs a newly synthesized gene product or products that are responding to ecdysone. Supported by these findings we concluded that *Hemolin* is a secondary response gene.

A final experiment tested the putative hormone responsive sites in the hemolin gene for binding factors. Nuclear extracts from the fat body of naïve, 20E-stimulated and Lepidoptera ringer solution (LR) injected control pupae of male and female *H. cecropia* were prepared, and electrophoresis mobility shift assay (EMSA) was performed using HRE-IR, HRE-M and MRE oligonucleotides as probes. All the probes gave rise to retarded high mobility complexes. Competition with unlabeled oligonucleotides showed that the binding was specific. The HRE-IR binding complex was clearly increased by ecdysone, which demonstrates the importance of this site in ecdysone activation of the gene.

Paper III: *In vivo* development of *Plasmodium falciparum* is inhibited by the moth immune protein hemolin

"In a few more months, perhaps in a year, or in two years, the death-dealing pests would begin to come under control, would begin to diminish entirely in favourable spots; and with them, slowly, the ubiquitous malady would fly from the face of civilisation – not here or there only, but almost throughout the British Empire-nay, further, in America, China and Europe" (Ross, 1923:365) [134].

This was the wish and belief of Dr Ronald Ross stated more than 80 years ago.

Dr Gro Harlem Brundtland, the former director general of the World Health Organization, said in 1998:

"Malaria is the single largest disease in Africa and a primary cause of poverty. Every day 3000 children die from malaria. Every year there are 500 million cases among children and adults" [134].

The bitter reality is that malaria has not been eradicated as Ross predicted (although he did not mention Africa in his prediction), but still kills millions of people every year. Indeed, malaria is reappearing in places that have been free from malaria [135-137].

One of the major international initiatives, The Roll Back Malaria (RBM), has set its goal as halving the incidences of malaria in the next 10-20 years, and a united effort has started (www.rbm.org) [138]. The Multilateral Initiative for Malaria (MIM) is also developed to fight malaria by building bridge between scientists working on malaria research in Africa but also other groups with similar interest. Moreover, it is one of the main tasks of MIM to lay pavement for lifting the know-how and competence of the researchers in Africa and other developing countries (http://www.mim.com). Many of the works invested in preventing malaria has not succeeded fully, but it has definitively contributed to understanding the complexity of malaria as a disease *per se,* and it lead to the understanding of other factors that have frustrated local and global malaria eradication efforts.

WHO has called for new ideas to prevent malaria transmission, and this call has stimulated new genetic approaches and biotechnological approaches. With the aim of screening known AMPs from non-vector insects for their anti-parasitic activity in general, and for the malaria parasite in particular, we explored the effect of hemolin on the development of the malaria parasite, *P. falciparum*.

Initially, we wanted to compare the hemolin gene in refractory strains with that in susceptible strains to see whether hemolin gene was altered, and, if

so, to link the alteration to refractoriness or susceptibility. Our strategy required that we clone hemolin from both the refractory strains and the susceptible strains.

We screened genomic and cDNA libraries **(**kind donation from Dr. Inga Sidén-Kiamos**)** by plaque assay, and we used PCR-based screening using hemolin cDNA as the probe and the template respectively. Degenerated primers derived from *H. cecropia* and *M. sexta* hemolin and the *Drosophila* neural cell adhesion molecule neuroglian were used in the PCR-based screening. Neuroglian was included in the primer design because it shares 38% identity with hemolin at nucleotide level [52]. We used degenerated primers derived only from hemolin gene in the second phase of screening. One more hemolin sequence appeared in PubMed at this time, hemolin from *Hyphantria cunea* [139]. We were able to make the primers more mosquito-like to facilitate the screening by introducing codons that are preferentially used by mosquitoes. Despite all efforts, we were unable to clone hemolin in mosquitoes. When the complete *Drosophila* genome sequence was published we realized that it did not contain hemolin. This led us to believe that hemolin is unique for lepidopteran species. It has to date not been found in other species except in Lepidoptera. The publication of the complete genome sequence of *An. gambiae* [140] contributed further to the assumption that hemolin is not present in dipterian insects.

In the meantime, we have carried out *in vitro* experiment, where we tested the effect of recombinant hemolin from *H. cecropia* on *P. falciparum* development. The addition of hemolin to the gametocyte containing blood enhanced the exflagellation but this effect could not always be seen. In contrast, in feeding experiments using the *Plasmodium falciparum*/*Anopheles stephensi* system, adding hemolin to the parasitized blood meal gave clearer results. The development of oocysts was repressed by about 90%. We could not detect morphological difference between the oocysts in the control group and those in the experimental group. Moreover, in long-term experiment no sporozoites

were found in the salivary gland of the hemolin treated group to be compared with the average of 13 600 in the control group. This very promising result is encouraging to use the non-vector immune protein hemolin in transgenic approaches to fight malaria transmission.

To gain more knowledge on the effects of hemolin in other Plasmodium species *P. berghei* and *P. gallinaceum* were also tested. An *in vitro* study of *P. berghei* ookinete formation after addition of hemolin produced immature ookinetes in higher numbers than in PBS treated controls (Assefaw-Redda et al., unpublished data). Exflagellation, the prerequisite for fertilization and ookinete formation, was not affected by the addition of hemolin to *P. berghei* culture in vitro. This suggests that hemolin affects pre-oocyst stages of *P. berghei*. The effect of hemolin in *P. gallinaceum* development was more defined. It stopped exflagellation completely and no sporozoites were observed 18 hours later (Männikkö et al unpublished data).

The idea of using transgenic mosquitoes has stimulated many scientists to explore the possibility. The feasibility of a para-transgenic approach was tested and shown to control effectively Chagas disease when symbionts were remodelled to express AMP gene in the vector *Rhodnius prolixus* [141]. Many molecules have been tested for their ability to stop the development of the malaria parasite with a final goal to generate transgenic or para-transgenic mosquitoes and some of these are listed in Table 1 [142-149]. This demonstrates the interest among scientists to screen for molecules that could be used in transgenics and that interesting new molecules may be in the pipeline. Table 1 brings up some of the work done in *Anopheles* mosquitoes, as part of the fight against malaria.

In much of the published work, the peptides have been administered by injection. Thus, feeding experiments will be necessary for the evaluation of how the peptide survives the passage through the mosquito gut and if it can still interfere with the lifecycle of the parasite. Some of the peptides used have

shown remarkable results [147-149]. Most of the work referred in Table 1 investigates the effects on ookinetes or oocysts production. None of the studies examine parasite development from microgamete exflagellation to sporozoites production as a whole. Nearly all studies so far used different mosquito-parasite combinations, different peptides, and different concentrations, and this makes it difficult to compare their usefulness. In Table 1 the original data from different studies has been recalculated to mole quantities, based on the assumption that mosquitoes feed about two microlitres.

Transgenic gene expression efforts that do not reach total inhibition of the parasite are difficult to use as the only tool for stopping malaria transmission. An efficient mosquito control program must be available at the same time to eliminate the whole parasite reservoir. A transgenic study like Moreira *et al*. would be more informative if the complete lifecycle of the parasite was tested rather than selected stages, Table 1 [147]. It is also noteworthy to mention that Moreira *et al* unfortunately used two different transgenic mosquito lines in their experiment during infecting naïve mosquitoes to screen for infection by blood smear and feeding of transgenic mosquitoes on infected mice to screen for oocysts Table 1. This makes the vector competence comparison difficult.

In our feeding experiment (Paper III), we studied the parasite throughout the development in the mosquito and found that no sporozoites developed although some oocysts were present in the hemolin treated groups. Development of transgenic/para-transgenic mosquitoes has been hampered due to lack of reliable gene transfer methods. The mosquito genome sequence has contributed to the discovery of many genes to be used in transgenic approaches[150]. The parasite genome sequence is expected to deliver information on pathways and enzymes that are suitable for intervention. Concerning hemolin we are currently making truncated hemolin constructs to dissect the molecular part(s) of hemolin with the plasmodium development

detaining effect. Moreover, the isolation of gut bacteria from *Anopheles* mosquitoes to be used in the para-transgenic approaches is proceeding in our lab.

Mosquitoes use many methods and molecules to combat the malaria parasite. Unfortunately, they are not successful, although they reduced the parasites number dramatically during parasite development in their gut [75]. It is not exaggeration to say that mosquitoes are generally refractory to malaria parasite since it is not more than ten percent of the *Anopheles* strains that true human malaria vectors. There are about 3000 mosquito species known and the *Anopheles* genus includes about 400 species (ca 10% of the total mosquito strains). Out of the Anopheles genus it is about 40 strains that are important malaria vectors. One could say that little is needed to stop the development of the parasite in susceptible mosquito strains and that transgenic/para-transgenic approach is one way to deny the malaria parasite development and transmission by the mosquito.

Table 1: The effects of AMPs on the development of *Plasmodium*

	Vector *Anopheles*	Parasite	Molecule	Conc. (nmole)	Time of treatment	Inhibition %	Stage	Referens
In vitro					**days**			
		Pf F32	CA(1-13)-M(1-13)	20µM	0	80-90	Re-inv.	Boman et al 1989
Injection					5.5-6			
	gambiae	*Pc,Pk,Pf***	Magainin 2	0.2	"	82-95	Oocysts	Gwadz et al 1989
	dirus	*Pc*	Magainin 2	"	"	91	"	"
	freeborni	*Pf***	Magainin 2	"	"	83-91	"	"
	gambiae	*Pc*	Cecropin B	0.13	"	81-92	"	"
	gambiae	*Pb*	Vida1	0.05	4	prev 75	Oocysts	Arrighi et al 2002
	gambiae	"	Vida2	"	"	" 86	"	"
	gambiae	"	Vida 3	"	"	" 65	"	"
	gambiae	"	ILF	"	"	" 77	"	"
	gambiae	"	P2WN	"	"	" 58	"	"
Membrane fed					**minutes**			
	freeborni	*Pf*	Propeptide	0.072	0	97-100	Oocysts	Bhatnagar et al 2003
	albimanus	*Pb*	Shiva-3	0.2	10, 20 30 mp	100	"	Rodriguez et al 1995
	stephensi	*Pf**	Hemolin	0.018	0	80	"	Assefaw-Redda et al
	stephensi	"	"	"	0	100	Sporozoite	"
	gambiae	*Pf*	PLA2η	0.03	0	95	Oocysts	Zieler et al 2001
	stephensi	"	"	"	0	97	"	"
	gambiae	*Pb*	Vida1	0.1	0	16	Oocysts	Arrighi et al 2002
	gambiae	"	Vida2	"	0	21	"	"
	gambiae	"	Vida3,ILF¤	"	0	38	"	"
	gambiae	"	P2WN	"	0	16	"	"
	gambiae	"	PBS	control	0	13	"	"
Transgenic					**days**			
	st AF1	*Pb*	PLA2ε	foi mouse	0	76.5-98.8	Oocysts	Moreira et al 2002
	st BM1	"	"	foi mouse	0	76.9-84.7	"	"
	st BF4	"	"	imfon mouse	20	80-100	blood smear	"
	st AM3	"	"	imfon mouse	20	100	"	"
Para-transgenic					**days♣**			
	stephensi	*Pb*	scFv		4	77-99	Oocysts	Yoshida et al 2001
	"	"	"		4	96	"	"

Published data has been recalculated for comparison of molars of the molecules used

st = *stephensi*
Pc = *Plasmodium cynomolgi*
Pk = *Plasmodium knowlesi*
* = *Pf* strain 3D7
** = *Pf* strain 3D7 and NF54
Pb = *Pb* strain Anka 2.34
Pf F32 = *Pf* strain F32
ε = bee venom phospholipase A2
η = sanke venom phospholipase A2

st AF1, BM1, BF4 and AM3 are *stephesi* transgenic lines
foi = fed on infected mouse
imfon = sporozoite infected mosquito feed on naive mouse
mpi = 10, 20, or 30 mimutes pre incubation with the peptide
¤ = Vida 3 and ILF showed the same protection
prev = prevalence of infected mosquitoes
Re-inv = reinvassion
♣ = days before blood feeding mosquitoes were allowed to feed on recombinant E. coli either with single chain antibody (Pbs21), scFv or fused with shiva-1, scFv-shiva-1

Conclusions and remarks

- Hemolin is an immune protein expressed during oogenesis and embryogenesis. It is also expressed in unfertilized and fertilized eggs.
- The expression of hemolin may have two roles in the insect. It may protect the embryo from infection at this fragile time and it may participate in the development of the embryo. This agrees with previous results on *Hemolin* dsRNA knockouts in the pupal stage, which showed embryonic lethality in the next generation. This confirms that hemolin is important during development.
- The *Hemolin* gene is indirectly regulated by 20-hydroxyecdysone.
- At least one ecdysone-induced factor is needed for the activation of hemolin expression. Future work will concentrate on identifying this factor or factors.
- Two putative hormone responsive elements (HRE-IR and HRE-M) are located in the upstream regulatory region of the *Hemolin* gene in *H. cecropia* and in *M. sexta*. One additional site (MRE) similar to the HRE is present in *H. cecropia* only. Other regulatory sites associated with the hemolin gene are one site with similarity to the interferon regulatory factor binding element (IRF-E) and the interferon stimulated responsive element (ISRE), and a fourth site is a κB motif.
- The HREs and MRE have an affinity for nuclear factors from fat body tissue. Binding to HRE-IR increases after ecdysone treatment, indicating that a pre-existing binding factor is induced or activated.
- The development of the malaria parasite *Plasmodium falciparum* is affected by hemolin. This leads to a complete lack of sporozoites in the salivary glands of the mosquitoes after feeding the mosquitoes with infected blood containing hemolin. Importantly, hemolin did not affect mosquito fecundity.
- Since hemolin does not kill or lyse bacteria, *Hemolin* is a candidate gene

for transmission blocking by paratransgenics. Work is proceeding to determine which part of hemolin confers its effect on *P. falciparum* development.

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