Comparative and functional characterization of An. gambiae and An. arabiensis infections with P. falciparum natural populations

Συγκριτική και λειτουργική ανάλυση της μόλυνσης των κουνουπιών An. gambiae και An. arabiensis με φυσικούς πληθυσμούς του παρασίτου της ελονοσίας P. falciparum

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1. Abstract

The cyclopropagative development of *Plasmodium* parasite inside its *Anopheles* vector comprises a critical step for malaria transmission. The current thesis provides a comparative analysis of the responses raised by the two major malaria vectors, *An. gambiae* and *An. arabiensis*, upon infections with *P. falciparum* natural populations. A combinatorial approach was implemented here, including: i) Genome-Wide Transcriptional Profiling of the bipartite mosquito-parasite systems, conducted upon geographically related field isolates, in conjunction with ii) functional characterization of potential regulators involved in the *An. gambiae* defense against the *P. berghei* rodent model sexual development. The parallel identification of both *An. gambiae* and *An. arabiensis* transcriptomes, exhibited in four distinct timepoints (1h, 6h, 10h and 24h post-infection) during parasite's sexual and sporogonic development, revealed significant inter-spieces and adjunct species-specific transcriptional responses that configure mosquitoes' defense. Nevertheless, *P. falciparum* displayed a robust transcriptional regulation that is hardly altered during infections with the two mosquito species, subsequently proposing that the parasite follows a tight developmental program during the sexual midgut stages. Further exploration and experimental interrogation on mosquito signaling components that demonstrated significant upregulation in the early stages was accomplished here, as well. Importantly, RNA interference (RNAi)-mediated gene silencing illuminated the significant role of a novel neuropeptide, Neuropeptide M (NPM), in the control of the mosquito antiparasitic responses. These evidence provide important information towards the recent efforts for complete construction of the parasite-vector interactome map, the cornerstone of the Transmission Blocking Interventions (TBI) against malaria.

2. Table of contents

3 Introduction

3.1 Malaria: one of the most devastating diseases in the world

3.1.1 Abridgement of history: From past to present

Malaria or a disease congener with malaria has been noted for more than 4,000 years. Originated in Ancient China (2700 BC), *Nei Ching* (Canon of Medicine) was the first document that described several characteristic symptoms of what would later be named malaria. Many years later, a physician born in ancient Greece and today regarded as the "Father of Medicine", Hippocrates, will be the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived. (*http://www.cdc.gov/malaria/about/history/index.html*)

Nevertheless, not until 1889 was the protozoal (single celled parasite) cause of malaria discovered by Alphonse Laveran, a French army doctor working in a military hospital in Algeria. Laveran set himself the task of explaining the role of the black pigmented corpuscles that he observed microscopically in the blood of people suffering from malaria. He therefore proposed that malaria is caused by this organism, the first time a protist was identified as causing disease (later known as of the genus *Plasmodium)*. For this and later discoveries, he was awarded the 1907 Nobel Prize for Physiology or Medicine (Figure 3.1.1). However, Laveran was not able to explain the problem of the existence of these parasites outside the patient's body. The answer would come some years later.

There was already an empirical association between malaria and stagnant waters (breeding grounds for the mosquitoes), as this connection led the Romans to begin drainage programs, the first intervention against malaria. Guided by this notion, Sir Ronald Ross, a British army surgeon working in India, will be the first to demonstrate that the parasite is transmitted between its vertebrate hosts by mosquitoes. Strongly influenced by the also British parasitologist Patrick Manson, Ross made his ground-breaking discovery by the use of an avian malaria model system. Following Ross, some other Italian scientists, Giovanni Battista Grassi, Amico Bignami, and Guiseppe Bastianelli, in 1898, will be the first to document the complete transmission cycle of the parasite inside the mosquitoes.

Today, it is well known that the protists causing malaria are of the genus *Plasmodium* and use a dual-host system, one vertebrate and one invertebrate. Plasmodia infectious to human are using female *Anopheles* mosquitoes.

Figure 3.1.1
Left Alphonse Laveran, Nobel Prize for Physiology or Medicine (1907) *"in recognition of his work on the role played by protozoa in causing diseases"*.

Right Sir Ronald Ross, Nobel Prize for Physiology or Medicine (1902) *"for his work on malaria, by which he has shown how it enters the organism and thereby has laid the foundation for successful research on this disease and methods of combating it"*.

3.1.2 Diagnosis – Symptoms - Clinical burden

Diagnosis

Malaria is usually diagnosed via microscopic examination of blood smears or by antigen-based rapid diagnostic tests (RDTs) (Kattenberg JH et al., *Malaria Journal* 2011). Microscopy remains as the most commonly used method to detect the malarial parasite, as approximately 165 million blood smears were examined for malaria in 2010. However, diagnosis by microscopy suffers from two main disadvantages: many rural settings are not equipped to perform the test, and the accuracy of the results depends not only on the skills of the person examining the blood smear, but also the levels of the parasite in the blood. Commercially available RDTs comprise a more modern and often more accurate diagnostic tool at predicting the presence of malaria parasites, but they are widely variable in diagnostic sensitivity and specificity depending on manufacturer, and are unable to tell how many parasites are present (Wilson ML, *Clinical Infectious Diseases* 2012). Nevertheless, in regions that cannot afford laboratory diagnostic tests, it has become routine to use only a history of subjective fever as the indication to treat for malaria. The drawback of this practice is overdiagnosis of malaria and mismanagement of non-malarial fever, as uncomplicated malaria is difficult due to its non-specific symptoms of fever, headache, sweats, chills, rigors (shaking or shivering) and joint pain (Bell D. and Winstanley B., *Br Med Bull* 2004). This diagnostic way wastes limited resources, erodes confidence in the health care system, and conduces to drug resistance (Perkins MD et al., *Malaria Journal S1-5* 2008)

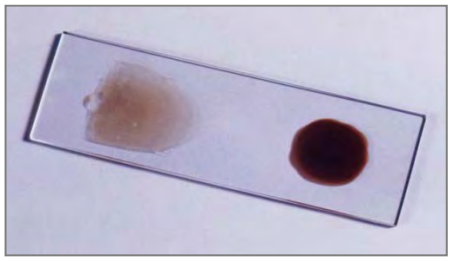


Figure 3.1.2 Blood film: the gold standard for malaria diagnosis *(CDC - Public Health Image Library (PHIL), #5901)*

Symptoms

As mentioned before, malaria is consisted of, not only specific, but also non-specific symptoms, which hinders and deludes the diagnosis. The typical incubation period is 10 to 15 days; however, in some cases it can take up to a year for symptoms to develop. Initial manifestations of the disease that are common to all malaria species resemble the flu-like symptoms, and can be similar to other conditions such as septicemia, gastroenteritis, and viral diseases (Nadjm B. and Behrens RH, *Infect. Dis. Clin. North Am.* 2012). The typical symptom of malaria is paroxysm—a cyclical occurrence of sudden coldness followed by rigor and then sweating and fever, occurring every two days or three days. Headache, shivering, fever, vomiting, arthralgia, hemolytic anemia, jaundice, retinal damage, hemoglobinuria, and convulsions may also configure the main presentation of malaria. However, about 30% of people will no longer have febrile signs upon presenting to a health care facility. Severe malaria is usually caused by *P. falciparum* (often referred to as falciparum malaria), whose symptoms arise 9–30 days after infection (Bartoloni A. and Zammarchi L., *Mediterr J Hematol Infect Dis* 2012).

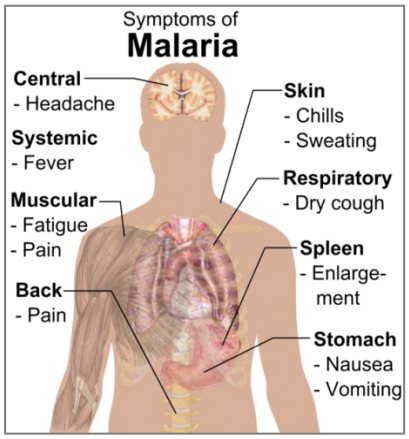


Figure 3.1.3 Clinical symptoms of malaria (*Fairhurst RM, Wellems TE, 2010*)

A serious type of malaria, cerebral malaria, frequently exhibits neurological symptoms, including abnormal posturing, opisthotonus, nystagmus, conjugate gaze palsy (failure of the eyes to turn together in the same direction), seizures, or coma. If it is not treated properly, malaria can lead to grievous complications, such as breathing problems, seizures, organ failure and severe anaemia.

Clinical Burden

Many decades after its identification, malaria still exists as one of the deadliest diseases affecting mankind. According to the *World Malaria Report 2012,* published by the WHO (World Health Organization) organization, summarizing data received from 104 malaria-endemic countries and territories for 2011, 3.3 billion people (half the world's population) live in areas at risk of malaria transmission. The latest estimates count 219 million cases of malaria in 2010 and an estimated 660 000 deaths, with children under five years of age and pregnant women most severely affected.
(*http://www.who.int/malaria/publications/world_malaria_report_2012/wmr2012_full_report.pdf*)

It is recorded that, the highest mortality rates are inextricably correlated with the highest rates of extreme poverty. More specifically, ninety-nine of the examined countries had on-going malaria transmission, with the African countries representing the main hotspot; approximately 80% of cases and 90% of all malaria deaths occur there (Figure 3.1.4). The six countries in the WHO African region (in order of estimated number of cases) with the highest burden are: Nigeria, Democratic Republic of the Congo, United Republic of Tanzania, Uganda, Mozambique and Cote d'Ivoire. These six countries account for an estimated 103 million (or 47%) of malaria cases. In South East Asia, which is the second most affected region in the world, India holds the highest malaria levels (with an estimated 24 million cases per year), followed by Indonesia and Myanmar. As stated in the World Health Assembly and Roll Back Malaria targets for 2015, fifty countries are on track to reduce their malaria case incidence rates by 75%. Nevertheless, these fifty countries only account for 3% (7 million) of the total estimated malaria cases.

Additionally, malaria has a negative socio-economic impact on both the countries and the individuals, including the high cost for purchasing drugs, maintenance of health facilities, health interventions against malaria, but most significantly, absence of education and work, obstructing the stability and growth of the countries.

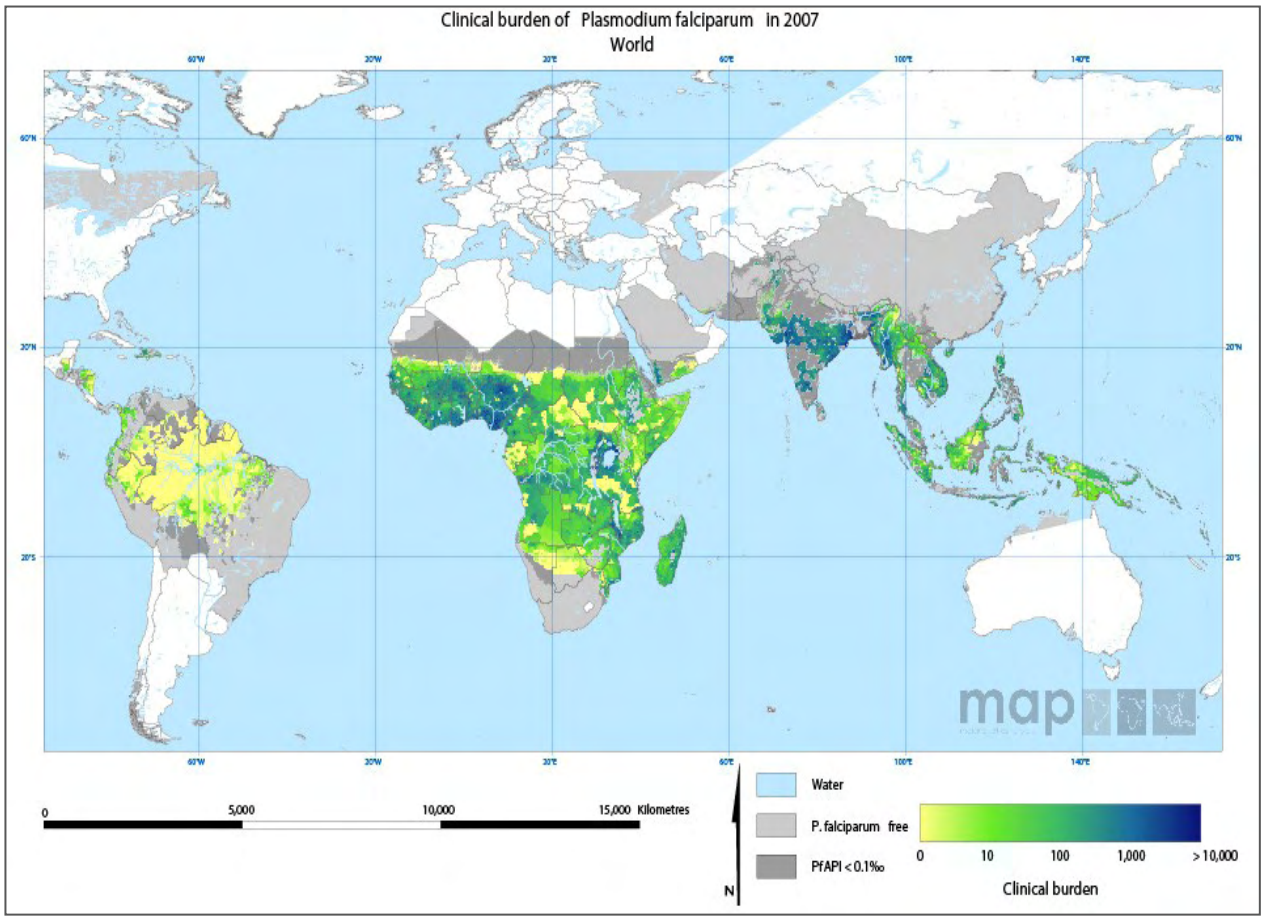


Figure 3.1.4 Estimated clinical burden of *Plasmodium falciparum* in 2007. Bayesian geostatistical estimates (posterior means) of the number of all-age clinical cases of *Plasmodium falciparum* infection on a logarithmic scale between 0 and 10,000 cases, within the stable limits of *P. Falciparum* transmission. Areas of no risk and unstable risk (*Pf*API < 0,01%) are also known. (Hay SI et al., *PLoS Medicine* 2010)

3.2 The natural ecology of malaria: the vertebrate host – the mosquito vector

3.2.1 Overview of the malaria lifecycle

Not only from a humanistic, but also from a scientific angle, malaria stimulates the interest of the scientific community. The malaria parasite, *Plasmodium*, is involved in an infrequent dual-host system, as its life cycle comprises two stages: one inside a vertebrate and one inside a mosquito.

In vertebrates, the parasites grow and proliferate first in the liver cells and then in the red cells of the blood. In the blood, successive corpuscles of parasites are multiplied inside the red cells and destruct them, releasing daughter parasites ("merozoites") that continue the cycle by invading other red cells. The blood stage parasites are responsible for causing the malaria symptoms. The intra-vertebrate stages are also mentioned as “asexual” stages, referring to the nature of the parasite’s development. When specific forms of blood stage parasites ("gametocytes") are picked up by a female Anopheles mosquito during a blood meal, they start another, different cycle of growth and proliferation in the mosquito, noted as “sexual” parasite stages. Nine to eighteen days later, the parasites are found (as "sporozoites") in the mosquito's salivary glands. When the Anopheles mosquito takes a blood meal on another vertebrate, a

new cycle begins, as the sporozoites are ejected along with mosquito's saliva and start another infection inside the vertebrate when they parasitize the liver cells. (Figure 3.2.1)
Thereby, the mosquito acting as a vector carries the disease from one human to another. Differently from the human host, the mosquito vector does not suffer from the presence of the parasites. (*http://www.cdc.gov/malaria/about/biology/index.html*)

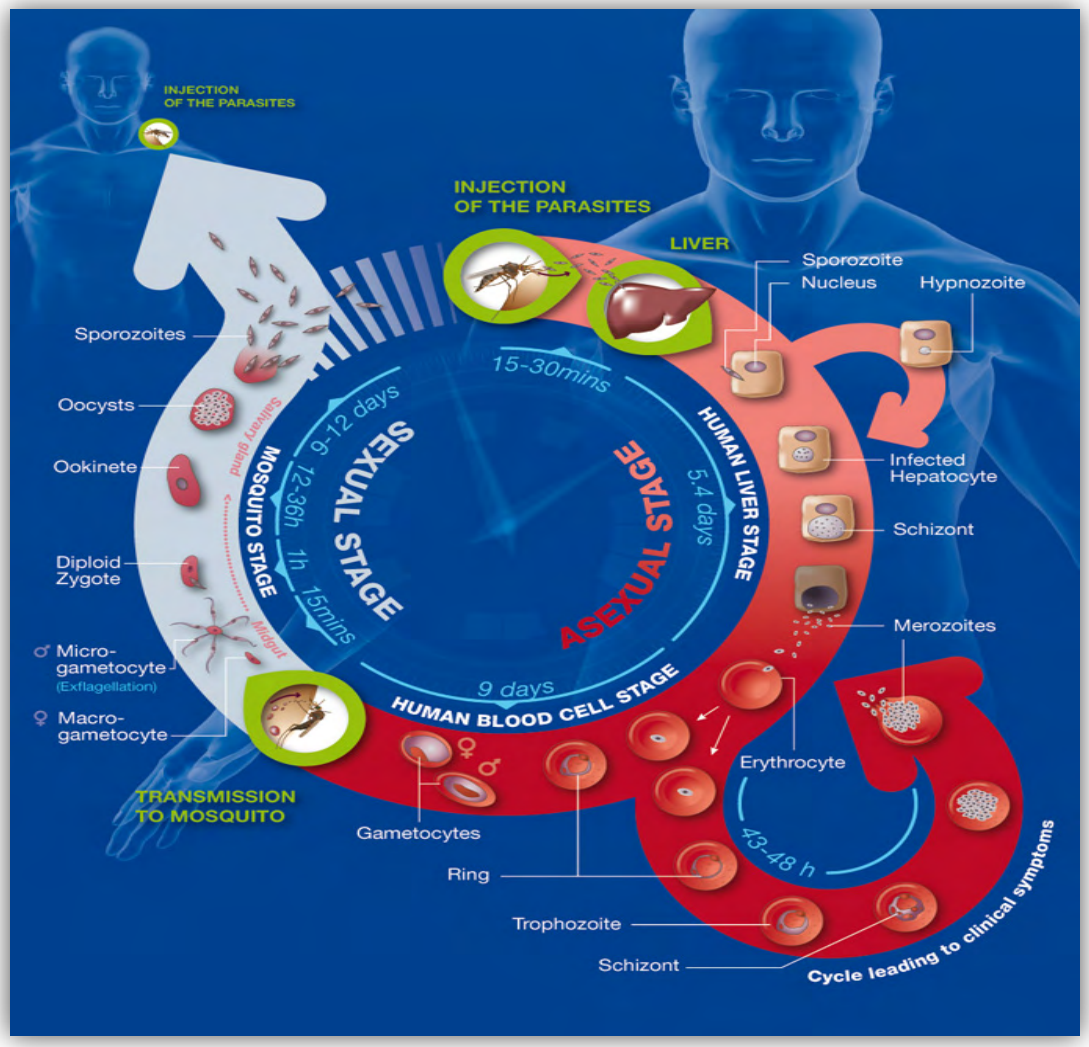


Figure 3.2.1. Cyclopropagative development of *Plasmodium* parasites inside both the human host (pink and red pathways) and the mosquito vector (cyan pathway). It must be noted that the timings are relevant only for *P. falciparum* (Wells TN and Pollem EM, *Discovery Medicine* 2010)

3.2.2 Plasmodium development inside the human host: Malaria Pathogenesis

Malaria pathogenesis is revealed under *Plasmodium's* development inside the vertebrate host, the human (Figure 3.2.1). Thus, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host through salivation during a blood meal. Then, the sporozoites circulate through the blood stream and reach the liver cells, in which they invade. There, they mature into schizonts, which rupture and release merozoites . In P. vivax and P. ovale there is a dormant stage (hypnozoites) that can persist in the liver and cause relapses by entering the bloodstream weeks, or even years later. After this initial proliferation in the liver, the so-called exo-erythrocytic schizogony, the parasites undergo asexual reproduction inside the erythrocytes, erythrocytic schizogony, mediated by red blood cell invasion from merozoites. Next, the ring stage trophozoites mature into schizonts and rupture releasing merozoites.

These merozoites can re-invade a red blood cell, displaying the clinical manifestations of malaria. However, some parasites differentiate into sexual erythrocytic stages; male and female gametocytes. (http://www.cdc.gov/malaria/about/biology/index.html)

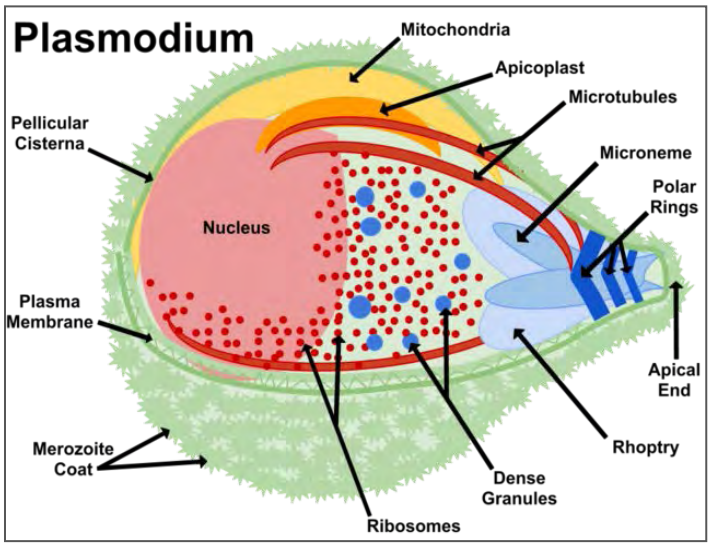
3.2.3 Plasmodium development inside the mosquito vector: Malaria transmission

3.2.3.1 Identification of the enemies: *Anophelines* and *Plasmodia*

The *Plasmodium* parasite

Plasmodium is a genus of *Apicomplexan* parasites which was described in 1885 by Ettore Marchiafava and Angelo Celli. Currently over 200 species of this genus are recognized and new species continue to be described (Chavatte J.M. et al, Parasite 2007). Of the over 200 known *Plasmodium* species, at least 11 species infect humans; some species infect other animals, including monkeys, rodents, birds, and reptiles. Five *Plasmodium* species cause malaria that affect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Malaria caused by *P. falciparum* is the most deadly form and dominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found infrequently.

As a protist, *Plasmodium* is a eukaryote. However, it displays unusual structural characteristics in contrast with general eukaryotes, including the rhoptry, micronemes, and polar rings near the apical end. Also, as *Apixomplexan*, these parasites contain a degenerated chloroplast called an apicoplast. These special features play a significant role in the several developmental functions of *Plasmodium.* (Figure 3.2.2)



Kingdom:	*Chromalveolata*
Superphylum:	*Alveolata*
Phylum:	*Apicomplexa*
Class:	*Aconoidasida*
Order:	*Haemosporida*
Family:	*Plasmodiidae*
Genus:	***Plasmodium***

Figure 3.2.2 Left: Representation of *Plasmodium's* significant cellular components Unusual structures of this organism in comparison to general eukaryotes include the apicoplasts, micronemes, rhoptry, and polar rings near the apical end. Right: Taxonomy of Plasmodium (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=418103)

The sequencing of several *Plasmodium* species' genome (*P. falciparum, P. knowlesi, P. vivax, P. berghei and P. yoelii*) gave a boost to the study of malaria biology. Thereby, it was revealed that the average genome size was approximately 25 megabases organized into 14 chromosomes, whose lengths vary from 500 kilobases to 3.5 megabases (Hall N., *Science* 2005).

Mosquito vector: the *Anophelines*

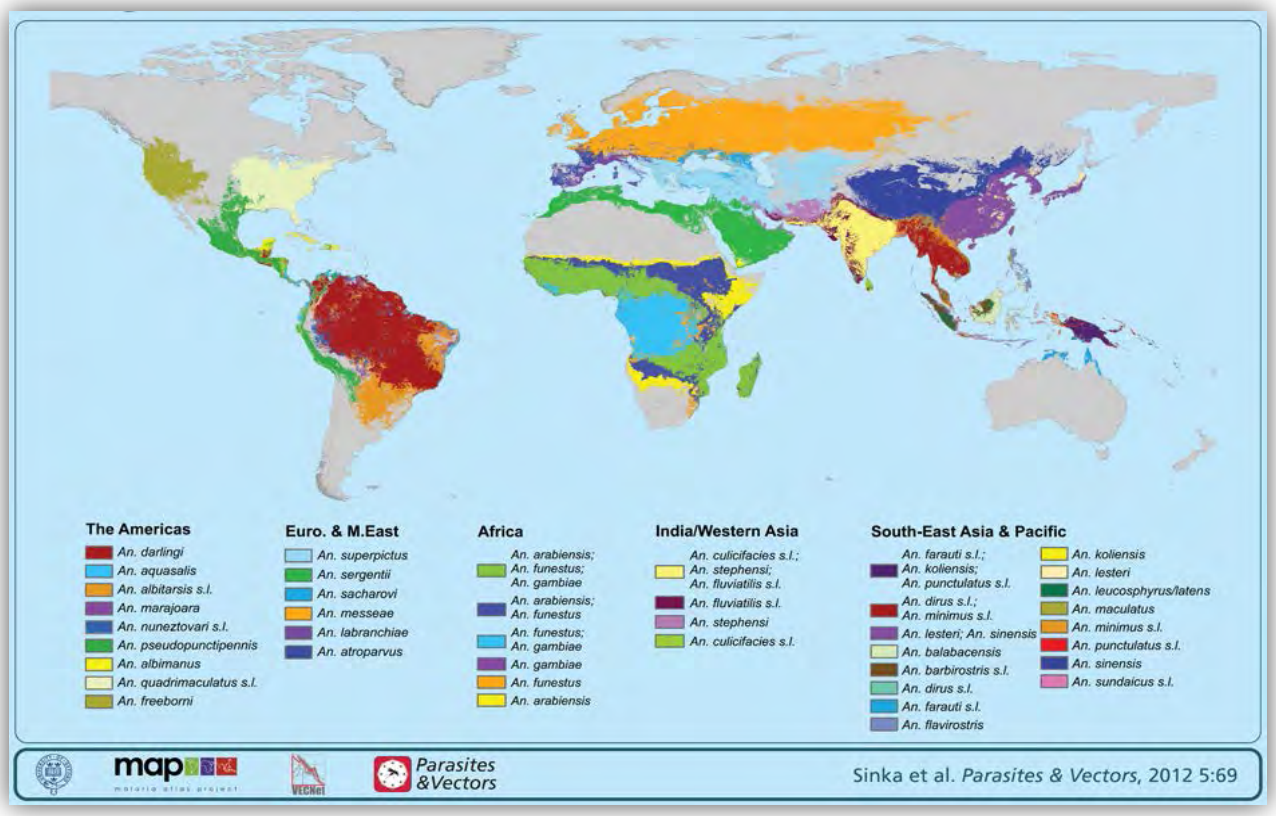


Figure 3.2.3 Global map of dominant malaria mosquito vectors (Sinka ME. et al., *Parasites & Vectors* 2012)

Anopheles, named from the Greek an ("not") + ophelos ("benefit"), is a genus of mosquito (*Culicidae*), many species of which are vectors of malaria. *Anopheles* mosquito was first described in 1818 by German entomologist Johann Wilhelm Meigen. However, Batista Grassi (and others) was the first to prove that the *Anopheline* mosquitoes, and not mosquitoes of other genera such as *Culex* or *Aedes*, were capable of transmitting the disease to humans. There are approximately 400 "Anopheles" species, of which 30-40 transmit five different *Plasmodium* species that cause malaria and affect humans in endemic areas. The best characterized of them is *Anopheles gambiae*, due to its predominant role in the transmission of the most dangerous *Plasmodium falciparum.* (Figure 3.2.3)

Some species of "Anopheles" also can play the vector role for the *Filariidae Wuchereria bancrofti,* canine heartworm *Dirofilaria immitis,* and *Brugia malayi*. Also, they can serve as vectors for some viruses, like the one that causes O'nyong'nyong fever. Mosquitoes in other genera (Aedes, Culex) can also serve as vectors of disease agents (*www.cdc.gov/malaria/about/biology/mosquitoes/*).

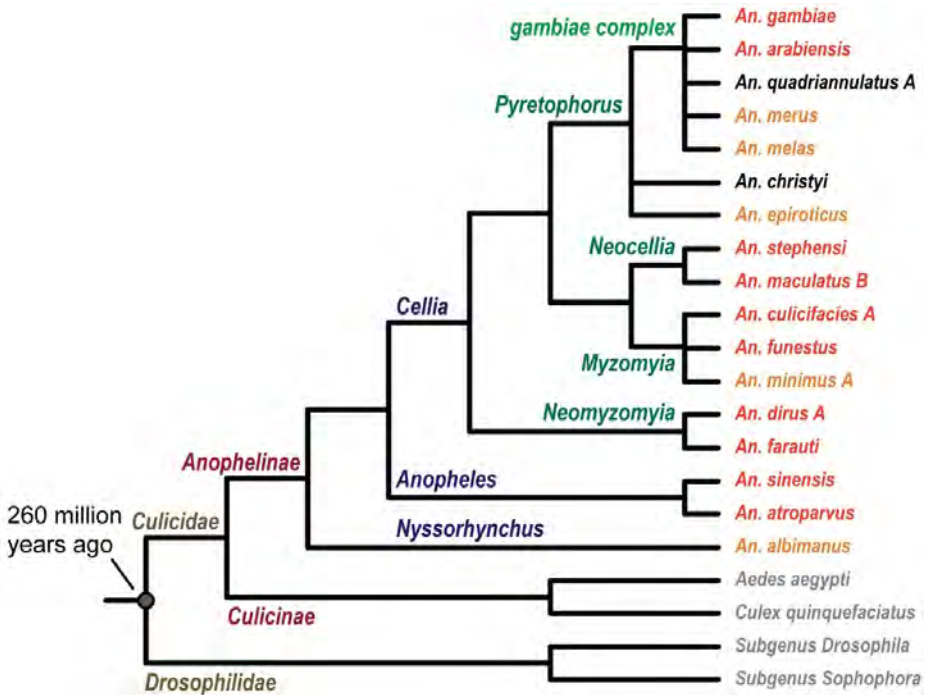


Figure 3.2.4 Dendrogramatic illusration of the 16 *Anopheline* species and their putative evolutionary relationships. The two sequenced *Culicines, Aedes aegypti* and *Culex quinquefasciatus*, and the sequenced *Drosophila* species are also demonstrated. *Anopheles* species that comprise the major human malaria vectors are labelled in red, minor vectors are labelled in orange, and species that are not human malaria vectors are labelled in black (Neafsey DE. et al., *G3* 2013).

Life cycle and anatomy

Anophelines, like all mosquitos, go through four stages in their life cycle; egg; larva; pupa; and imago (adult). The first three stages are aquatic and last approximately 5-14 days, depending on the species and the environmental conditions (temperature, humidity etc.). Female *Anopheles* mosquitoes act as malaria vectors during the adult stage. The adult females' life span is up to a month, or more in captivity, although they do not live more than 1-2 weeks in nature (http://www.cdc.gov/malaria/biology/mosquitoes).

Eggs

Adult females lay 50-200 eggs per oviposition. Eggs are laid singly directly on water and are unique in having floats on either side. Eggs are susceptible in drying and hatch within 2-3 days; in colder climates hatching may take up to 2-3 weeks.

Larvae

The larval stage is always aquatic. Mosquito larvae anatomy includes a well-developed head with mouth brushes used for feeding, a large thorax and a segmented abdomen. In contrast to other mosquitoes, *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is parallel to the water surface. Larvae feed on bacteria, algae and other microorganisms in the surface microlayer; sometimes they even present a cannibalistic behavior. They absorb O_2 through spiracles located on the 8th abdominal segment and dive below the surface only when disturbed. Larvae develop through four stages, or instars, after which they metamorphose into pupae. At the end of each instar, the larvae molt, discarding their exoskeleton, or skin, to allow for further growth. Larvae occur in a wide range of habitats; however, most species prefer clean, unpolluted water. *Anopheline* larvae have

been found in fresh or salt-water marshes, mangrove swamps, grassy ditches, rice fields, the edges of streams and rivers, and small, temporary rain pools.

Pupae

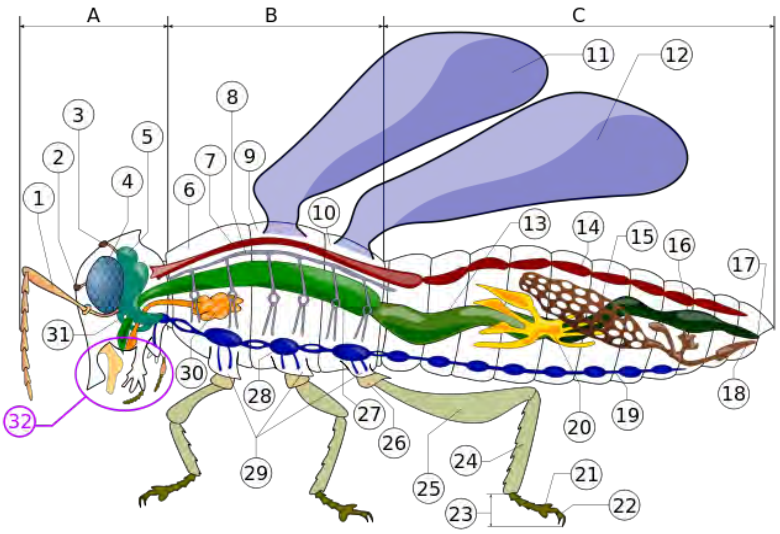
The pupa is comma-shaped when viewed from the side. The head and thorax are merged into a cephalothorax with the abdomen curving around underneath. Like larvae, pupae must frequently come to the surface in order to breathe, which they do through a pair of respiratory trumpets on the cephalothorax. Unlike most other insect species, they do not feed, despite the fact that they are extremely active.

Adult

Adult *Anopheles* mosquitoes have bodies constituted by three distinctive sections: head, thorax and abdomen (Figure 3.2.5). The head functions are dedicated in acquiring sensory information and feeding. It contains the eyes, a pair of long antennae for detecting host odours as well as odours of breeding sites where females lay eggs, and an elongated, forward-projecting proboscis used for feeding. The thorax is specialized for locomotion containing three pairs of legs and a pair of wings attached. The abdomen is focused on food digestion and egg development.

Male and female adult *Anopheles* can be distinguished by the size of their antennae; it is larger in males than females. The two sexes usually mate a few days after emerging from the pupal stage. Males live for about a week, feeding on nectar and other sources of sugar. Although females also feed on sugar sources for energy, they generally require a blood meal for the eggs' development. After obtaining a full blood meal, the female rests for a few days while the blood is digested and the eggs are developed. Once the eggs are fully developed, the female lays them and resumes host seeking.

One significant behavioral factor is the degree to which an *Anopheles* species prefers to feed on animals such as cattle (zoophily) or humans (anthropophily). It is more probable that malaria parasites are transmitted from one person to another via Anthropophilic *Anophelines*. Most *Anopheles* mosquitoes are not exclusively anthropophilic or zoophilic. Nevertheless, the major malaria vectors in Africa, *A. gambiae* species complex (including *A. arabiensis*) and *A. funestus*, are strongly anthropophilic and, ergo, the most efficient malaria vectors in the world.



Insect anatomy scheme
A- Head
B- Thorax
C- Abdomen
1. antenna
2. ocelli (lower)
3. ocelli (upper)
4. compound eye
5. brain (cerebral ganglia)
6. prothorax
7. dorsal artery
8. tracheal tubes (trunk with spiracle)
9. mesothorax
10. metathorax
11. first wing
12. second wing
13. mid-gut (stomach)
14. heart
15. ovary
16. hind-gut (intestine, rectum & anus)
17. anus
18. vagina
19. nerve chord (abdominal ganglia)
20. Malpighian tubes
21. pillow
22. claws
23. tarsus
24. tibia
25. femur
26. trochanter
27. fore-gut (crop, gizzard)
28. thoracic ganglion
29. coxa
30. salivary gland
31. subesophageal ganglion
32. mouthparts

Figure 3.2.5 Adult insect anatomy. (Piotr Jaworski, 2008)

3.2.3.2 Malaria transmission biology: Plasmodium sexual development

Sexual developmental stages of Plasmodium should be accorded the same importance as the asexual (blood) stages, as the parasite undergoes distinct and substantial changes, essential in order to follow its original goal: infect the vertebrate hosts. As outlined above, during the asexual stages, a small number of parasites do not undergo schizogony but differentiate into the sexual stage male or female gametocytes. Afterwards, a female Anopheles draws a blood meal from an individual infected with malaria, dragging along the male and female gametocytes of the parasite. During ingestion, they find their way into the gut of the mosquito. The ensuing phase, which is called sporogony, includes morphological, cellular and molecular transformations that are consisted of five major steps: gametogenesis, zygote formation and locomotion, ookinete-to-oocyst transition, oocyst maturation and sporozoites formation, sporozoites motility and salivary gland invasion (Vlachou D. et al., *Curr Opin Genetics Dev* 2006). (Figure 3.2.1)

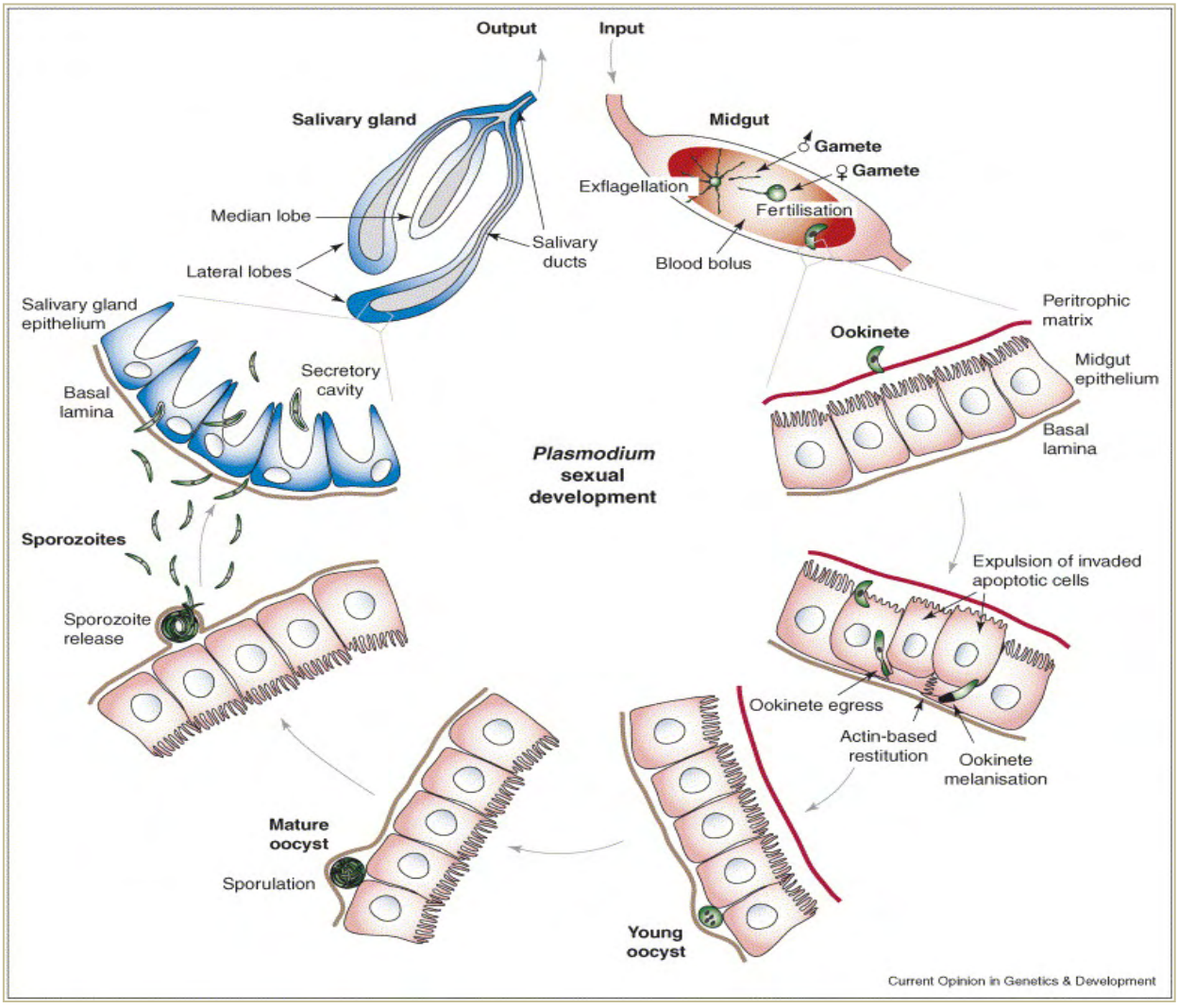


Figure 3.2.6 *Plasmodium* sexual development (sporogony). During this phase, the parasite undergoes through five different stages: gametogenesis, zygote formation and locomotion, ookinete-to-oocyst transition, oocyst maturation and sporozoites formation, sporozoites motility and salivary gland invasion (Vlachou D. et al., *Curr Opin Genetics Dev* 2006).

Gametogenesis

After the ingestion of the infectious blood meal, the Plasmodium gametocytes mature into male and female gametes (the sexual reproductive stage of the parasite), in the mosquito midgut lumen, under environmental and mosquito-specific factors that vary for different Plasmodium species. These factors involve a decrease in temperature by 5^{o}C, a rise in the pH, and exposure to xanthurenic acid (XA). A release of calcium in the cytoplasm of the gametocyte, induced by a signal transduction cascade, causes them to begin development and to emerge from the red blood cells.

The female gametocyte
The maturation process of the female gametocyte is a preparative stage for the extensive protein synthesis associated with female gametocyte activation. Specifically, mRNA (a substantial sub-set of which is subject to translational repression), mitochondria, apicoplasts and ribosomes accumulate in the cytoplasm, whereas the female nucleus remains small in size. Also, the development of an extensive endoplasmic reticulum ensues the increased ribosome production is ER) in the female gametocyte. The mature female gametocyte is enriched in membrane bound vesicles known as osmophilic bodies (Sinden, *ASM press* 1998).
After gametogenesis, the activated, free female (macro) gamete enlarge in size (<3 fold size increase) during a process that occurs in absence of DNA replication.

The male gametocyte
On the other side, and despite the absence of genome replication, a nucleus enlargement is detected in the male gametocytes, with its genome taking upon a highly organized and ingathered state. The kinetochores of each chromosome are attached, through a nuclear pore, to the Microtubule Organising Centre (MTOC) situated on the cytoplasmic face of the nuclear envelope. The mature male gametocyte is largely devoid of ER and contains few mitochondria, osmophilic bodies and apicoplasts (Sinden, *ASM press* 1998). Then, CDPK4, a calcium-dependent protein kinase is activated, translating the XA-induced calcium signal into regulation of the cell cycle and triggering the beginning of the gamete production (Vlachou D. et al., *Curr Opin Microbiol* 2005). Directly, the activated male undergoes three subsequent rounds of mitosis, expediently replicating its genome to that of an octoploid value, which results in the release of eight haploid flagellated microgametes (a process known as “exflagellation”). The release of the flagellated gametes is substantially modulated by Pbmap2, a mitogen-activated kinase. Exflagellation and subsequent fertilisation includes the generation of exflagellation centres, where the emerging male (micro) gametes bind to uninfected RBC, infected RBC and macrogametes, causing them to cluster around the exflagellating male gametocytes. The exflagellation centres are important for the establishment of infection in the vector, (Eksi et al., *Mol Microbiol* 2006) despite the fact that their exact function is not well understood.

Zygote formation and locomotion

Soon after gamete production, fertilization occurs. It results in the fusion of the plasma membrane of the two cells, with the male nucleus and its axoneme entering the macrogamete cytoplasm (Sinden et al., *J Protozool* 1985). There, the male and female nuclei fuse and produce a diploid zygote which undergoes meiosis, in absence of nuclear division or cytokinesis, and is connected with an increase of the genome value to that of tetraploid. The female-specific expression of two NIMA (never in mitosis/Aspergillus) related kinases, Nek2 and Nek4, regulate the DNA replication during the zygote meiotic event (Reininger et al., *J Bio Chem* 2009).

Thereupon, the primarily sphere-shaped zygote gradually elongates and transforms into the rod-shaped ookinete via the retort stage, distinguished by a growing apical protrusion.

Twelve to twenty four hours later, the zygote has developed into the crescent-shaped mature ookinete. This process involves the generation of an extensive network of subpellicular microtubules lining the interior of the ookinete, and forming their associated microtubule organizing centres (MTOCs), the apical polar ring. In the meantime, apical secretory organelles known as micronemes are synthesised (Canning and Sinden, *Parasitology* 1973). Ookinete maturation is accompanied by *de novo* protein synthesis, some of which transcripts are translationally repressed in the female gametocyte and others which transcription is ookinete specific (Mair et al., *Science* 2006). The events described above, configure the motility and invasive ability that is required for the ookinete to escape the bolus and migrate over the midgut epithelium.

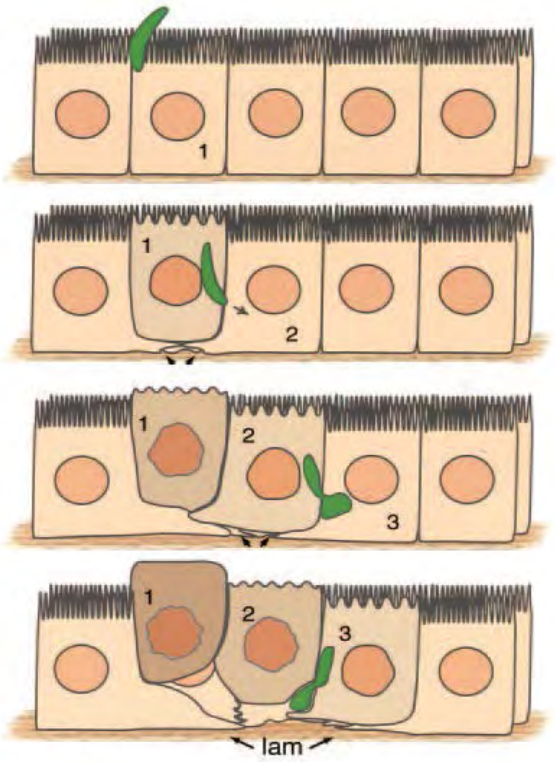


Figure 3.2.7 Model for midgut invasion. Numbers indicate the sequence of cell invasions and the ookinete route.
A. An ookinete invades a midgut epithelial cell, probably at the apical crevices between midgut cells, adjacent to the cell junctions (Zieler and Dvorak, 2000).
B. Re-invasion of a neighbouring cell via the basolateral plasma membrane. The first invaded cell looses microvilli, undergoes apoptosis and is expelled towards the midgut lumen. Adjacent cells extend lamellipodia (lam) and cover up the area beneath the protruding cell, to maintain the epithelium barrier.
C. Both invaded cells are expelled towards the midgut lumen; however, extensive lamellipodial protrusions seal the generated wound, while the ookinete re-invades a third cell.
D. Serial cell invasion shortens the distance that the parasite traverses intercellularly, before the lamellipodia can restore the epithelial barrier. (Figure and legend extracted from *Vlachou D. et al., Cellular Microbiology 2004*)

<u>Ookinete-to-oocyst transition</u>

Approximately 22-24h post-infection, the motile ookinete penetrates the midgut epithelium, and, upon arrival at the basal side, begins to transform into the sessile oocyst. (Vlachou et al., *Cell Microbiol* 2004).

As mentioned above, the microneme is an organelle, located in the anterior of the ookinete, implicated in host cell recognition and attachment, but also in gliding motility. It secretes proteins, many of which are associated with its surface and which, by interacting with immobile mosquito ligands and translocating to the rear end of the parasite, cause a “grab-and-push”-style, forward parasite motion.

The first barrier that the invading ookinete needs to hurdle is the chitineous peritrophic matrix (Figure 3.2.6), coating the apical side of the midgut epithelium. Traverse of this gel-like matrix is catalyzed by the function of a chitinase (CHIT1), (Dessens et al., *Infect Immun* 2001). Moreover, CDPK3, a *P. berghei* calcium-dependent protein kinase, seems to play a significant role in the early events of midgut invasion, as well, facilitating the ookinete motility. Targeted disruption of CDPK3 significantly impairs, but does not nullify rodent malaria transmission (Siden-Kiamos et al., *Mol Microbiol* 2006). A second

identified signalling component, essential for *P. berghei* transmission, is the cGMP signalling molecule guanylate cyclase β (GCβ) (Hirai et al., *J Biochem* 2006). Interplay between calcium and cGMP signalling, working in parallel or alternatively, could suggest a combined theory about the consistent role of these pathways to ookinete motility and invasion ability.

Next, the ookinete must attach to the apical surface of the midgut epithelium, penetrate the cytoplasm of the epithelial cells by disrupting their cell membrane, to finally reach the basal lamina (Figure 3.2.6). Some members of a conserved *Plasmodium* perforin like protein (PPLP) family conduct this permeating process in accordance with a signature membrane-attack complex and perforin (MACPF)-related domain. Two members of the *pplp* gene family, PPLP1 in sporozoites and PPLP3 in ookinetes, located to the micronemes, induce the secretion of PPLPs during parasite cell invasion, while PPLP5 and PPLP3 (also described as membrane attack ookinete protein, MAOP) help the parasite to enter the cells. (Ecker et al., *Exp Prasitol* 2007).

During its complicated journey through the mosquito midgut epithelium, the *P. berghei* ookinete invades multiple epithelial cells before it reaches the basal lamina and transforms into the oocyst (Figure 3.2.6). The parasite's route through the epithelial barrier is initially intracellular, during which the ookinete glides on the cell membranes in foldings of the basolateral domain. (Figure 3.2.7) There have been described three distinctive types of ookinete movement and their potential role to the midgut invasion; first, there is a stationary rotation mostly occured in the midgut lumen and might, but not obligatory, for invasion. Next, there is a combinatorial movement, spiraling, which is based on rotational motility combined with translocation steps and orientation changes and is believed to take place in the apical crevices of the midgut, (Vlachou et al., *Curr Opin Genet Dev* 2006).

The molecular mechanisms underlying gliding motility have been elucidated for sporozoites and are thought to be similar in ookinetes. A protein termed cell-traversal protein for ookinetes and sporozoites (CelTos) mediates the ookinete's navigation across the cytoplasm of the invaded midgut epithelial cells. The major ookinete surface proteins P25 and P28, among other *Plasmodium* surface or secreted proteins, are also known to mediate the invasion process. These proteins are conserved across *Plasmodia* and include three or four epidermal growth factor (EGF) domains. EGF domains are not only widespread in eukaryotes, but also proteins that encode them participate in numerous receptor-ligand interactions. Ookinetes lacking both P25 and P28 showed increased sensitivity to proteases in the mosquito midgut, a significant decline in their invasive ability and reduced ability to transform into oocysts (Tomas et al., *Embo J* 2001).

Another gene expressed in the mature ookinete is WARP (von Willebrand factor A domain-related protein). In contrast to P25/28, WARP protein expression is detectable eight hours after fertilisation, with a micronemal localisation in the mature ookinete. Nevertheless, WARP is not essential for *P. berghei* transmission. An additional protein, which is also localized in micronemes and involved in ookinete midgut invasion, is the secreted ookinete adhesive protein (SOAP), (Dessens J et al., *Mol Microbiol* 2003). SOAP and P25/28 bind to the laminin, the major component of the mosquito basal lamina, facilitating the ookinete stabilization and inducing the oocyst formation. Additional laminin-binding proteins can be found at the TRAP protein family, whose founding member is the thrombospondin related anonymous protein (TRAP protein). CTRP, the circumsporozoite and TRAP-related protein, also belongs to that family, localises to the micronemes in the ookinete and is thus secreted during invasion in order

to accumulate at the point of contact between the ookinete and basal lamina (Vlachou et. al, *Cell Microbiol* 2004).

It is substantial to underline that the interaction of ookinetes with basal lamina components is pivotal for oocyst development. Experiments that included ookinete injections into the mosquito haemocoel pointed their ability to form oocysts at the basal lamina of not only the midgut but also the fat body and the malphigian tubules (Weathersby AB, *Exp. Parasitol* 1954). Likewise, despite the fact that Plasmodium is incapable of development while fed to Drosophila melanogaster, ookinetes inoculated into the D. melanogaster haemocoel are able to attach to the basal lamina and transform into oocysts (Schneider D. et al., *Science 2000*). Ergo, it seems that the actual interaction with the basal lamina is the major factor that triggers oocyst development.

Oocyst maturation and sporozoites formation

As mentioned before, the ookinete traverses the midgut epithelium and encounters the haemolymph (residing in the sub-epithelial space) and the basal lamina. There, it is transformed into oocyst, interacting with the basic components of the basal lamina; laminin, collagen (IV), entactin and perlecan (Nacer et al., *Parasit Vectors* 2008). Inside the developing oocyst, many nuclear divisions occur, which, by not being coupled to cytokinesis, lead to the formation of a multinucleated parasite that gradually grows in size. Simultaneously, the oocyst plasma membrane is folded inwards forming crevices that divide the cell into compartments termed sporoblasts. Inside the sporoblasts, and in accordance with a process that includes mobilization of the nucleus and other cellular organelles, the sporozoites develop and eventually bud off. Cytokinesis of the budding sporozoites mediates the formation of a mature oocyst that comprises haploid sporozoites.

To date, there are only few proteins identified to play a significant role in Plasmodium sporogony. Nevertheless, it is known that cytokenisis and sporozoites formation are mainly induced by the circumsporozoite protein (CSP), which is the main surface protein of the oocyst and the salivary gland sporozoites. Other significant for oocyst development proteins are the LglI (LCCL)–lectin adhesive-like protein (LAP) family and the limulus clotting factor C, Coch-5b2.

As previously mentioned, growth and partition of each oocyst produces thousands of haploid forms, the sporozoites. These active sporozoites debouch from the oocyst and migrate to the mosquito salivary glands, a process mainly facilitated by the egression cystein protease, ECP.

Sporozoites motility and salivary gland invasion

After the sporogonic phase (8-15 days post-infection), the sporozoites are released into the body cavity of the mosquito, as a result of the oocyst eruption, finding their way to the salivary glands. It is though unclear how sporozoites achieve to migrate towards the salivary glands.

The involvement of CTRP and TRAP proteins to the midgut and salivary gland cells invasion consequently suggest that, motility-related proteins are used by parasite stages to invade two different types of cells, in both the vertebrate host and the invertebrate vector (Vlachou et al., *Curr Opin Genet Dev* 2006). Moreover, it is known that an actomyosin motor, located apically between the plasma membrane and the inner membrane complex, promotes the Plasmodium motility. The cytoplasmic tail of TRAP and the interplay between the tail of Myo-A and the Myo-

A tail domain-interacting protein (MTIP) are involved in actomyosin motor function. Interestingly, this structure generates the forward-gliding parasite motility, not only for the salivary-gland invasion stage, but also for penetration of the host-cell membranes.

In contrast to the midgut invasion, which includes the ookinete's route from the apical to the basal surface, the salivary gland invasion involves sporozoites that follow the reverse route (from the basal to the apical side). Therefore, salivary gland penetration must recruit different molecular mechanisms. Indeed, it is proposed that the interaction with the salivary glands' basal plasma membrane results in the formation of a parasitophorous vacuole, which decomposes inside the cytoplasm through endocytosis, eventually releasing the parasites (mechanism that has not been observed in midgut invasion process). However, these two processes display some common attributes; SM-1 (salivary gland and midgut peptide 1), inhibits both the invasive ability of the ookinete and the salivary gland invasion. This finding suggests that the recognition and initial attachment of the salivary gland epithelium is mediated by a mutual, unknown to date, ligand (or ligand paralogues).

A second parasitophorous vacuole is considered to encompass the sporozoites during their exit to the secretory cavity of the salivary glands. Inside the cavity, the decomposition of the vacuole releases the sporozoites, which migrate by gliding motility into the fine secretory channel that then converge into the common ducts. Only sporozoites that traverse through the ducts can be transmitted upon mosquito salivation, as the great majority of sporozoites, mainly found in the secretory cavities, are either stored or lost.

Review

Sexual developmental stages are fundamental for Plasmodium's life cycle. During these stages, innumerable infecting forms of the parasite occur within the mosquito, which induce pathogenesis in the human host following their injection with the mosquito bite. The five major steps described before analyze the complicated processes and transitions that Plasmodium undergoes in order to sustain, reproduce and expand its infectious force into the nature. Therefore, passage through the mosquito is obligatory for the parasite, not only for transmission, but also for its evolution and adaptation. *(*Christophides et al., *Immunol Rev* 2004*)*

The figure below (Figure 3.2.8) summarizes the temporal patterns of gene expression displayed among the several sexual developmental stages of Plasmodium. A recent survey integrated genomic, transcriptomic and proteomic analyses upon *Plasmodium falciparum* and *Plasmodium yoelii* species, revealing a conserved core of 4500 Plasmodium genes distributed in the central regions of the 14 chromosomes. Thus, there were illuminated four major strategies for gene expression during parasites' life cycle: a) housekeeping, b) host related; c) strategy-specific related to invasion, asexual replication and sexual development and d) stage-specific (Hall N. et al., *Science* 2005). Generally, it was known that *Plasmodium* transcripts are essentially produced when needed ("transcripts to go" model). Interestingly, in the same survey it was observed that posttranscriptional gene silencing (PTGS) induced translational repression on the mRNA during sexual development, illuminating a novel mechanism of the regulation of gene expression in *Plasmodium.*

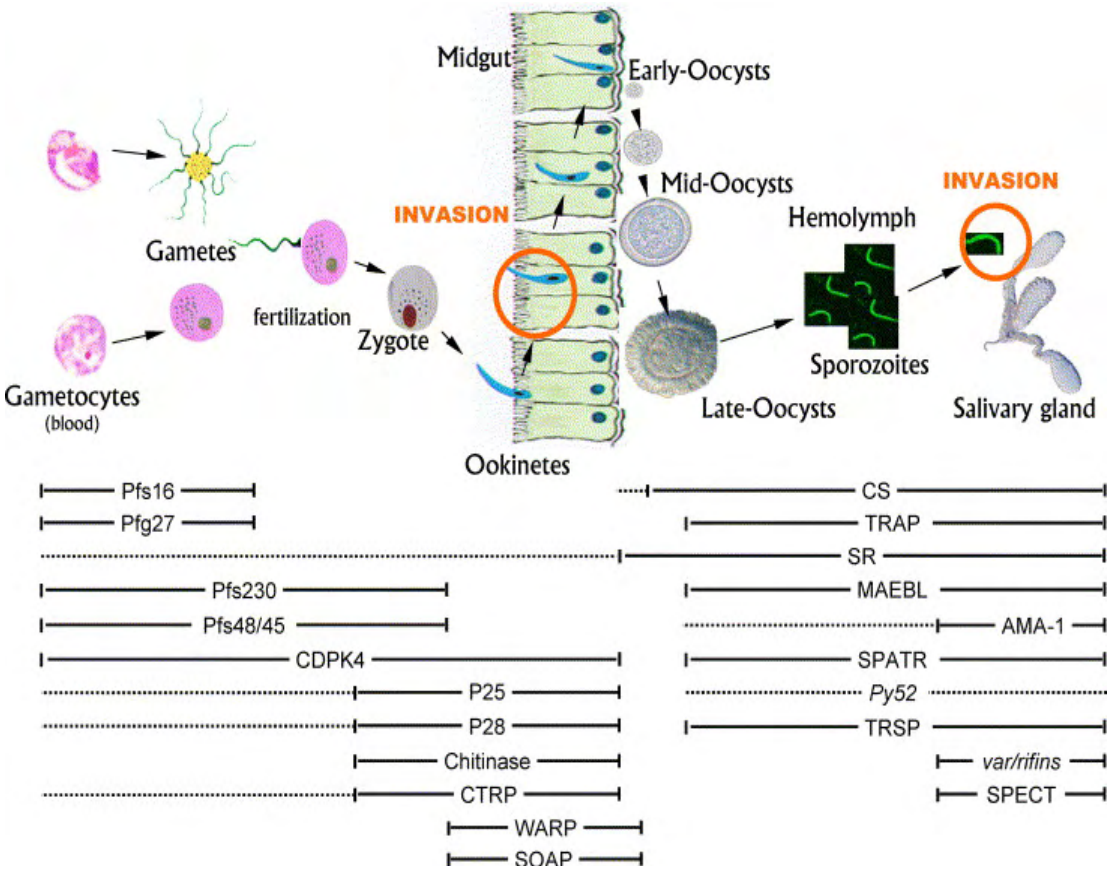


Fig. 3.2.8 *Plasmodium* sexual (sporogonic) life cycle and temporal patterns of gene expression. Temporal patterns of expression of genes discussed above are summarized in the lower portion of the figure. The solid lines indicate parasite stages at which protein was detected, and the dashed lines indicate parasite stages at which mRNA, but not the corresponding protein, was detected (Moreira et. al, *Int J Parasitol*. 2004)

3.2.3.3 Conserved Mosquito responses upon *Plasmodium* infection

During sporogonic (sexual) development, *Plasmodium* interacts with the mosquito vector at multiple stages and locations, and the nature and efficacy of this bi-directional interaction determines the success of transmission. The susceptibility or refractoriness of various *Anopheles* taxa against various *Plasmodia* is caused not only by the diverse ecoethological characteristics of these mosquitoes but also by the impact of cellular and biochemical interactions between the two organisms.

As mentioned above, *Anopheles gambiae* is the major vector for human malaria. In general, invertebrates' defense system involves only innate immunity, and thus is vitally important. The effectiveness of the innate immune system and its evolutionary flexibility for tackling diverse sets of invaders are clearly testified by the evolutionary success of many insects, the most species-rich and morphologically diverse class of invertebrates. During its biological evolution, *Anopheles* managed to obtain an effective innate immune arsenal to fight infections, including *Plasmodium* invasion. The basic knowledge about *An. gambiae* immune system has derived mainly from studies included infections with the rodent model *P. berghei* (Christophides et al., *Immunol Rev* 2004).

In general, *Anopheles* defense responses upon *Plasmodium* invasion take place in various mosquito tissues: the lumen and epithelial cells of the midgut, the hemolymph and the salivary

glands. The hemolymph, which represents the open circulatory system of insects, propagates many of the humoral and systemic immune reactions in mosquitoes. The general view of insect immunity is that it largely originates in the insect analog of liver, the fat body. Nevertheless, the hemolymph is the carrier of what is recognized as a crucial though dispersed immune tissue, the hemocytes (blood cells). Some of these cells provide circulating immune factors while others are the basic cellular components of the innate defense. Finally, regarding to *Plasmodium* invasion, local epithelial immune responses are highly important in parasite-invaded tissues (Christophides et al., *Immunol Rev* 2004*,* Vlachou D. et al., *Curr Opin Microbiol* 2005). The previews years has been recorded an abundance of studies trying to identify the mosquito responses, as they play a key role in the molecular interplay between the developing parasite and the infected vector.

The developing *Plasmodium* spends approximately a day in order to transform into ookinete and begin the invasion through mosquito's midgut epithelium. During the first stages, mosquito's main molecular mechanisms include positive regulators of parasite's development, as the mosquito tries to identify the presence of the invader, providing though an appropriate environment for its development (XA and low pH in the mosquito midgut induce gametogenesis).

However, the main parasite-mosquito interactions eventuate approximately 20h later, when the motile ookinete begins to invade the midgut epithelial cells.
The invading *P. berghei* ookinete causes severe damage to the midgut epithelium and invaded cells undergo programmed cell death, apoptosis. Apoptosis is associated with a loss of microvillus, extensive DNA fragmentation and protrusion of the invaded cells towards the midgut lumen. Thus, the invaded dying cells are deported from the epithelium and wound healing mechanisms such as extensive lamellipodia crawling by neighbouring cells mediates the restoration of the epithelial barrier (Vlachou et al., *Cell Microbiol* 2004), (Figure 3.2.7). It is still unclear whether or not midgut invasion is receptor-mediated (Vlachou D. et al., *Curr Opin Microbiol* 2005). However, recent studies pointed that annexins, which are strongly expressed in the midgut, bind to *P. berghei* ookinetes. In toto, there are three major mechanisms that configure the mosquito response during midgut penetration: parasite lysis, melanisation and local epithelial reactions. (Figure 3.2.9)

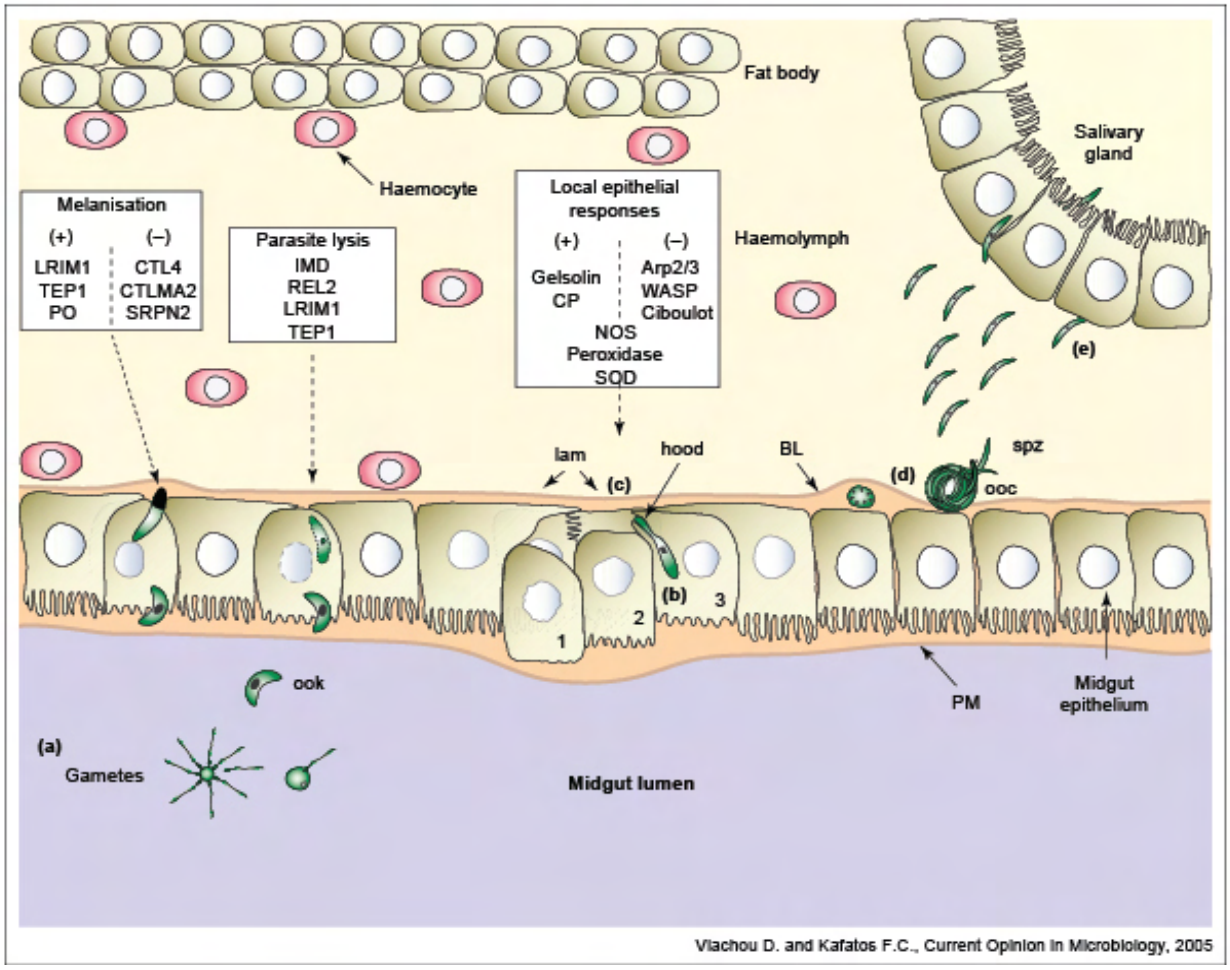


Figure 3.2.9 The parasite-vector interactions during *Plasmodium* sexual development inside *An. gambiae* midgut. Successful transmission of the malaria parasite *Plasmodium* requires complete sexual development in the mosquito before it can be transmitted to the vertebrate host. Mosquito molecules putatively involved in positive (+), negative (−) and unclassified reactions to the parasite are indicated in boxes. Such reactions include parasite melanisation, lysis and local epithelial responses. Following gamete development and fertilization **(a)**, *Plasmodium* ookinetes (ook) serially traverse the cytoplasm of several midgut cells (indicated as 1, 2 and 3) before accessing the basal lamina by emerging basolaterally into the extracellular space **(b)**. During midgut invasion, the parasite provokes strong responses of the epithelial cells, including the induction of directional lamellipodia protrusions (lam) beneath the invaded apoptotic cells and the formation of cytoplasmic lamellar protrusions (ookinete hood) of the invaded cells, which tightly embrace the parasite as it exits from the midgut **(c)**. In the space between the midgut epithelium and the basal lamina (BL), the parasite develops into an oocyst (ooc), in which some thousands of sporozoites (spz) are produced **(d)**. Some days later, the oocyst ruptures and sporozoites are released into the haemolymph and then invade the salivary gland **(e)**. During this journey, the parasites are also exposed to the immune response of the fat body (the analog of vertebrate liver), which together with the haemocytes are considered to be the main immune organs of insects. CP, capping protein; IMD, immune deficiency gene; PM, peritrophic matrix; PO, phenoloxidases; REL2, Relish 2; SOD, superoxide dismutase. (Christophides et al., *Immunol Rev* 2004)

Parasite lysis and melanisation

Substantial parasite losses are documented to be mediated by mosquito innate immune system. Anopheles genome sequencing revealed genes potentially involved in mosquito immune reactions to the invasion of the midgut by ookinetes (Holt RA et al., *Science* 2002, Christophides et al., *Science* 2002*).* Interestingly, unambiguous experiments provided evidence for two *An. gambiae* genes, LRIM1 (leucine-rich repeat immune protein 1) and TEP1 (thioester-

containing protein 1), which are involved in the killing and extermination of up to 80% of the invading P. berghei ookinetes, even in parasite-susceptible mosquitoes *(*Osta et al., *Science* 2004*,* and Povelones et al., *Science* 2009*)*. TEP1 is a recently identified complement C3-like protein and is secreted into the haemolymph by haemocytes, acting as recognition receptor that binds to bacteria and Plasmodium and thereby promotes two distinct immune reactions: phagocytosis of bacteria and lysis of Plasmodium ookinetes. LRIM1, the leucine-rich immune factor, is also involved in both reactions as it is strongly upregulated during bacterial and Plasmodium infections. The knock-down of *LRIM1* or *TEP1* via dsRNA mediated RNAi results in a significant increase in oocyst numbers (Osta et al., *Science* 2004). Recent studies revealed that LRIM1 and APL1C, two members of the LRIMs family, circulate in the haemolymph along as a disulfide-bonded complex that specifically interacts with the mature form of TEP1, taking part in the mosquito complement-like pathway as crucial players of the immune defense against Plasmodium parasites (Povelones et al*., Science* 2009*,* and Povelones et al*., Plos Pathog* 2011*)*.

Some of the ookinetes that survive lysis might be blocked by a humoral immune reaction that is important in arthropods, melanisation. In melanisation process, microorganisms are enclosed in a melanin capsule and are exterminated by the production of toxic reactive oxygen intermediates or by asphyxiation (Kumar et al*., PNAS* 2003). In general, melanisation in insects requires proteolytic cleavage of prophenoloxidases into active phenoloxidases, which catalyze melanin synthesis. The regulation of this reaction is based on diverse proteins, including clip-domain serine proteases and their inhibitors, serpins (SRPNs), inactive serine protease homologues that are missing catalytic triad residues, and C-type lectins (CTLs) (Christophides et al*., Immunol Rev* 2004). It has been established that at least one serpin, SRPN2, plays a negative role in ookinete killing and melanisation: its depletion causes spontaneous melanisation and a strong attenuation of oocyst numbers. Also, despite the generally positive role of insect CTLs in activation of melanisation, two *An. gambiae* proteins, CTL4 and CTLMA2, prevent melanisation of *P. berghei* ookinetes in the mosquito midgut, inducing a significantly augmentation of the mosquito's vectorial capacity.

The majority of the proteins mentioned above are detected in the haemolymph, indicating the central role of this open circulatory system in systemic anti-parasitic reactions. It appears that the parasite confronts an attack of circulating immune factors while exiting the basal side of the midgut epithelium. However, additional factors might fire reactions of the immune system and facilitate parasite killing, before or during invasion. Recent data have indicated immune signalling pathways that lead to the production of antimicrobial peptides (e.g. cecropins) and regulate melanisation (Meister et al., *PNAS* 2005). Transgenic overexpression of a mosquito cecropin in the midgut leads to a significant reduction in oocyst numbers.

Local epithelial reactions

Apart from the classic innate immune reactions, there has been recently proposed a number of other responses fired by the mosquito against *Plasmodium* ookinetes. During intracellular midgut invasion, the parasite triggers a plethora of local epithelial reactions, comprising apoptosis of the invaded cells followed by epithelial repair mechanisms based on actin cytoskeleton and microtubule remodeling. The parasite "hood" (figure parapanw), an interesting actin-based structure which is extended by the invaded cell, embraces the parasite as it exits the hostile midgut environment. It is proposed that the hood resembles the phagocytic cup formed around pathogens (e.g. bacteria) destined for phagocytosis by vertebrate blood cells. This resemblance in association with the confirmed involvement of TEP1

and LRIM1, two key parasite antagonists in bacterial phagocytosis, derives the suggestion that the hood might be part of a local epithelial immune reaction. Consistent with this hypothesis are the data derived from RNAi-induced silencing of two regulators, Wiskott–Aldrich syndrome protein (WASP) and Apolipophorin II/I (ApoII/I). The transcriptional depletion of WASP, which is a local activator of actin cytoskeleton reorganization, induces significant increase of *P.berghei* infection loads (Vlachou D et al., *Curr Biol* 2005). On the other hand, *P. berghei* leads to upregulation of the mosquito precursor ApoII/I, a significant circulating lipid transport regulator whose silencing disrupts mosquito egg development and drastically decreases parasite oocyst numbers.

Microbial pathogen invasion and infection mediate the manipulation of the actin cytoskeleton of host cells. Similarly, the massive remodeling of the midgut cell cytoskeleton that has been detected during ookinete penetration might be triggered either indirectly, e.g. by epithelial apoptosis, or directly, e.g. by Plasmodium components. Thereafter, Subtilisin 2, a serine protease exported by *P. berghei* ookinetes inside the midgut cells, appears to be associated with actin filaments.

Another mechanism observed during ookinete invasion involves the reported enhanced activity of nitric oxide synthase (NOS) in midgut cells, which catalyzes the production of NO that can be readily converted to nitrite. The same cells display strong peroxidase activity, which, in the presence of H_2O_2, induces tyrosine nitration that results in extensive invaded-cell degeneration and can damage the parasites (Kumar et al., *J Bio Chem* 2004*)*. A time delay between the activation of NOS and peroxidase possibly gives an advantage to the majority of ookinetes to exit the cells before tyrosine nitration begins, in susceptible mosquito strains. However, in refractory L3-5 mosquitoes, the extensive *Plasmodium* killing and melanisation might be explained, to some extent, by the early transcriptional induction of superoxide dismutase in the midgut, which is accompanied by high haemolymph levels of hydrogen peroxide. Actually, a recent study highlighted a heme peroxidase (HPX2) and NADPH oxidase 5 (NOX5) as critical inducers of midgut epithelial nitration and antiplasmodial immunity that enhance nitric oxide toxicity in *Anopheles gambiae*. It was revealed that epithelial nitration and TEP1-mediated lysis work sequentially, suggesting that epithelial nitration induces activation of the mosquito complement cascade, working as an opsonization-like system (Oliveira et al., *Science* 2012).

Epilogue: The transmission bottleneck – Mosquito's vectorial capacity

There have been enormous steps in the last decades upon understanding the malaria transmission biology and the identification of the conserved mosquito-parasite interactions, as they play a key role on malaria obliteration. The rapid technological advance on the past decade provided a significant number of novel techniques, such as microarray technology and dsRNA mediated RNAi, which facilitated malaria study and revealed a plethora of new data about parasite development, vector responses and, the most intriguing, their cellular and molecular interactions (Vlachou D. et al., *Current Biology* 2005 and Povelones et al., *Science* 2009).

Throughout Plasmodium sporogony, significant losses are mainly observed in two important stages: gametogenesis and ookinete-to-oocyst transition. The efficiency of gamete production largely depends both on the combination of Anopheles-Plasmodium species, and the presence and amount of gametocyte activation factors in the blood meal. During the second limiting step 90-99% of the ookinetes are lost and the number of parasites reaches the minimum at this critical stage, often in single digits (Christophides et al., *Immunol Rev* 2004). Thus, a bottleneck

effect is observed as the parasite traverses the midgut epithelium in order to form oocysts (Figure 3.2.10). This population bottleneck is highlighted as one of the major phenomena that could provide molecular targets for transmission blocking interventions (Sinden et al., *Plos Pathog* 2007). However, it is notable that the mosquito becomes infective even if only few oocysts develop inside its midgut, as the amplification factor in the oocyst-to-sporozoite transition is between 2000 and 8000-fold (Christophides et al., *Immunol Rev* 2004).

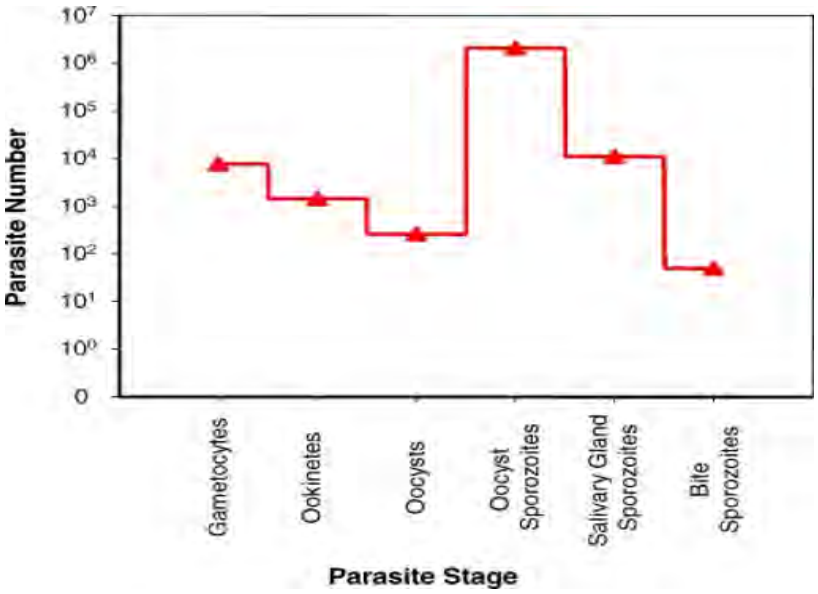


Figure 3.2.10
Changes in *Plasmodium* population density during development within the mosquito.
Reported changes in numbers of *P. berghei* as it develops in *An. stephensi*, starting from an intake of 10^4 macrogametocytes. Note the use of a log-scale on the *y*-axis. Figure adapted from *Sinden et al., Plos Pathog 2007.*

It was mentioned above that not only refractory and but also susceptible mosquitoes launch an anti-parasitic immune response. Therefore, it is intriguing the observation that the immune response of susceptible mosquitoes, although does not lead to sterile immunity (transmission blockage), is strong enough to severely decimate the parasite population at the midgut bottleneck. There are three hypotheses attempting to explain this only partially effective immune response paradox. First of all, it is possible that the mosquito actively restrains the immune response inasmuch as to protect its reproductive fitness, curbing the immune attack (for the parasite population as a whole) at a sub-lethal level. Secondly, it is possible that the parasite has developed protective mechanisms which allow them to withstand this immune response (e.g. enhanced protease sensitivity observed for the *pb25/28* double KO). Finally, it has been proposed that the timing of invasion and exit may be crucial to parasite survival, as suggested by the Time Bomb model. Accordingly, during midgut cell invasion, the ookinete must rapidly traverse and exit the increasingly hostile environment of the cell in order to survive proposing that survival may consequently be a matter of timing, with early ookinetes successfully completing the migration before “the detonation of the time-bomb” (adapted from *Bushell Ellen, Imperial College London PhD thesis 2009*). Regardless of any scenario, it is undeniable that the mosquito-parasite ecological and molecular interplay consists a highly complicated biological system; thus, the mosquito defense, the parasite development and their respective interactions should be examined through an overall evolutionary aspect, considering the mosquito-parasite species combination, their co-adaptation time and place, the local selective pressures etc.

3.3 Control Measures

3.3.1 Malaria control and obstacles to eradication

In retrospect, malaria has not always infected the poor tropical regions of the world.
However, after the end of the Second World War, the world-wide malaria eradication campaign managed to successfully annihilate malaria from many temperate zone countries, including former Soviet Union, the U.S. and the Mediterranean. Also, in some tropical regions (such as South America, parts of South East Asia and India, and some African nations), a significant reduction in disease burden has been achieved (Mabaso et al., *Trop Med Int Health* 2004).

In contrast to many other infectious diseases, malaria has a determinant ecology which involves the parasitism upon a vertebrate host and a mosquito vector, which may be regarded as a second host. This dual-host system reflects the complexity for its management, but also creates additional targets for disease control. Malaria control measures can be broadly divided into two basic categories, based on the targeted organism:
1) Parasite obliteration from the human host.
2) Vector control

3.3.2 Parasite obliteration from the human host

3.3.2.1 Anti-malarial medications

Anti-malarial medications are drugs designed to prevent or cure malaria. They are mainly dispensed in order to treat actually or potentially infected individuals, to prevent immunity-deficient individuals visiting a malaria-endemic region from infection, or as a part of the Routine intermittent treatment, foremost as Intermittent Presumptive Therapy (IPT) for pregnant women (White, *Plos Med* 2005).

Based on their parasite subcellular and biochemical targets, anti-malarial medications disrupt four major structures/processes: cytoplasm (folate synthesis), mitochondrion (electron transport chain), food vacuole (haem-detoxification), and the apicoplast (protein synthesis), (Greenwood et al., *j Clin Invest* 2008). The most significant approved drugs are quinine and its derivatives, which are effective against the mature trophozoite and inhibit the parasite haem-detoxification process, and the artemisinin, a compound extracted from the plant *Artemesia annua,* which is successfully active against all asexual blood stages.

Despite the global pharmacological efforts against malaria, there are two severe obstacles in the efficiency of malaria chemotherapy: the necessity for new drug discoveries and the wide-spread resistance against existing drugs. Regarding to the last problem, a combinational drug therapy of different forms of Artimisin (ACT) has been proposed as first-line treatment for all P. falciparum malaria in endemic countries, which also has the advantage of reduced treatment time (from seven to three days), thus increasing the rate of patient compliance (White, Science 2008).

3.3.2.2 Prophylactic vaccination

Despite the progress that has been made in the last decade toward developing malaria vaccines, there is currently no effective malaria vaccine on the market (http://www.cdc.gov/malaria/malaria_worldwide/reduction/vaccine.html). In general, there are three conceivable strategies for vaccine development (Matuschewski, *Curr Opin Immunol* 2006):
1) Liver stage targeting (Pre-erythrocytic),
2) Blood-stage targeting (Erythrocytic), and
3) Mosquito-stage targeting vaccines (Transmission blocking).

The first category includes the use of irradiated, live attenuated sporozoites, genetically engineered sporozoites, and/or parts of them, such as the circumsporozoite protein CSP (Matuschewski, *Curr Opin Immunol* 2006). Until today, live attenuated sporozoites remain the gold standard for malaria vaccines. Nevertheless, after irradiation, the result is an undefined mixed population of sporozoites, with a random selection of mutations.

Regarding blood-stage vaccines, there are two complementary antibody targeting strategies: anti-parasitic (anti-merozoite) and anti-disease (anti-cytoadhesion) vaccines. Inhibition of merozoite RBC invasion via antibodies strategy has focused on targeting the members of the merozoite surface protein (MSP) family, most notably MSP1 but also MSP3. Natural resistance is associated with the production of protective antibodies against the complete *Pf*EMP1 repertoire. Thus, targeting cyto-adherence by anti-*Pf*EMP1 antibodies could induce protection against severe malaria. Despite their intriguing theoretical base and the great efforts upon them, to date, blood-stage vaccines are not efficient enough to diminish malaria. The last strategy (transmission blocking vaccines) will be thoroughly discussed in the next section.

3.3.3 Vector control

3.3.3.1 Host-mosquito contact reduction

It has been observed that most *Anopheline* vector species display a nocturnal feeding pattern; thus, it is important to reduce the biting attacks during this period. Studies have shown that the use of insecticide-treated nets (ITNs) in sub-Saharan Africa causes a significant decline in malaria related morbidity and mortality in children under five. Moreover, use of insecticide-treated bednets by pregnant women also reduces anaemia among mothers, the number of babies with low birth weight and infant mortality before delivery (http://www.cdc.gov/malaria/malaria_worldwide/reduction/itn.html).

The biggest problems that ITN-based malaria control is currently facing are the low coverage of people at risk and the issue of re-treatment (Guyatt et al., *Trends Parasitol* 2002). Both issues are substantially connected with the low socioeconomic status of endemic regions; however, international programmes provide an assistance to some extend by both distributing and re-treating bed-nets for free of charge. (Curtis et al., *J Am Mosq* Control Assoc 2006).

3.3.3.2 Elimination of larval and adult stage mosquitoes

Before the massive usage of DDT, environmental engineering programs, such as draining breeding grounds of water, and larvicidal methods, such as oil or Paris green (copper acetoarsenate) spreading onto the surface of breeding pools, were the most popular methods for mosquito population controlling. Additionally, further larvicidal approaches, such as the introduction of larvivorous fish (primarily *Gambusiaf finis*), have been successfully established.

However, in the Global Malaria Eradication campaign (WHO), launched in 1955, large scale indoor residual spraying (IRS) using dichlorodiphenyltrichloroethane (DDT) was the gold standard for mosquito vector elimination. The effectiveness of DDT is based on its lethal toxicity against mosquitoes, even after exposure in a low concentration. Thus, the DDT spraying campaigns conducted in after World War II, in combination with chloroquine treatment, succeeded in malaria disappearance from many temperate and tropical low-transmission zones (Sadasivaiah et al., *Am J Trop Med Hyg* 2007). Nevertheless, by the 1960s, the emergence of DDT resistance along with operational, administrative and economic problems in the developing nations, gradually led to the abortion of this control strategy. Consequently, the malaria eradication project in the high-transmission tropical zones remained incomplete.

Figure 3.3.1 World War II poster, U.S. National Archives and Records Administration

Some years later, the incoming advance in Biotechnology and Molecular Biology provided a plethora of new perspectives and techniques, boosting the scientific research and illuminating a broader spectrum of strategies in the fight against malaria.

One simple conception towards adult mosquito elimination is the massive sterile male release (*sterile insect techniques, SIT*) into an area, which has been successfully applied in several small-scale areas. However, due to the need for massive mosquito releases and the low mating competence of released sterile males, this approach appears to be impractical for most areas. Nevertheless, based on the same conception, modern studies recruit Genetically Modified Mosquitoes (GMMs) in order to suppress vector populations, boosting the selection that drives to male offspring production (Christophides G. K*., Cell Microbiol* 2005). More interestingly, a second GMM strategy involves the introduction of mosquito vectors with robust refractoriness against *Plasmodium* infection in the field, ideally aiming to the total replacement of the wild natural populations. Following that thought, a recent study revealed that genetically manipulated *An. stephensi,* which integrated a transgene encoding the IMD pathway-controlled NF-kB Rel2 transcription factor in the midgut and fat-body tissue, had higher resistance against Plasmodium and bacterial infection (Y. Dong et al*., Plos Pathog* 2011). However, the introduction of a desired trait into a wild population has to be also supported by a strong driving mechanism. A synthetic homing endonuclease-based gene (HEG), which is a simple selfish genetic element, appeared to be an applicable driving system in laboratorial *An. gambiae* populations, as it led to domination of the transgenic individuals in only few generations (Windbichler N., *Nature* 2011). Last but not least, a recent interesting conception

involves the use of genetically modified bacteria that, in symbiosis with the mosquito vector, could deliver the anti-malarial effector modules to its midgut lumen. Significantly, engineered *P. agglomerans* strains that secreted anti-malarial effectors inhibited *P. falciparum* and *P. berghei* development by up to 98% (Wang S. et al., *PNAS* 2012).

3.3.3.3 Transmission blocking vaccines: the "altruistic vaccine" getaway

A potential strategy that aims to repress malaria transmission is that of Transmission Blocking Vaccines (TBV). The principle of TBV is to use the human adaptive immune response to produce antibodies that are subsequently transferred to the mosquito midgut during uptake of a blood meal, via vaccination of individuals with mosquito-stage parasite proteins (Dinglasan and Jacobs-Lorena, *Trends Parasitol* 2008). The antibodies produced bind to the parasite within the mosquito midgut and thereby facilitate antibody or complement mediated killing. Alternatively, they could obstruct the function of parasite proteins, leading to development abortion and transmission blockage (Saul, 2007). The goal of these vaccines is to nullify the prevalence of the targeted pathogen in the host pool, diminish its communicability and subsequently protect the community from contracting the disease (herd immunity). In contrast to the classical vaccine design, the TBV approach does not directly provide an immune protection against the disease contraction; it is an "altruistic vaccine" which indirectly protects individuals within a vaccinated population by eliminating the number of infection carrying vectors (Dinglasan and Jacobs-Lorena, *Trends Parasitol* 2008).

According to their expression pattern, *Plasmodium* protein candidates for TBV development can be separated into two different groups: pre- and post- fertilization (Saxena et al., *Eukaryot Cell* 2007). Antibodies against two gametocyte expressed proteins Pfs48/45 and Pfs230 showed successful transmission blockade by killing the parasite during gametocyte activation, when the proteins become exposed on the gamete surface. On the other hand, Pfs25 and Pfs28, which are expressed and transferred on the parasite surface after mosquito midgut invasion, display minimal polymorphisms and immunity is thereby not strain-specific (Saxena et al., *Eukaryot Cell* 2007). Finally, a recent study revealed a novel formin-like protein expressed in male gametocytes, MISFIT, which regulates the dynamic remodelling of actin and microtubule networks and represents a potential target for TBV.

A negative issue associated with TBV strategy is that the antigens are never naturally expressed in the human host, and thus antibody responses may be short-lived, as there is no natural boosting. Moreover, the vaccine will be efficient only in a local transmission area, implying that a significant number of carriers must be vaccinated and develop a robust immune response in order to achieve adequate transmission blocking levels (Saul A., *Curr Opin Infect Dis* 2007).

3.3.3.4 Synopsis - Obstacles in eradication

In conclusion, every current malaria control strategy is associated with its congenital benefits and drawbacks. The two main limitations that severely hamper both host and vector control methods concern the low number of currently available effective compounds and the rapid emergence of resistance upon them (Egan and Kaschula, *Curr Opin Infect Dis* 2007). Importantly, the socioeconomic background and the lack of proper infrastructure in endemic countries have also restrained eradication efforts. However, no method should be considered

in its isolation, as a combinatorial strategy, in accordance with specific regional circumstances, appears to be the key in malaria eradication (Bruce-Chwatt, *Annu Rev Public Health* 1987).

In overall, malaria is currently a both preventable and curable disease. However, its eradication has not yet been possible due to several complicating factors. The continued loss of innumerable lives every year illuminates the unambiguous necessity for higher funding in malaria research (Miller and Greenwood, *Science* 2002).

4 Project Aim

4. Project Aim

The aim of this final year project is to study the responses of the two major malaria vectors, *Anopheles gambiae* and *Anopheles arabiensis*, when infected with sympatric *Plasmodium falciparum* natural populations. This investigation is comprised of two binary biological systems (*An. gambiae/P. falciparum* and *An. arabiensis/P. falciparum*) that are examined in a specific time frame, during which, *Plasmodium* undergoes several developmental changes. The ultimate goal is not only to identify similarities and differences between the two mosquito species during infection with the same sympatric parasite, but also to explore possible differences in the development of the same *Plasmodium* parasite inside the two different mosquito vectors.

In order to reach this goal, two experimental strategies are followed: (i) a high-throughput approach, and (ii) a low throughput approach. According to the first approach, transcriptional profiles of both bipartite systems are identified through analysis of Transcriptomic Microarray data. The fundamental hypothesis that structures this approach is that transcripts exhibiting a significantly up- or down- regulation, are probably implicated and contributing in the molecular and cellular processes occurring inside the systems; on a reductive approach, the regulated expression profile (of an organism) is directly correlated with the functional profile (of the same organism). As an offshoot of the first approach, candidate genes that exhibit intriguing profiles are further characterized via Reverse Genetics. In this low throughput approach a second hypothesis is constructed, according to which, RNAi-induced transcriptional silencing of a significant gene produces changes in the phenotype that are associated with the gene's function.

The overall target of this project is to detect and characterize the molecular and biochemical interactions between the mosquito vector and the parasite invader, during malaria transmission, in order to reveal potential targets for Transmission Blocking Interventions (TBI).

5 Materials & Methods

5.1 Biological Materials and Operating Procedures

5.1.1 Mosquito Breeding

Anopheles gambiae N'gousso-strain mosquitoes were cultivated according to standard methods (Sinden et al 2002) and reared by T. Habtewold and S. Katarzyna. In summary, adult mosquitoes were maintained enclosed in netted buckets, at 28°C ambient temperature, 70% relative humidity, in 12-hour light/12-hour dark conditions. Mosquitoes are fed on autoclaved and sterile filtered 10% fructose solution, before and after the operating procedures.

5.1.2 Adult mosquito Micro-Injections

This protocol describes the Standard Operating Procedure "Microinjection of adult *Anopheles* mosquitoes", recorded in the Laboratory of Immunogenomics, Imperial College London (Protocol Number: *LIM 0025*, generated by: *Fanny Turlure*, approved by: *George K. Christophides*, 17/10/2008). The same protocol can also be found published as "Garver L. and G. Dimopoulos, *Protocol for RNAi Assays in Adult Mosquitoes (An. Gambiae)*, J Vis Exp. 2007; (5): 230." supplied with audiovisual material:

- *Scope and field of application:*
  Inject adult mosquitoes with double-stranded RNA to induce RNAi

- *Principle:*
  Injection of dsRNA into adult *Anopheles* mosquitoes causes silencing of the targeted gene allowing the subsequent molecular and cell biological study

- *Sample:*
  On one day post-emergence, uninfected adult females (*A. gambiae Ngousso* strain)

- *Main Apparatus:*
  Dissecting microscopes, Nanoject II (Drummond Scientific Company)

- *Materials and Reagents:*
  Double-stranded RNA of the targeted genes (GFP and NPM), glass capillary tubes

- *Procedure:*
  Anaesthetized mosquitoes are placed onto a CO_2 pad. Next, a needle is created from a melting point pulled glass capillary tube by breaking the very tip of the glass using a pair of forceps. Next, the needle is filled with the desired inoculum: GFP or NPM dsRNA; using the Nanoject II (Drummond). An individual mosquito is impaled with the needle and the foot-pedal is used to administer the desired output volume of inoculums (69nl). Then, the mosquito is pulled off the needle by a gentle push using a paintbrush. This step is followed until all mosquitoes are injected. At the end, the mosquitoes are gently placed into netted cups and left for incubation according to the standard methods (see 5.1.1).

- *General mosquito handling procedures:*
  Live mosquitoes should be contained in netted plastic or paper pots. Mosquitoes may only be removed from the pots after anaesthesia. For CO_2 anaesthesia, the CO_2-pad (5 mbar) is placed over the netted pot for 2 min or until mosquitoes are absolutely still. Net is removed

from pots and mosquitoes are placed onto CO_2-pad, allowing CO_2 flow to continue throughout the procedure. Maximum recommended time of CO_2 exposure is 15 min. For ice anaesthesia, the netted pot is placed on ice for 5 min or until mosquitoes are absolutely still. Net is removed from pots and mosquitoes are placed onto icecold glass Petri dish, dissection glass or plate held on the ice.

- Safety notes and Warnings
  Injury from forceps, dissection needles and scissors must be avoided by storing them in capped in appropriate containers.
  Protective clothing: Designated lab coats, and gloves must be worn when carrying out these procedures. Protective clothing must be removed before leaving the insectary.

5.1.3 Mosquito Blood feeding - Infections

This protocol describes the Standard Operating Procedure "Blood feeding of adult *Anopheles* on mice infected with Plasmodium", recorded in the Laboratory of Immunogenomics, Imperial College London (Protocol Number: *LIM 0021*, generated by: *Tibebu Habtewold*, approved by: *George K. Christophides*, 17/10/2008):

- *Scope and field of application:*
  Infection of female *Anopheles* mosquitoes with rodent malaria parasites, *Plasmodium*
- *Principle:*
  Rodent malaria parasites are safe, versatile and convenient model organisms to study transmission of malaria in human malaria vectors mosquitoes. The laboratory mouse is a very convenient rodent host for rearing these parasites. Blood feeding of mosquitoes in infected mice ensures natural infection.
- *Sample:*
  On one day post-emerging, uninfected adult females (*A. gambiae Ngousso* strain); *Mus musculus* mice strain *TO*, infected with *Plasmodium Berghei Pb*GFPcon parasite line (*Gfp* gene is knocked-in along with the constitutive *eef1-α* promoter, Franke-Fayard et al., 2004)

- *Main Apparatus:*
  N/A

- *Materials and Reagents:*
  Anaesthetic (Rampun:Ketaset:PBS 1:2:3); 1mL syringes and 20G 1½ needle (yellow); 5% dextrose solution
- *Procedure:*
  Fructose pads are removed from cages 0-3 hrs prior to blood feeding. Mice with 4-7% parasitemia are injected with the anaesthetic IP or IM as instructed (0.25 ml per 10g body weight) and put in plastic boxes until fully sedated. 1-3 mice are placed directly on the net that covers the mosquito cage, and mosquitoes are allowed to feed on them for 15-20 min. After feeding is completed, mice are subjected to euthanasia by cervical dislocation while still sedated. If blood needs to be collected, sedated mice (before culling) are subjected to heard puncture using needle and syringe. Last, fructose pads are returned to the mosquito netted buckets, and the buckets are placed in a 19^{o}C-temperature incubator, with 70% relative humidity, in a 12-hour light/dark cycle.

- *Important mice handling procedures - Safety notes and Warnings:*
  In addition to the project license held by the PI, a personal license is needed before carrying out these procedures. Ketaset can be extremely hazardous. It must not be swallowed or injected.
  Protective clothing: Designated lab coats, FFP2 face mask or respirator, mop caps and gloves must be worn when carrying out these procedures.
  The current procedure was performed by licensed and highly trained personnel (*Sala Katarzyna* and *Habtewold Tibebu).*

5.1.4 Separation of Blood and Non-blood fed mosquitoes

One to two days post-infection, mosquitoes that have taken an infectious blood meal must be separated from the non-fed ones. This could be easily accomplished by following the ensuing steps:

I) Anaesthetized mosquitoes are gently placed onto an ice-cold glass Petri dish.
II) Then, with the use of forceps and under a light source, the blood-fed mosquitoes are separated from the non-fed mosquitoes. Mosquitoes can be easily identified from their abdomen: blood-fed have red-coloured abdomens (sign of a blood meal); non-fed don't.
III) Mosquitoes that had taken a blood meal are returned to their netted buckets, back to the same incubation conditions. Ten to fifteen non-fed mosquitoes are being stored for Q-PCR analysis, and the rest are killed.

- *Notes:*
  In order to distinguish the blood meal in mosquitoes' abdomens before digested, it is suggested to follow this procedure 24h-48h (maximum) after infections. For CO_2 anaesthesia, CO_2-pad (5 mbar) is placed over netted pot for 2 min or until mosquitoes are absolutely still. The net is removed from pots and the mosquitoes are placed onto CO_2-pad, allowing CO_2 flow to continue throughout the procedure. Maximum recommended time of CO_2 exposure is 15 min. For ice anesthesia, the netted pot is placed on ice for 5 min or until mosquitoes are absolutely still.
  Moreover, extra attention should be given on this operation, as at this point the parasite will start to form oocysts. Possible mosquito maltreatment will probably cause oocyst ruptures and degeneration.

5.1.5 Mosquito midgut Dissections

The current protocol describes the Standard Operating Procedure "Dissection of *Anopheles* tissues", recorded in the Laboratory of Immunogenomics, Imperial College London (Protocol Number: *LIM 0024*, generated by: *Fanny Turlure*, approved by: *George K. Christophides*, 17/10/2008):

- *Scope and field of application:*
  Midgut dissections from blood-fed/infected mosquitoes to be used for molecular and cell biological experiments
- *Principle:*
  To study the biology of mosquitoes and their responses to microorganisms including the malaria parasites it is required that midguts are dissected and examined in isolation.

- *Sample:*
  Infected with Plasmodium adult females (*A. gambiae Ngousso* strain)

- *Main Apparatus:*
  Dissecting microscopes, operating forceps
- *Materials and Reagents:*
  1X phosphate-buffered saline solution (PBS); 4% PFA solution (v/v) (2mL 16% formaldehyde solution, 5,2mL dH_2O, 0,8mL 10X PBS); Vectashield® (VectorLabs); dissecting forceps, CO2 main supply
- *Procedure:*
  Place anesthetized mosquito in a drop of phosphate buffered saline (PBS). Midgut dissection: the lower abdominal part is grasped with one forceps or dissection needle and the lower part of the thorax/upper abdomen with another forceps, the midgut is gently teased out and separated from malpighian tubules and foregut. The procedure is repeated for the required number of mosquitoes. Directly after extraction, the midguts are placed and stored in 1X PBS solution. Light exposure must be avoided. At the end, the midguts are fixed in microscope glass slides, according to the following fixing procedure:
  -Midguts are placed in 1X PBS solution
  -Then, midguts are transferred in 4% PFA solution for 45min (fixation step)
  -Next, they are transferred in 1X PBS solution for 15min (1^{st} wash)
  -The previous step is repeated again (2^{nd} wash)
  The midguts are mounted in Vectashield® (VectorLabs) on a microscope slide under sealed cover-slips. Slides are stored in the dark at 4ºC until processing.

- *General mosquito handling procedures:*
  Live mosquitoes should be contained in netted plastic or paper pots. Mosquitoes may only be removed from the pots after anesthesia. For CO_2 anesthesia, CO_2-pad (5 mbar) is placed over netted pot for 2 min or until mosquitoes are absolutely still. The net is removed from pots and the mosquitoes are placed onto CO_2-pad, allowing CO_2 flow to continue throughout the procedure. Maximum recommended time of CO_2 exposure is 15 min. For ice anesthesia, the netted pot is placed on ice for 5 min or until mosquitoes are absolutely still. Net is removed from pots, mosquitoes are placed onto ice-cold glass Petri dish, dissection glass or plate held on the ice.

- *Safety Notes and Warnings*
  Formaldehyde is a severely hazardous mutagen and thus should be treated according to the respective safety rules.
  Injury from forceps, dissection needles and scissors must be avoided by storing them in capped in appropriate containers.
  Protective clothing: Designated lab coats, and gloves must be worn when carrying out these procedures.
  Protective clothing must be removed before leaving the insectary.

5.1.6 Microscopy

Microscope slides with fixed midguts stored after dissections are used for oocysts enumeration via light and fluorescent microscopy. This process is implemented with the Zeiss AxioImager M2 (Carl Zeiss, Inc) microscope in conjunction with Zeiss AxioCam HRc camera coupled with Zeiss Axiovision40, 4.6.1.0 version, software (Carl Zeiss, Inc) as described in Bushell ES et al., *Plos Pathogens* 2009. Images were captured at 100X and 200X final magnifications both in bright field and under ultraviolet (UV) light.

5.2 Nucleic acids manipulation

5.2.1 RNA extraction

The protocol used for this experiment is based on the research of Chomczynski P, Sacchi N. 1987 and reviewed by the authors again in 2006. The ensuing steps describe the main protocol for mosquito total RNA extraction:

1. 10-20 CO_2-anesthetized adult mosquitos (1-2 days old) or separated non-fed mosquitoes (see 5.1.4) are placed into an RNAase free eppendorf tube along with 200 μl TRIzol® (Invitrogen) (*Hummond et. Al, BioTechniques 2007).*
2. The solution is homogenized and 400 μl of TRIzol® are also added
3. Then, the solution is centrifuged for 10 min at 12 Krpm in 4^{o}C, followed by the transfer of the clear supernatant into a fresh eppendorf tube and incubation at Room Temperature (15-30^{o}C) for 5 minutes.
4. Next, 120 μl $CHCl_3$ are added and the mix is being shaken vigorously for a few seconds and incubated at Room Temperature for 5 minutes.
5. The solution, then, is centrifuged again for 20 min at 12 Krpm in 4^{o}C, followed by the transfer of the upper aqueous phase into a fresh eppendorf tube.
6. Afterwards, 350 μl isopropanol are inserted inside the solution. The ensuing steps include a gentle mix and 10-minute incubation at Room Temperature.
7. Then, the sample is centrifuged for 15 min at 12 Krpm in 4^{o}C.
8. Next, the supernatant is removed and the pellet is being washed with 600μl of 75% ethanol solution. The solution is mixed via vortex for a few seconds.
9. Finally, the sample is centrifuged for 5min at 8 Krpm in 4^{o}C followed by an air- or speed-vacuum dry of the pellet. The pellet is dissolved in 50μl RNAase free H_2O and the solution is incubated for 20min in 37^{o}C.

Notes:

☑ The final solution's quantity and quality should be evaluated before proceeding to the next steps (see 5.2.7).

☑ RNA samples and whole anaesthetized mosquitoes are stored in -80^{o}C.

5.2.2 In vitro cDNA synthesis

The current cDNA synthesis protocol describes the in vitro single-stranded RNA conversion into double-stranded DNA (cDNA), as demonstrated in "*SuperScript® III First-Strand Synthesis System for RT-PCR"* protocol, documented in *Invitrogen™ by life technologies™* manuscript with Part Number: *18080051.pps* and Publication Number: *MAN0001346, Rev. 3.0*:

The following 20-µl reaction volume can be used for 10 pg–5 µg of total RNA or 10 pg–500 ng of mRNA:

1. The following components are added to a nuclease-free microcentrifuge tube:
   -1 µl of oligo(dT)$_{20}$ (50 µM),
   -10 pg–5 µg total RNA
   -1 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH).
   -Sterile, distilled water to 13 µl.
2. Next, the mixture is heated to 65°C for 5 minutes and incubated on ice for at least 1 minute.
3. The contents are collected of the tube by brief centrifugation, followed by the addition of:
   -4 µl 5X First-Strand Buffer
   -1 µl 0.1 M DTT
   -1 µl RNaseOUT™ Recombinant RNase Inhibitor (Cat. no. 10777-019, 40 units/µl)
   Note: When using less than 50 ng of starting RNA, the addition of RNaseOUT™ is essential.
   -1 µl of SuperScript™ III RT (200 units/µl)*
4. Afterwards, a brief mixing by pipetting gently up and down is needed.
5. Then, the solution is incubated at 50°C for 30–60 minutes.
6. Finally, the reaction is inactivated by heating the tube at 70°C for 15 minutes.

The cDNA can now be used as a template for amplification in PCR.

Notes:

☑ *If generating cDNA longer than 5 kb at temperatures above 50°C using a gene-specific primer or oligo(dT)20, the amount of SuperScript™ III RT may be raised to 400 U (2 µl) to increase yield.

5.2.3 DNA purification

The protocol used for purification of the PCR product is achieved with the QIAquick PCR Purification kit (QIAGEN), as described in the kit's handbook[1]. Before proceeding to the main protocol, ethanol 100% must be added to Buffer PE. Also, all centrifugation steps are carried out at 10,000 g (≈13,000 rpm).

1. 5 volumes of Buffer PB (250 µl) are added to 1 volume (50 µl) of the PCR sample and followed by mix.

2. To bind the DNA, the sample is applied to a QIAquick column in a 2 ml collection tube and then is centrifuged for 30-60.

3. The flow-through is discarded and washed by adding 0.75 ml Buffer PE to the column. Centrifuge for 30-60 is ensuing.

4. Next, the flow-through is discarded again and the sample is being centrifuged for an additional 1 min at maximum speed.[2]

5. QIAquick column is placed in a clean 1.5 ml microcentrifuge tube.

6. For elution of the DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O are inserted to the center of the QIAquick membrane ensued by a centrifugation the column for 1 min. Alternatively, for increased DNA concentration, 30 µl of elution buffer are inserted to the center of the QIAquick membrane, the column is left to stand for 1 min, and then it is centrifuged.[3]

Notes:

1.http://devbio.wustl.edu/krolllab/Kroll_Lab_Protocols/Molecular%20Biology%20protocols/Cloning%20protocols%20folder/PCR%20purification-Qiagen.pdf

2. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

3. IMPORTANT: It must be clear that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, it must be clear that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

5.2.4 RNA purification

There are two steps for the purification of the dsRNA: the digestion of the DNA template and the clean-up of the sample with the RNeasy kit (QIAGEN)✧‡.

1. *Digestion of the DNA with DNAaseI:* 1 μl DNAase (Ambion kit) is added to the 20m μl reaction followed by a 15 min incubation at 37^{o}C.
2. 80μl RNAase-free H_2O is added to the sample in order to increase the volume to 100 μl. Next, follows an addition of 350 μl Buffer RLT and the solution is mixed thoroughly.
3. Then, 250 μl of 100% ethanol are inserted to the diluted RNA and the sample is mixed gently by pipetting. The next step must follow immediately.
4. The sample (700 μl) is transferred to an RNeasy mini column in a 2 ml collection tube. The sample is centrifuged for 15 sec at 8,000 g (≈10,000 rpm). The flow-through and the collection tube are discarded, and the column is transferred into a new 2 ml collection tube.
5. Next, 500 μl of Buffer RPE are pipetted onto the RNeasy column. The ensuing steps include a centrifugation for 15 sec at 8,000 g (≈10,000 rpm), in order to wash the column, and the flow-through is discarded.
6. The last step is repeated, but the centrifugation time is adjusted to 2 min, in order to dry the silica-gel membrane.
7. The column is placed in a fresh 2 ml collection tube and it is centrifuged at full speed for 1 min, in order to eliminate any chance of ethanol carry over.
8. For elution, the RNeasy column is placed in a new 1.5 ml RNeasy free collection tube.
9. Then, 30 μl RNase-free water is added directly onto the silica-gel membrane of the column and the sample is centrifuged for 1 min at 8,000 g (≈10,000 rpm).
10. The last step should be repeated (Final volume: 60 μl) ¶
11. If not used immediately, the sample should be stored at -80^{o}C

Notes:

✧ The protocol described above is an abridgment of the QIAGEN's handbook "RNeasy® Mini Handbook, Fourth edition, September 2010" (http://www.genome.duke.edu/cores/microarray/services/rna/qc/documents/RNeasy_Mini_Handbook.pdf)

‡ The addition of β-Mercaptoethanol is not necessary and should be avoided. (β-Mercaptoethanol (β-ME) must be added freshly to the Buffer RLT before use. 10 μl β-ME are added per 1 ml Buffer RLT (only 350 μl of the Buffer RLT per reaction are necessary). Also, it should be clear that 4 volumes of ethanol have been added to 1 volume of Buffer RPE.

¶ After finalizing the purification, there should be a quantity and quality control of the product, as these are described in chapters 5.2.8, 5.2.9.

5.2.5 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a biochemical technique in molecular biology that amplifies a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. (Saiki R.K. et al., *Science* 1988)
The primers designed for PCR should enhance a 200-600 bp section of the gene of interest. The targeted region should not be located in high homology regions, and preferably located as close to the 3' prime end of the gene as possible. Both primers need a T7 promoter sequence tail at their 5' end. Standard purification (desalt) of the primers is acceptable.
The targeted fragment is amplified by PCR using the following concentrations:

Reagents	**Quantity**
10x PCR Buffer	5 µl
Taq polymerase	0.7 µl
dNTP mix (10 mM)	1 µl
DNA template (0.5 µg/µl)	1 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
ddH_2O	40.3 µl
Total	**50 µl**

The PCR cycle program includes:

Stages	**Temperature**	**Time**	
Initiation:	95 ^oC	5 min	
Denaturation:	95 ^oC	30 sec	x40
Annealing:	58 ^oC	30 sec	x40
Elongation:	72 ^oC	1 min	x40
Termination:	72 ^oC	5 min	
Store:	4 ^oC	forever	

Notes

☑ cDNA should be used as PCR template, but genomic DNA (1µg/µl) is acceptable if the targeted region does not contain an intron and the gene structure is suitable.

☑ Primers used:
Forward primer: 5'-taatacgactcactatagggCGGGGGATTATGACACTAGC-3'
Reverse primer: 5'-taatacgactcactatagggGTCTCCGTGTCTGCACCAG-3'
(small letters represent the T7 sequence)

5.2.6 Double-stranded RNA (dsRNA) synthesis

The ensuing steps synopsize the double-stranded RNA synthesis procedure with the MEGAscript® T7 kit (*Ambion* by *life technologies™*), as it is described in the company's user guide "MEGAscript® Kit, Publication number 1330M, Revision G" (http://tools.invitrogen.com/content/sfs/manuals/1330M_G.pdf).

1. The reagents are added into an RNAase free tube in the following order:

Reagents	**Quantity**
DEPC treated H_20	4 μl
ATP	2 μl
CTP	2 μl
GTP	2 μl
UTP	2 μl
Buffer (warm to 37^{o}C)	2 μl
Cleaned PCR product	4 μl
Enzyme mix	2 μl
Total	**20 μl**

2. The mixture is gently stirred and spun down.
3. Next, the sample is incubated at 37^{o}C for 12-16 hours (preferably with constant shaking). Incubation times may vary; the viscosity of the sample will increase with time.
4. The process can be interrupted and continued at a later time (in the meantime the sample needs to be freezed at -80^{o}C).
5. Once incubation finishes, the sample proceeds for quality control in order to evaluate the product size, quality and quantity. It is crucial that the final solution contains only the desired product in concentration equal to 3mg/μl.
6. The dsRNA can be stored in -80^{o}C for a long time before use.

5.2.7 Quantitative Real-Time PCR (Q-PCR)

Accurate quantification of starting amounts of DNA, cDNA, and RNA targets can be accomplished via Real-Time PCR (Q-PCR). Gene specific primers* and the SYBR-Green detection and amplification reagent (Applied Biosystems) were used along with ABI PRISM® 7000 Sequence Detector System (Applied Biosystems), according to the manufacturer's guidance (http://www2.udel.edu/ctcr/sites/udel.edu.ctcr/files/ABI%20Prism%207000%20Sequence%20Detection%20System%20User%20Guide.pdf). The experimental strategy is the exact one, as previously described (Vlachou et al., *Current Biology* 2005), and was performed by specialized in qRT-PCR performance and analysis personnel (Stathopoulos Stavros, PhD student).
Compendiously, total RNA extracted from mosquitoes in both KD and control groups converted into cDNA and used as a template in order to quantify the mRNA levels of the targeted transcripts. A 100 bp fragment discrete from the KD targeted region was enhanced and quantified based on both a standard curve created by the use of solutions with specific concentration (absolute quantification) and the expression of S7 gene, a housekeeping gene which is consistently expressed in adult mosquitoes (relative quantification). (Dimopoulos et al., *PNAS* 2000) The final knock down efficiency calculation results from the integration of all the

methods above (first absolute quantification, then relative quantification and finally average of the values occurred from the three different biological replicates).

* Forward primer: 5'- GCAGCGGCCAGAGTTCGGAG -3'
  Reverse primer: 5'- GCCCAGCTGTCGCGTGCTAT -3'
  amplicon length: 137bp

5.2.8 Agarose Gel Electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. Here, was used for quality control of the nucleic acids (both DNA and dsRNA) produced from the protocols above (J. Sambrook, *Cold Spring Harbour Laboratory Press* 2001).

To create 1% agarose gel, the following reagents are necessary:

I. TBE buffer solution*:H_2O in 1:9 proportion
II. 1X (final concentration) SYBR safe Gel Stain (Invitrogen^{TM}), for visualization
III. 1kb Plus DNA ladder (Invitrogen^{TM})
IV. 5X GelPilot DNA Loading Dye, (Qiagen), for loading and tracking of DNA

*For TBE preparation: 10.8 g Tris and 5.5 g Boric acid are dissolved in 900 ml distilled water, adding 4 ml 0.5 M Na_2EDTA (pH 8.0). The volume is adjusted to V_F= 1liter and then is stored at room temperature.

5.2.9 Nucleic Acid Quantification and Quality Assessment - Spectrophotometry

Nucleic acid samples can be readily checked for concentration and quality using the NanoDrop® ND-1000 Spectrophotometer (Nanodrop technologies Inc., V3.5 User's Manual) along with its respective software.

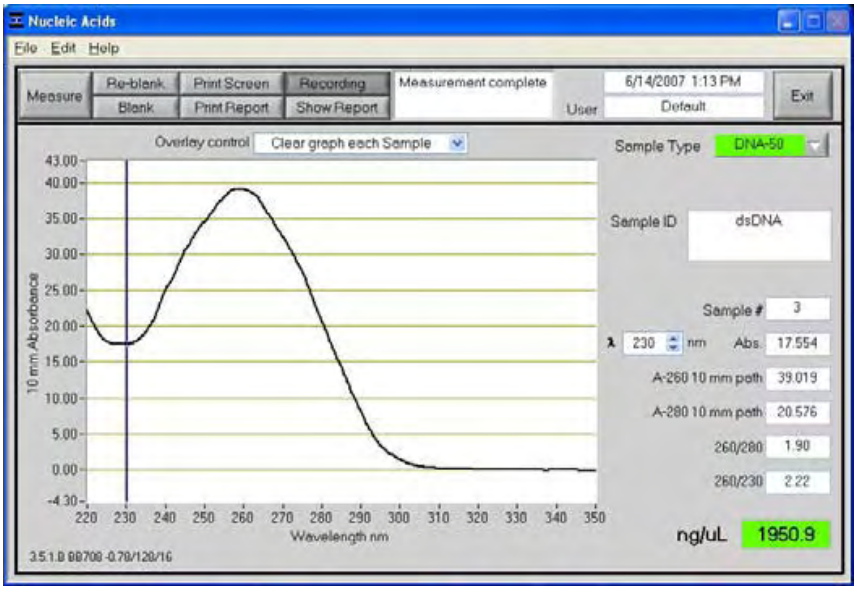


Figure 5.2.1 DNA quality and quantity control via NanoDrop® ND-1000 Spectrophotometer

Method
In the begging, the suitable software settings must be selected (Nucleic Acids -> 'DNA-50' for dsDNA and dsRNA, 'RNA-40' for RNA, 'ssDNA-33' for single-stranded DNA, or 'Other' for other nucleic acids. The default is DNA-50.
Next, in order to initiate measurements, 1 μl of H_2O is placed in the suitable position of the "case" followed by selecting the BLANK button. This step is essential for adjusting the measurements. Similarly, 1 μl of H_2O is placed again in the correct place and the measurement begins by clicking the MEASURE button.

Output
Based on the measurement, the software calculates the following parameters:

Sample concentration: sample concentration is calculated in ng/ul based on absorbance at 260 nm and the selected analysis constant. The NanoDrop® ND-1000 Spectrophotometer will accurately measure dsDNA samples up to 3700 ng/ul without dilution. The instrument automatically detects the high concentration and utilizes the 0.2mm pathlength to calculate the absorbance.

260/280 ratio: ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to evaluate the purity of DNA and RNA. A ratio of ≈1.8 is generally accepted as "pure" for DNA; a ratio of ≈2.0 is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

260/230 ratio: ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

5.3 In silico analysis and online data extraction

In order to extract online information, analyze data, apply statistical tests and construct graphs, the ensuing software was used:

- ***VectorBase***, Bioinformatics Resource for Invertebrate Vectors of Human Pathogens, release VB-2013-02 - February 2013© (www.vectorbase.org)

  VectorBase is a Bioinformatics Resource Center (BRC) which includes a powerful database of genomic, transcriptomic, proteomic etc. data about invertebrate vectors of human diseases.

  In the present study, it was used to extract information about the under examination gene (NPM). The data mined include the total gene sequence and location in the genome, cDNA sequence (ORF sequence), exon sequences, and the protein sequence. Moreover, further annotation details such as Gene Ontology (GO) function, functional domain features (InterPro domains and descriptions), external (already published) data, and genetic variation/population comparison information upon *Anophelines* and neighbor genera.

- ***E-RNAi, design of RNAi constructs, 3.2 online version,*** German Cancer Research Center (*dkfz.*), a web application for the multi-species design of RNAi reagents, 2010 update (Thomas Horn*, Michael Boutros, Nucleic Acids Res* May 2010*)*

  E-RNAi is an online tool for the design and evaluation of RNAi reagents for a variety of species. It can be used to design and evaluate long dsRNAs (including esiRNAs) as well as siRNAs.
  In the current experimental approach it was used in order to design T7 primers for long dsRNA construction for the targeted gene, following the webpage manufacturer's instructions.

- ***Geneious 5.6*** *(*Biomatters Ltd)

  Geneious 5.6 (latest release, Geneious 6.1) is a powerful software tool that allows basic in silico analysis on DNA, RNA and protein sequences, such as sequence manipulation, alignment, primers design etc.
  For the current study, it was used in order to design the suitable primers needed for Q-PCR amplification of the targeted transcribed locus (…), as described on the company's official Manual
  (http://www.geneious.com/assets/documentation/geneious/GeneiousManual.pdf).

- ***GraphPad Prism 5*** (© 2013 GraphPad Software, Inc)

  GraphPad Prism, available for both Windows and Mac Operating Systems, combines scientific graphing, comprehensive curve fitting (nonlinear regression), understandable statistics, and data organization.
  The usage of this software provided statistical analyses of the resulted data and scientific graphing visualization. Therefore, the following tools were used, according to the manufacturer's manual set:
  (*FAQ#1759,* http://www.graphpad.com/support/faqid/1759/)

  I. Mann-Whitney test (U-test) and scatter plot graphing of the oocysts distribution (KD versus Control)
  II. Linear graphs for identification of developmental trends
  III. Pearson correlation test combined with best linear distribution fit, with 95% freedom interval, scatter plot for mosquito and parasite regulated transcripts between *Gambiae* and *Arabiensis* datasets.

5.4 Microarray analysis

Data resulted from gene expression microarray samples were analyzed and surveyed by the use of the ensuing tools:

∇ ***GeneSpring GX*** version 12.5 ©Agilent Technologies Inc. 2012, (last revision October 2012),

GeneSpring GX software provides powerful, accessible statistical tools for fast visualization and analysis of transcriptomics, genomics, proteomics and metabolomics data. Additionally, GeneSpring GX offers an interactive desktop computing environment that promotes investigation and enables understanding of microarray data within a biological context. (http://genespring-support.com/files/12_5/GeneSpringNGSforSureSelect-Userguide.pdf)
It was utilized in this study for Data Normalization: Raw, unfiltered data extracted from microarray scanning analysis were subjected to normalization via Locally Weighted Linear Regression (Lowess) method. Due to the disproportion between the mosquito midgut and parasite transcriptome load, mosquito transcripts were normalized with 50.0 threshold and parasite transcripts with 10.0. (Quackenbush J., *Nat. Genet.* 2002).

∇ ***Microsoft Excel*** *(*Microsoft Office Package 2007):

Microsoft Excel is a popular and handful tool for simple and complicated data process, analysis and graphing. It provides an appropriate environment for microarray analysis and data mining.
The normalized microarray data were extracted in Microsoft Excel files in order to perform manual analysis. The filters applied to the values qualified transcripts that:

- Exhibit expression in at least 66,6% of all the biological replicates (>=66,6%)

- Display Coefficient of Variation value equal or lower that 50% (CV=<50% or Average>=2xSD)

- Manifest an expression higher that 0.7, in $\log_2$ scale, upfolds or downfolds, indicating the significantly upregulated and downregulated transcripts, respectively.

In addition, further analysis and graphing of the valid (filtered) data, such as bar graphs, was accomplished using Microsoft Excel platforms.

∇ ***GeneCluster 3.0*** (Open Source Clustering Software, Human Genome Center, Institute of Medical Science, University of Tokyo)

GeneCluster 3.0 was originally developed by Michael Eisen (Stanford University) and can be used to analyze gene expression data. Routines for hierarchical (pairwise simple, complete, average, and centroid linkage) clustering, *k*-means and *k*-medians clustering, and 2D self-organizing maps on a rectangular grid are included. (de Hoon M.J. et al., *Bioinformatics* 2004).
Significantly regulated mosquito and parasite transcripts resulted from the analysis were subjected in *k*-means clustering via “euclidian distance” similarity metric method. In addition, parasite transcripts with significantly differential regulation upon timepoints (>0.7

in log_2 scale) were organized in four distinct groups, based on their developmental pattern with Self-Organizing Map clustering, via "euclidian distance" similarity metric method (*initial tau value: 0.02*).

∇ ***Java Treeview 1.1.6.*** Open Source Software (© Free Software Foundation, 1991)

Java Treeview renders gene expression data into several interactive views and it can. The main treeview application considers these views interchangeable, and it is possible to define additional views. Moreover, it can be farther utilized for scientific visualization of microarray data, often analyzed with clustering methods. (Saldanha A.J., *Bioinformatics* 2004)

∇ ***GraphPad Prism 5*** (© 2013 GraphPad Software, Inc)

(Already mentioned at "*5.3, GraphPad Prism 5, II, III"*)

∇ ***Vectorbase*** Bioinformatics Resource for Invertebrate Vectors of Human Pathogens, release VB-2013-02 - February 2013© (www.vectorbase.org) **– *PlasmoDB***, Plasmodium Genomics Resources, Version 9.3, © The EuroPathDB Project Team (*www.plasmodb.org*)

Vectorbase (as previously mentioned) and *PlasmoDB* are databases hosting integrated genomic, transcriptomic and proteomic information about *Anopheles* and *Plasmodium* genus organisms, respectively. The whole transcriptomes of *Anopheles gambiae*, *Anopheles arabiensis* and *Plasmodium falciparum* in conjunction with further annotation details, such as GO Functions and InterPro Domains and Description, were extracted for the requirements of this survey. At this stage, it is significant to consort Oligo IDs (IDs that determine the probe in the array spots) with their respective Transcript IDs (ID names registered in transcriptomic databases).

6 Results

6.1 Experimental Design – General Overview

The current survey aims at the assessment of the impact made by the human malaria parasite, *P. falciparum,* upon infections of the two major malaria vectors, *An. gambiae* and *An. Arabiensis*. The general strategy is divided in two main parts: i) Genome Wide Transcriptional Profiling (High throughput approach); and ii) Functional Characterization (Low throughput approach). (Figure 6.1)

The first step designates the basic strategic line which includes the analysis of transcriptomic microarray assays conducted in Burkina Faso in 2008. Accordingly, after hybridizations and scanning, the expression data of both mosquitoes and parasite genes are analyzed separately, in order to investigate different hypotheses. Gene expression profiles from different mosquito species are studied both comparatively and individually in order to explore conserved and species-specific responses upon infection with sympatric parasite populations, respectively. Likewise, gene expression profile from the same parasite is assessed for identification of differences and similarities upon infection with different mosquito species. Ultimately, the integration of the information above could illuminate the biological interplay inside the variable mosquito-parasite transmission system, revealing key molecular interactions between the two organisms.

The second perspective includes the utilization of the data resulted from the analysis above, in order to identify novel genes that are implicated in conserved mosquito responses during parasite Plasmodium infections. This was achieved inducting dsRNA mediated RNAi, a Reverse Genetics approach.

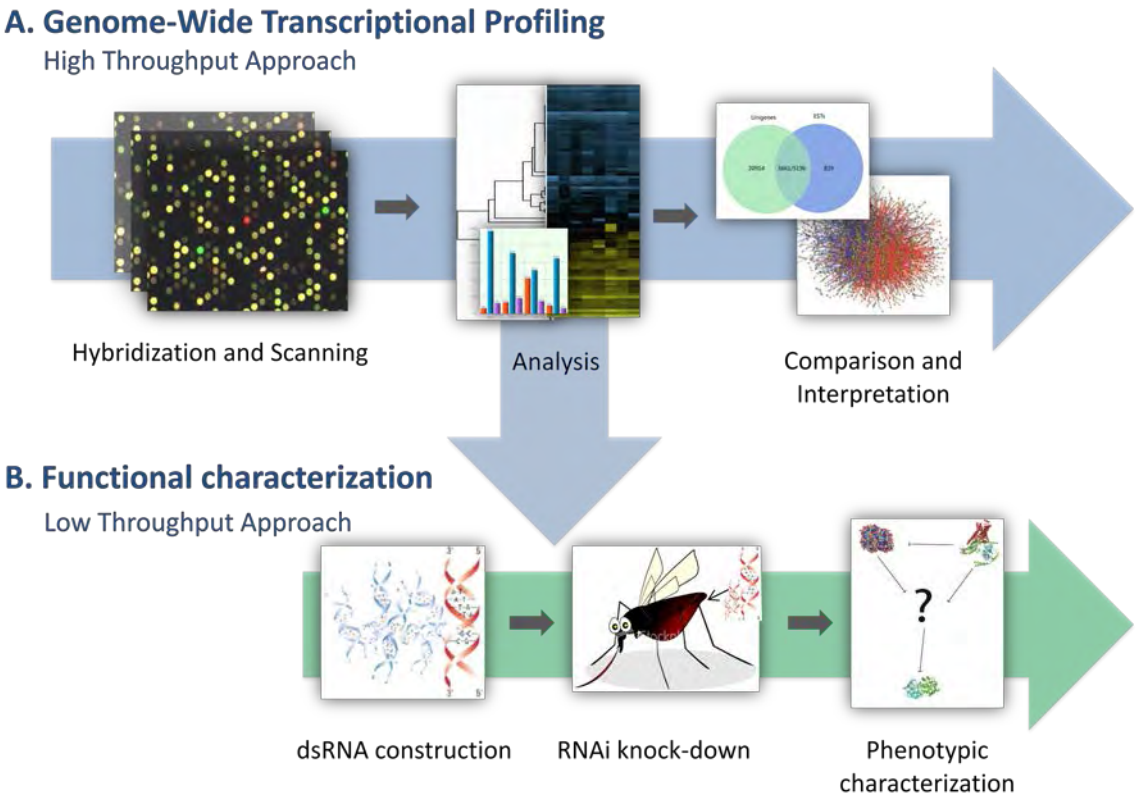


Figure 6.1.1 Project strategy- General overview: A. Genome-Wide Transcriptional Profiling (High Throughput approach) of the bipartite biological systems in order to reach a general interpretation, and B. Functional characterization (Low throughput approach) via Reverse Genetics with the view of revealing novel mosquito regulators during parasite infections.

6.2 Genome-Wide Transcriptional Profiling (High throughput approach)

The microarray assay implemented in the current survey initiated in 2009 by D. Vlachou's Research Team (Imperial College London). The first experimental steps included parasitological surveys for gametocyte carriers conducted in collaboration with local medical groups in primary schools in Bobo Dioulasso, Burkina Faso, where malaria is endemic. The collected blood samples that contained only one *Plasmodium* species (and not a mix of species) subjected to serum replacement in order to eliminate transmission blocking factors included in the blood plasma. Then, the samples were divided in two experimental groups: the infection group, in which mosquitoes were infected via membrane feeding with infectious parasites, and the control group, in which mosquitoes were infected with heat-inactivated (non-infectious) gametocytes. Infected mosquitoes from both groups were isolated in four distinct timepoints, and their midguts were dissected for total RNA extraction. The four timepoints correspond to the sexual developmental stages that *Plasmodium* undergoes in the mosquito midgut lumen: gametogenesis and zygote formation (T1); zygote maturation and early motility (T2); ookinete locomotion (T3); midgut invasion and ookinete-to-oocyst transition (T4). The previous procedure was accomplished two more times, so to result with three independent successful infections, representing the three independent biological replicates. During sample selections, it is significant to assess the gametocytemia in the blood, and infection prevalence and intensity 10 days after infections, in order to reduce the biological variance. The current experimental assay was implemented for both *An. gambiae* and *An. Arabiensis* infections with sympatric populations of *P. falciparum*.

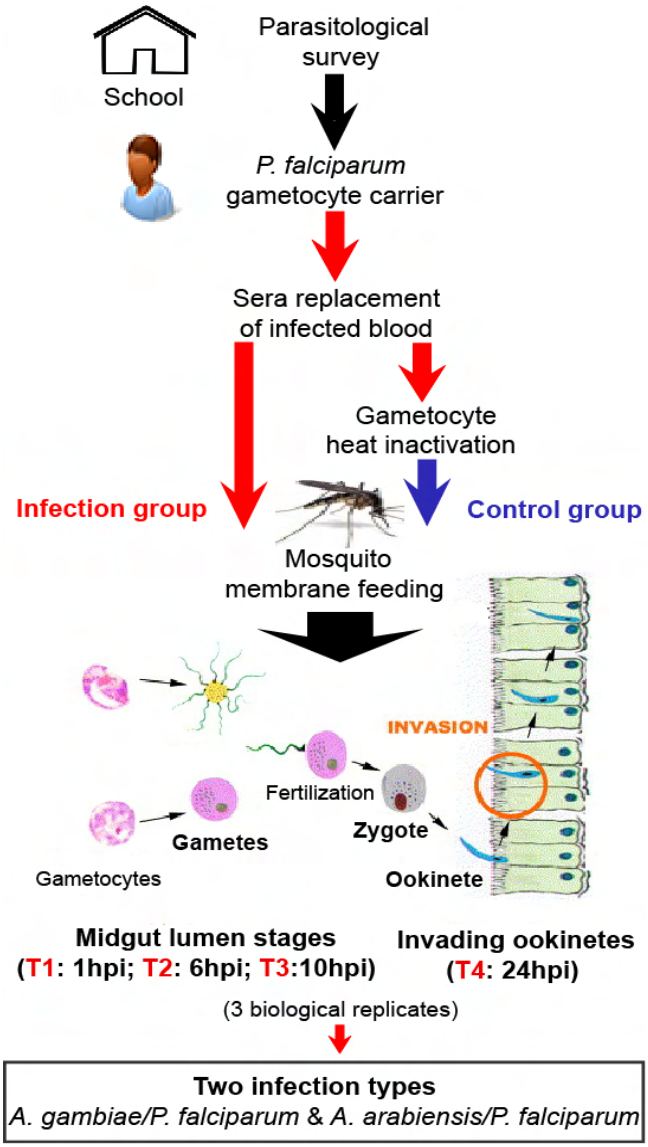


Figure 6.2.1 Schematic illustration of Microarray experimental design. *P. falciparum* gametocytes were isolated from infected carriers through parasitological survey. After blood serum replacement, two different experimental groups occurred: naïve mosquitoes were infected with sympatric infectious parasites (Infection group) and heat-inactivated parasites (Control group). Mosquito midguts were collected in four distinct timepoints (1h, 6h, 10h and 24 post-infection), representing the *Plasmodium* developmental landmarks in the midgut lumen stages.

In the ensuing phase of this research, the global transcriptional profiles of both the vector and the parasite were monitored simultaneously using DNA microarrays. In brief, this microarray platform included 44,000 oligonucleotide probes representing almost all the genes in the genomes of the two organisms

plus negative and positive control probes that allowed noise subtraction and intensity normalization.

Thus, total RNA extracted from the mosquito midguts was converted to cDNA and then used as a template for in vitro translation - cRNA synthesis. During this procedure, the total cDNA was translated to fluorescent RNA (cRNA), where Cytosines were labeled with a fluorescent dye. The two different biological groups (midguts with infectious parasites and midguts with inactivated parasites) were labeled with two different dyes, cy3 (red) and cy5 (green), respectively. (Figure 6.2.2) The following steps were comprised of hybridizations of the samples to the microarray slides and scanning.

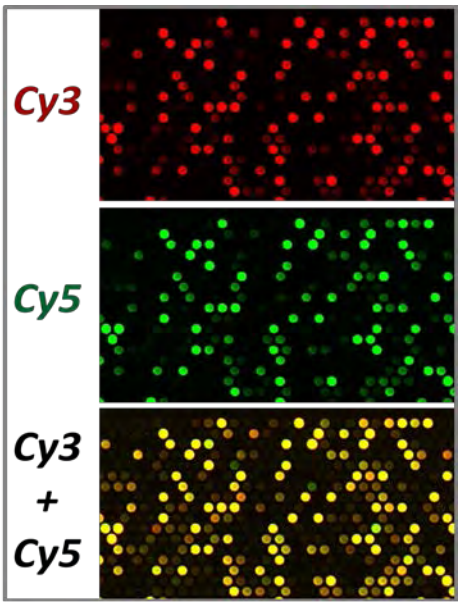


Figure 6.2.2 Images resulted from microarray slides scanning. The three images illustrate the fluorescence of the spots under cy3-stimulating laser beam, cy5-stimulating laser beam and a merged condition. In the last image, spots biased to red indicate upregulated expression and spots biased to green indicate downregulated expression.

The principal of this method is actually based on the competition between the labeled transcripts for hybridization with the spotted probe. Accordingly, the more abundant transcript between the two biological groups will dominate in the spot, producing higher signal intensity. However, this microarray technology does not aim at the absolute quantification of the transcripts, but provides a comparative perspective between two different conditions. Thus, the output signal intensity ratio of the two dyes in each spot represents the respective ratio of abundance of each transcript in the biological condition. The adjacent figure (Figure 6.2.2) illustrates the images resulted from the scanning. The first two images represent the fluorescence of the two dyes independently. The last image is a merged view of the slide and subsequently illustrates the comparative gene expression of the two transcriptomes. Spots that are biased to the red colour (cy3) imply an upregulated expression of the transcript upon infection, whilst spots biased to the green colour (cy5) imply a downregulated expression of the transcript upon infection. Yellow coloured spots indicate a consistent gene expression between infection and control groups; spots without signal intensity indicate no expression (e.g. transcripts that are not produced in the current conditions or developmental stages).

Consequently, the microarray scanning resulted in a massive set of data included raw signal intensities of the investigating samples. The figure below (Figure 6.2.3) depicts the final datasets obtained from the scanning process. As can be distinguished, there are two datasets, Gambiae and Arabiensis, implying the respective infection types hybridized (Gambiae dataset: *An. gambiae/P. falciparum* infections; Arabiensis dataset: *An. arabiensis/P. falciparum* infections). Each dataset is comprised of four-timepoint samples, every one of which includes three biological replicates. Due to some issues during this experimental process, all three T1 timepoint (1h) replicates from Arabiensis dataset and one T2 timepoint (6h) replicate from Gambiae dataset were excluded. Moreover, in every array set there are involved two spotted probes complementary to 5,557 parasite transcripts and two to 14,329 mosquito transcripts; some arrays controls are also spotted on the slides, as they are important for the analysis.

Therefore, the output raw signal intensity data are subjected to computational analysis, in order to extract the desirable information. The first step of analysis includes data normalization, using algorithms implemented via GeneSpring GX software. Normalization is a fundamental process before initiating the main analysis, as it adjusts the data values and provides noise reduction. After normalization, signal intensity ratios (converted to $\log_2$ scale) undergo manual analysis, by the use of Microsoft Excel software (in this step, array controls are excluded). Thereupon, parasite and mosquito gene expression data are questioned separately, and the two datasets are analyzed individually and comparatively.

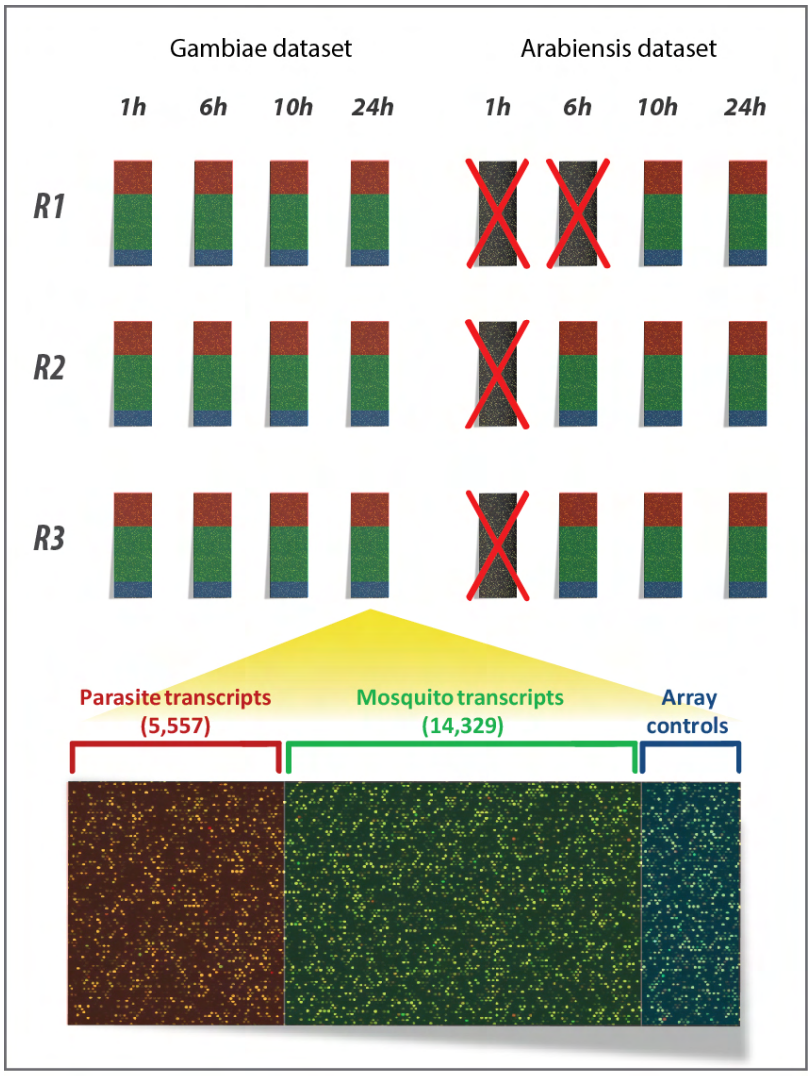


Figure 6.2.3 Overview of the microarray assay that illustrates the experimental arrangement of the samples. There are two major datasets, Gambiae and Arabiensis, representing *An. gambiae/P. falciparum* and *An. arabiensis/P. falciparum* infections, respectively, arranged in four timepoints and three biological replicates. Each array set contains 5,557 parasite transcripts, 14,329 mosquito transcripts and array controls.

Accordingly, the first steps include the detection of the regulated expression profile in *An. gambiae* and *An. Arabiensis* mosquitoes upon infection with the same sympatric *P. falciparum* parasite. In Figure 6.2.4, regulated transcripts in both species are illustrated via Eisengram (heatmap) visualization and Venn diagrams. Heatmaps (resulted from k-means clustering analysis) assist in visualization of regulation patterns per each mosquito and Venn diagrams in comprehension of how is the regulation distributed among timepoints (yellow colour implies upregulation and blue colour downregulation; the Venn circles' size is proportional to the number of transcripts included). In overview, upregulated transcripts (>0.7 in $\log_2$ scale or >1.5 folds) in both mosquitoes are more abundant than the downregulated genes. Interestingly, the majority of upregulated transcripts are distributed in T1 (1h pi) and T4 (24h pi) for the *An. gambiae,* and in T4 for the *An. Arabiensis*. However, *An. arabiensis* exhibits a regulation pattern (in upregulated genes) that suggests a transcriptional tuning, in accordance with the time of exposure to the infection, implying that the response is increased proportionally with time. On the other hand, *An. gambiae* exhibits a more complicated expression pattern that does not consort with the previous implication.

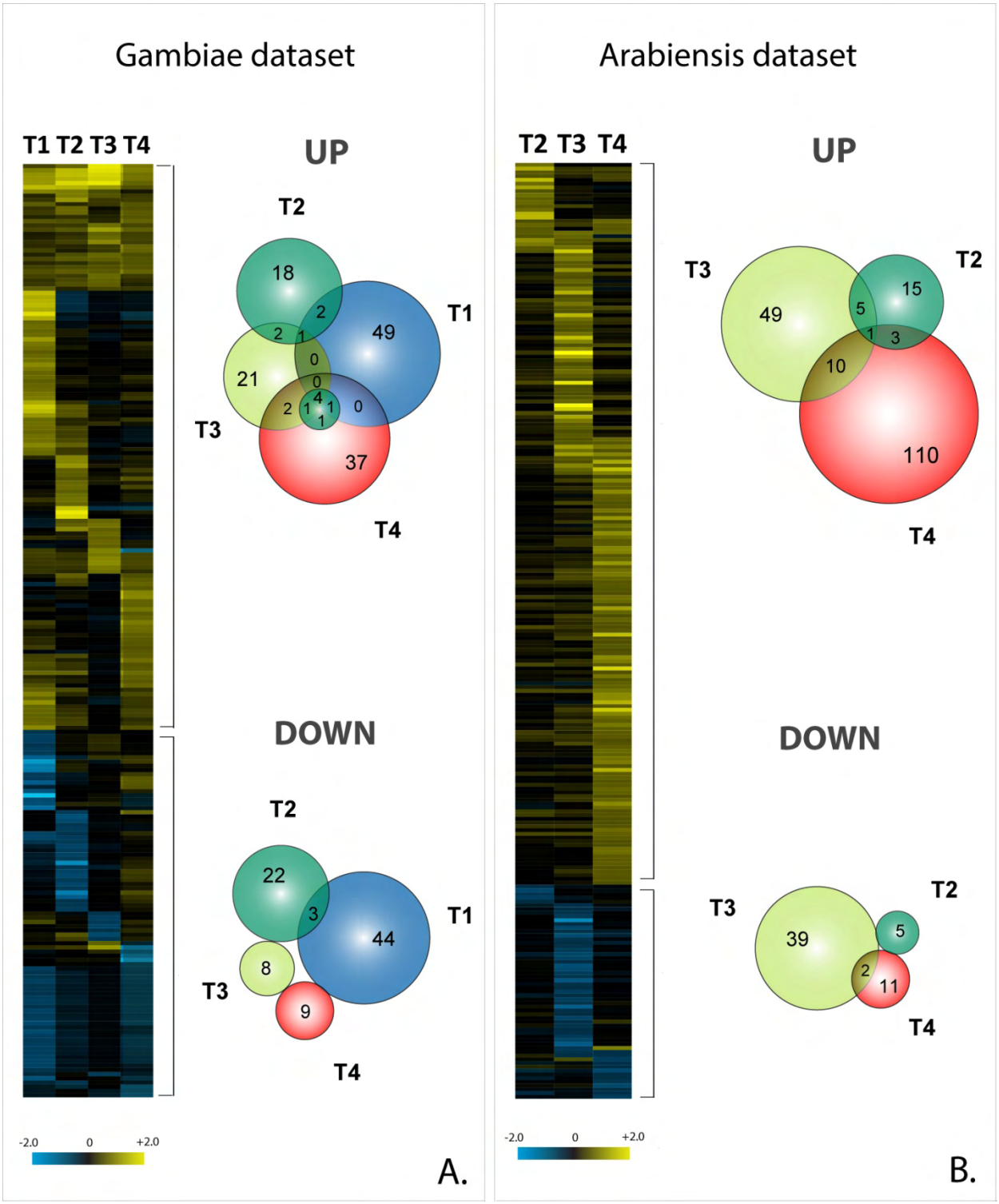


Figure 6.2.4 Expression profile and distribution of regulated transcripts in (A) *An. gambiae* and (B) *An. arabiensis* midgut upon infections with sympatric *P. falciparum* parasites. Transcripts that exhibit significant regulation are grouped in distinct co-regulation clusters (k-means algorithm) and visualized via heatmaps (Eisengrams); yellow colour represents upregulated (> 0.7 in $\log_2$) transcripts and blue colour downregulated (<-0.7 in $\log_2$). Venn diagrams display the distribution of the expression among the four distinct developmental timepoints: T1 (1h pi); T2 (6h pi); T3 (10h pi); T4 (24h pi) represented with four different colours: blue, green, yellow and red, respectively. The circles' diameter is proportional to the number represented.

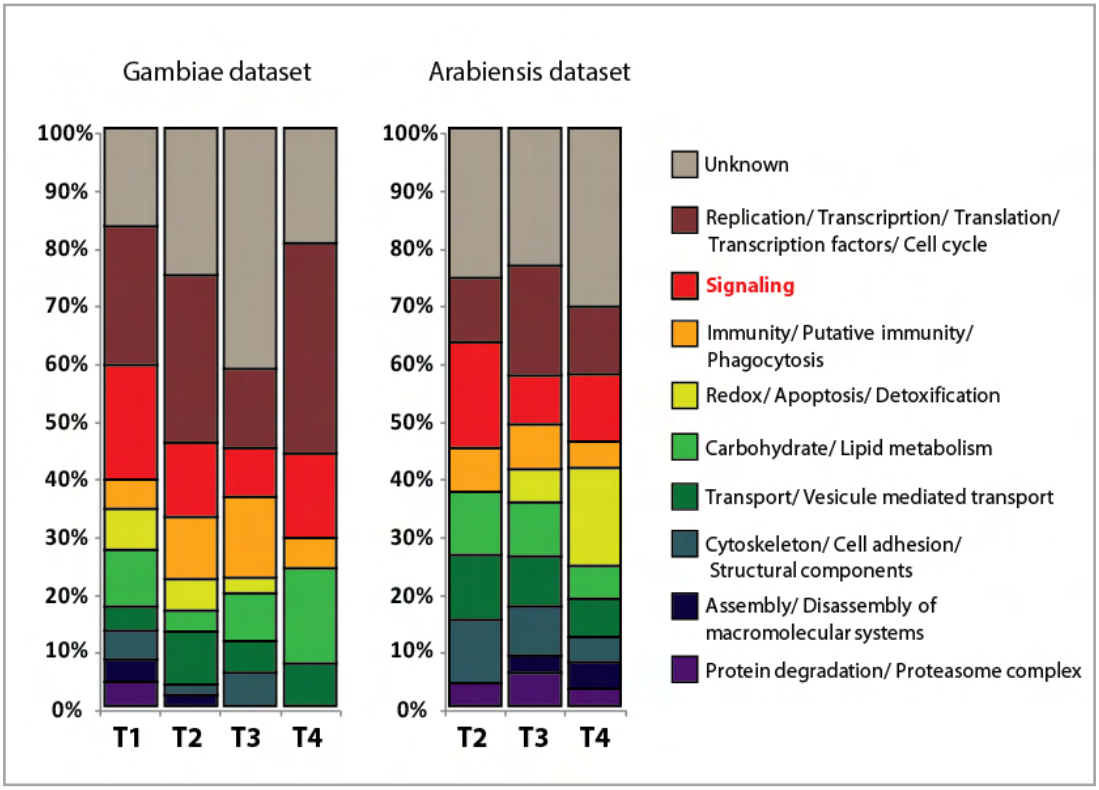


Figure 6.2.5 Functional classification of regulated transcripts in *An. gambiae* and *An. arabiensis* midguts upon P. falciparum infections. The coloured fractions are representing the main functional classes. Signaling process is highlighted, as exhibits an intriguing over-representation in the early temporal stages in both Gambiae and Arabiensis datasets.

For further investigation, the regulated transcripts from each dataset were apportioned in ten distinct classes, based on their annotated function (obtained from Vectorbase), reliant on a similar classification described in *Mendes et al., Infection and Immunity 2011*. (Figure 6.2.5) A significant proportion of the expression is allotted in housekeeping processes (such as Replication, Transcription, Translation, etc.), Signaling stimulation, Immune responses and Reduction-Oxidation/ Detoxification processes. Moreover, a considerable proportion of transcripts has not been functionally categorized, yet (unknown function). Intriguingly, about 20% of T1 regulated transcripts in *An. gambiae* and approximately 15% in T2 transcripts in *An. gambiae* and *An. arabiensis* appear to trigger molecular signaling reactions. Furthermore, in 24h post-infection (T4), *An. gambiae* exhibits an expression profile that is mainly comprised of housekeeping, signaling and metabolism processes. Contrastingly, *An. arabiensis* presents a more complicated profile in the same timepoint, including mainly Reduction-Oxidation/ Detoxification processes, apart from housekeeping and signaling.

The second aspect of the analysis attempts to comprehend the way that the same parasite, *P. falciparum,* infects two different mosquito species, *An. gambiae* and *An. arabiensis*. Again, the expression profile in the two datasets is studied based on heatmap visualization and Venn diagrams resulted from k-means analysis (Figure 6.2.6). Unambiguously, over-expressed transcripts in *P. falciparum* are more abundant than the under-expressed ones. Specifically, their abundance presents to be correlated with time, as the regulation seems to increase from one timepoint to the other. Moreover, although differentially distributed, parasite's expression profile appears to follow the same trend both in Gambiae and Arabiensis datasets. Observing the heatmap, there can be easily detected a common over-expressional peak that some parasites display in both datasets' T3 and T4 timepoints.

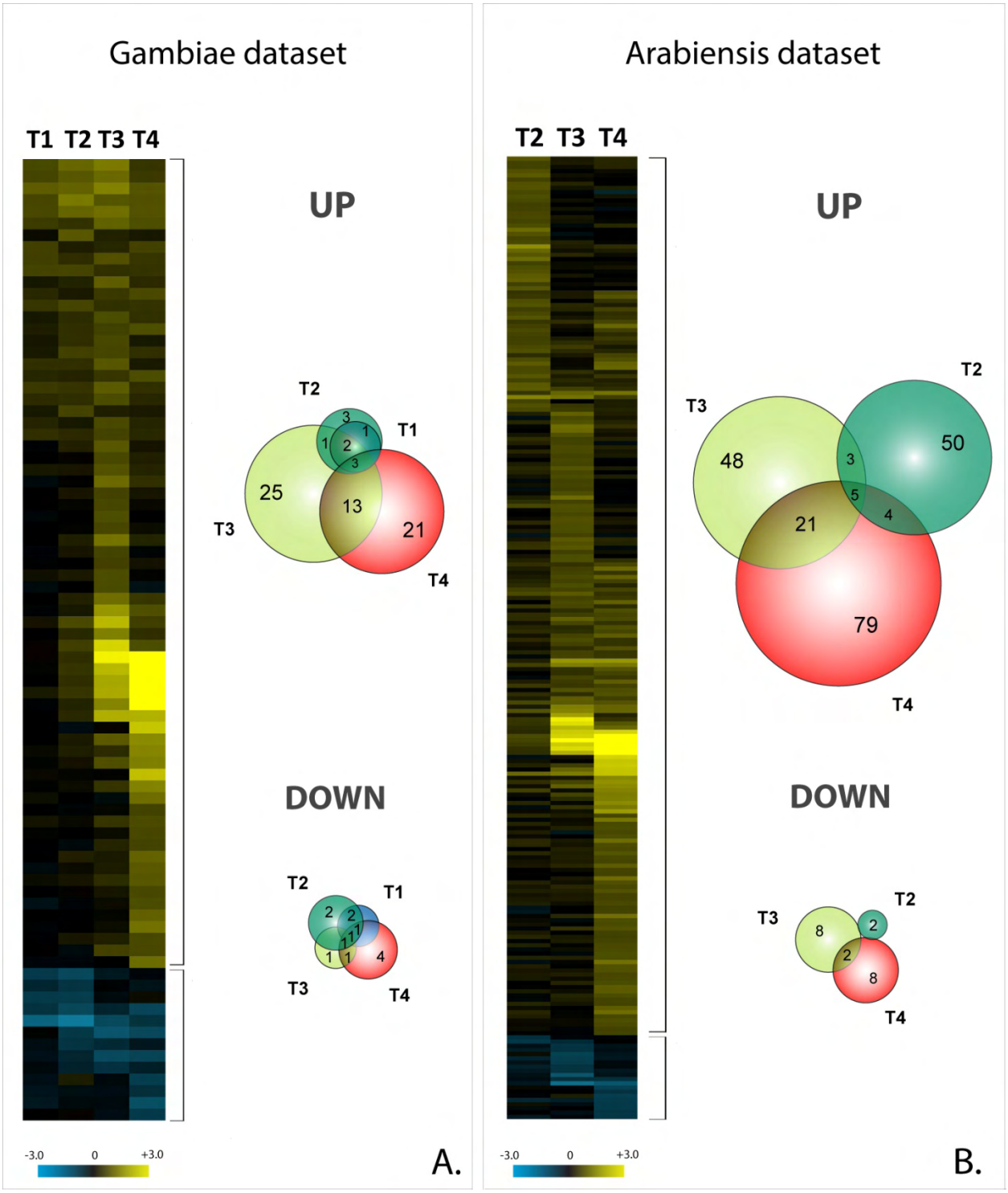


Figure 6.2.6 Expression profile and distribution of regulated transcripts in *P. falciparum* during sexual development in (A) *An. gambiae* and (B) *An. arabiensis* mosquitoes. Transcripts that exhibit significant regulation are grouped in distinct co-regulation clusters (k-means algorithm) and are visualized via heatmaps (Eisengrams); yellow colour represents upregulated (> 0.7 in $\log_2$) transcripts and blue colour downregulated (< -0.7 in $\log_2$). Venn diagrams display the distribution of the expression among the four distinct timepoints: T1 (1h pi); T2 (6h pi); T3 (10h pi); T4 (24h pi) represented with four different colours: blue, green, yellow and red, respectively. The circles' diameter is proportional to the number represented.

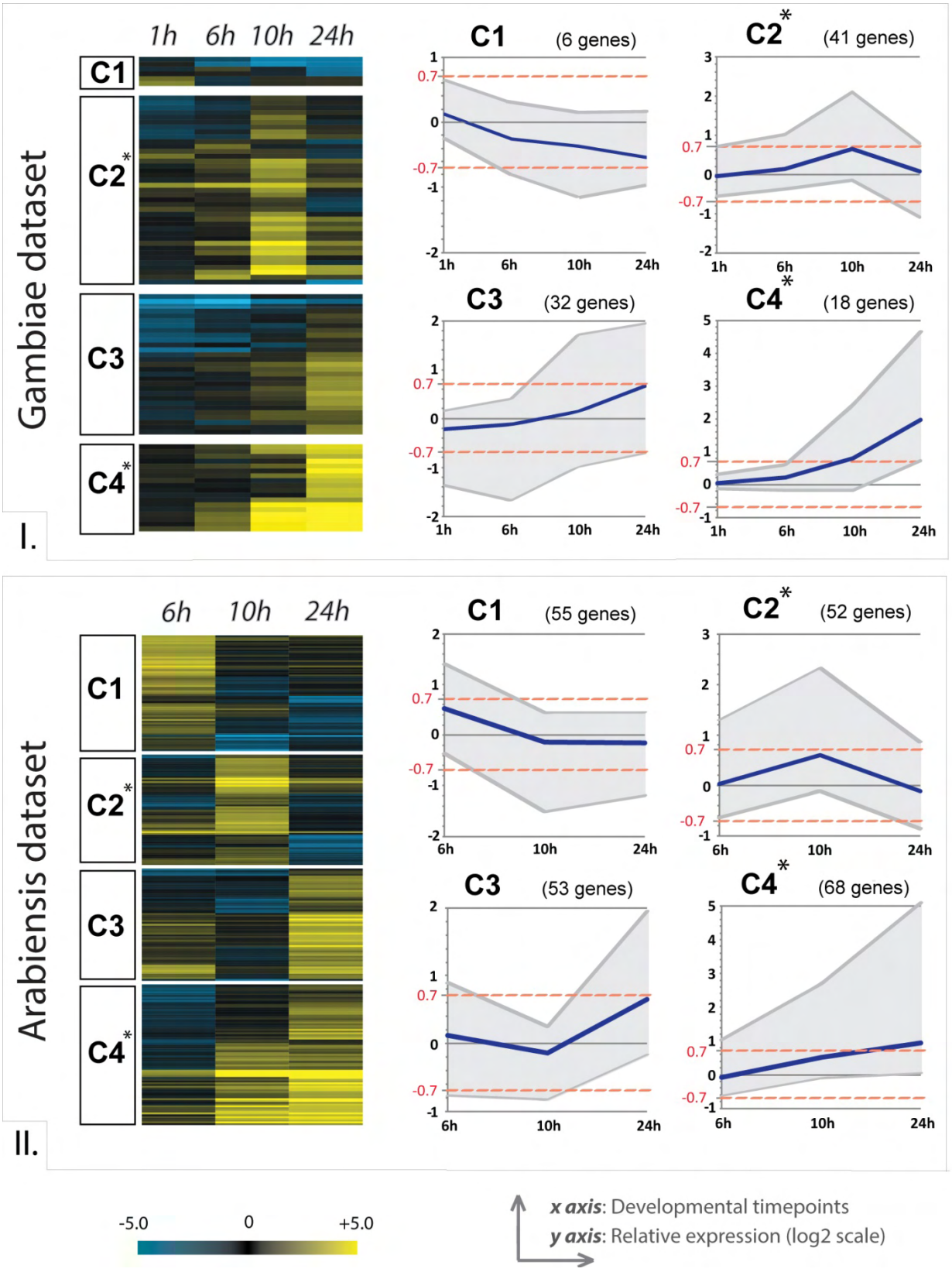


Figure 6.2.7 Significantly differential expression of *P. falciparum* regulated transcripts in (I) *An. gambiae* and (II) *An. arabiensis* infections. Transcripts that exhibit significant differential (>0.7 in $\log_2$ scale) regulation among timepoints in both infection systems are subjected to SOM-based analysis. The four clusters (Self Organising Maps) resulted from the analysis are visualized through heatmaps (left) and developmental line graphs (right). The bold blue line represents the average expression of the transcripts included in the cluster, and the gray area depicts the range of their expression (Maximum to Minimum). Stars indicate co-clustered transcripts that present a time-specific peak in their expression.

Next, for detailed examination of the expression patterns, the same transcript list is subjected to SOM-based analysis, producing four major clusters (SOMs: Self Organizing Maps) in each dataset (Figure 6.2.7). These clusters are demonstrated via Eisengrams and developmental line graphs that depict the gene expression alterations being occurred throughout parasite's development inside the two mosquito species (thick blue line represents the average expression, and the gray background represents the range of expression of the entities included in each cluster). Interestingly, the developmental trends represented in all four clusters are respectively similar between the two datasets. Furthermore, C2 and C4 clusters in both datasets display a developmental peak in 10h and 24h post-infection, respectively. Genes included in these particular clusters underwent further interrogation through functional characterization experiments included targeted gene disruption (knock-out) strategy and phenotypic analysis of the generated mutants (Taxiarchi C., *Final Year Project thesis*, 2013).

The consequent succession of the analysis above is to study comparatively the regulation of the two datasets in both mosquito and parasite transcripts. Thus, the transcriptional profile of over- and under-expressed transcripts in *An. gambiae* and *An. arabiensis* are subjected to k-means analysis and the output is visualized again through heatmaps and Venn diagrams. As displayed in Figure 6.2.8, each timepoint is comprised of regulation which is common or different in the two mosquito species. Definitely, there can be distinguished a common core in the regulation of the two vectors, logically representing the conserved responses, and significantly differential expression presented mainly in *An. arabiensis* and secondarily in *An. gambiae*, which suggest spieces-specific reactions.

The same comparative analysis performed in the *P. falciparum* transcripts reveals a more robust common core of regulation among the two types of infections (Figure 6.2.9). Specifically, it appears that the majority of *P. falciparum* over-expressed transcripts are stimulated upon infection to both *An. gambiae* and *An. arabiensis*. According to the heatmaps, the general trend of expression in both cases appears to follow the same pattern.

Finally, a supplemental statistical analysis was implemented via GraphPad Prism 5 software for further comparison of the two datasets. The last figure above (Figure 6.2.10) illustrates the correlation plots between Gambiae and Arabiensis datasets in T2, T3 and T4 timepoints for both mosquito (green dots) and parasite (purple dots) sets of transcripts. Moreover, Pearson correlation combined with best linear distribution fit analysis is applied in order to designate the statistical significance. Therefore, it is clear that gene expression in *An. gambiae* and *An. arabiensis* exhibits a profound deviation. However, in T2 the two mosquito profiles appear to be correlated ($P = 0.006$), albeit not significantly (Pearson $r= 0.22$). On the other hand, the parasite transcripts display a more conserved regulation during development inside the two different hosts. Actually, in all three timepoints the regulation is significantly correlated (P value < 0.001), and subsequently indicates that *P. falciparum* follows a strict developmental program that is scarcely affected by the *Anopheles* species vector (when referring to *An. gambiae* and *An. arabiensis* species).

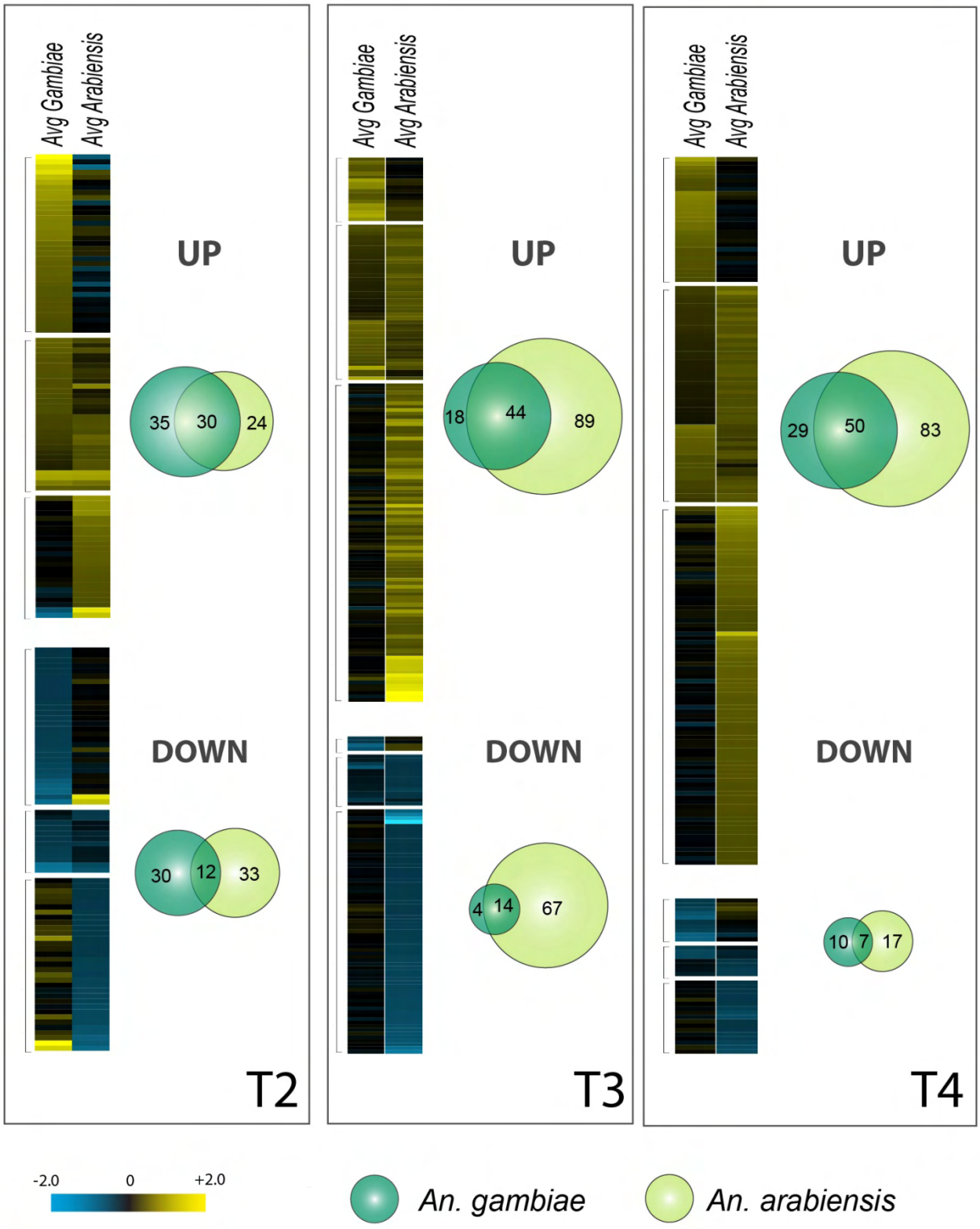


Figure 6.2.8 Comparative analysis of *An. gambiae* versus *An. arabiensis* regulated expression profile displayed 6h (T2), 10h (T3) and 24h (T4) after infections with *P. falciparum* natural populations. Significantly regulated transcripts are grouped in distinct co-regulation clusters (k-means algorithm) and are visualized via heatmaps (Eisengrams); yellow colour represents upregulated (> 0.7 in $\log_2$) transcripts and blue colour downregulated (< -0.7 in $\log_2$). The allocation of the common and differential regulation of the two mosquito species is illustrated via Venn diagrams. The circles' diameter is proportional to the number represented.

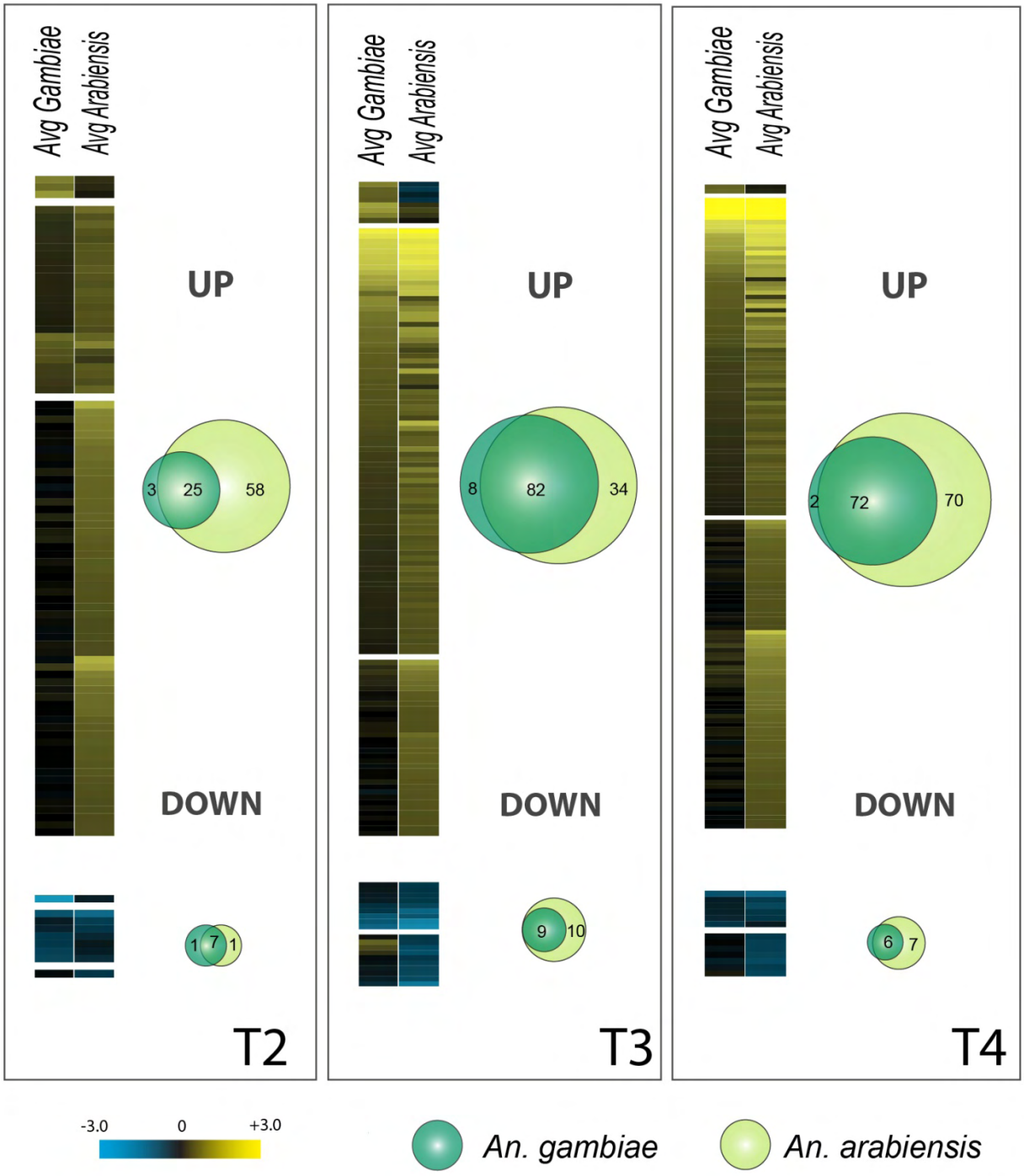


Figure 6.2.9 Comparative analysis of *P. falciparum* regulated expression profile displayed during sexual development 6h (T2), 10h (T3) and 24h (T4) post-infection inside *An. gambiae* versus *An. arabiensis* mosquitoes' midgut. Significantly regulated transcripts are grouped in distinct co-regulation clusters (k-means algorithm) and are visualized via heatmaps (Eisengrams); yellow colour represents upregulated (> 0.7 in $\log_2$) transcripts and blue colour downregulated (< -0.7 in $\log_2$). The allocation of the common and differential regulation of the parasite during development inside the two mosquito species midguts is illustrated via Venn diagrams. The circles' diameter is proportional to the number represented.

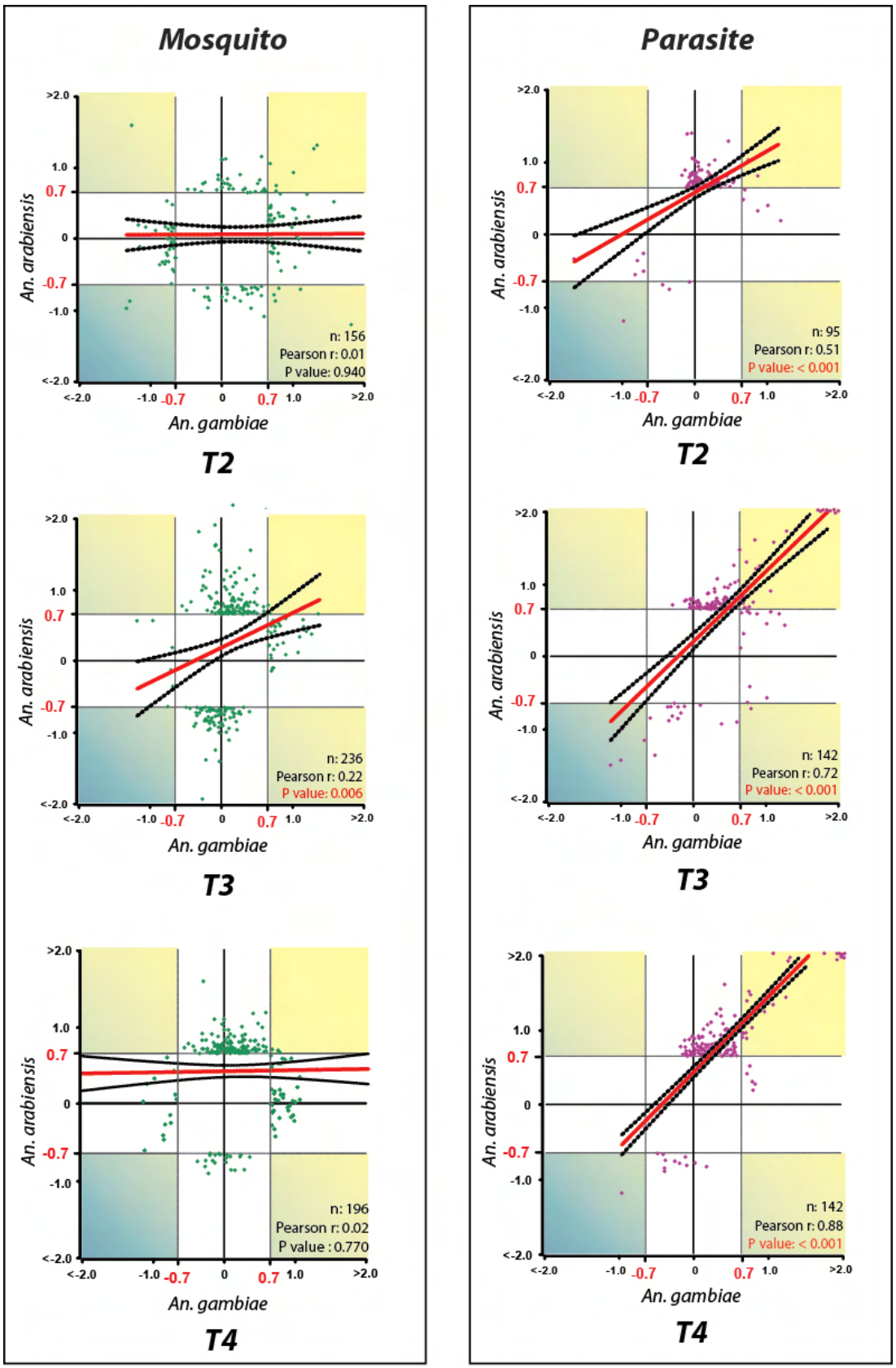


Figure 6.2.10 Statistical comparison of mosquito (left) and parasite (right) transcriptional profiles between *An. gambiae/P. falciparum* and *An. arabiensis/P. falciparum* infection groups during three distinct timepoints: T2 (6h pi); T3 (10h pi); T4 (24h pi). Scatter plots display the expression profile of differentially regulated transcripts (expression ratio greater than 0.7 $\log_2$) in Gambiae (x axis) versus Arabiensis (y axis) datasets. The total number of transcripts and the Pearson correlation significance between the expression ratios of the two datasets are displayed at the lower right of the panels. Red line represents the best linear distribution fit and black dashed lines indicate the 95% freedom interval.

6.3 Functional Characterization (Low Throughput approach)

The second part of the current study is referred to the functional characterization of candidate genes resulted from the microarray analysis. The strategy followed is actually a Reverse Genetics approach that indicates the (short-term) expressional disruption of transcripts that displayed an intriguing profile in infected mosquitoes, according to the previous analysis. Then, the phenotype of the impaired individuals is compared with the wild type ones', in order to reveal novel regulators of the mosquito defense against *Plasmodium* infections (Figure 6.1.1).

Thereby, in reliance to the individual analysis of the mosquito transcripts (Figure 6.2.4), their functional classification (Figure 6.2.5), their domains and special features, and their genetic architecture (obtained from Vectorbase database), candidate genes are selected for further investigation. As a result, three genes that demonstrated up-regulated expression in both datasets (>0.7 in $\log_2$ scale), are directly or indirectly involved in signaling processes and have an appropriate structure, are proceeded for functional analysis. Specifically, there are interrogated the Neuropeptide M-like gene (NPM), a latrotoxin G-protein coupled receptor gene (GPCRlar), and a putative signaling gene (Bab31). However, henceforth, due to practical reasons, there will be discussed only the first gene's characterization procedures and results.

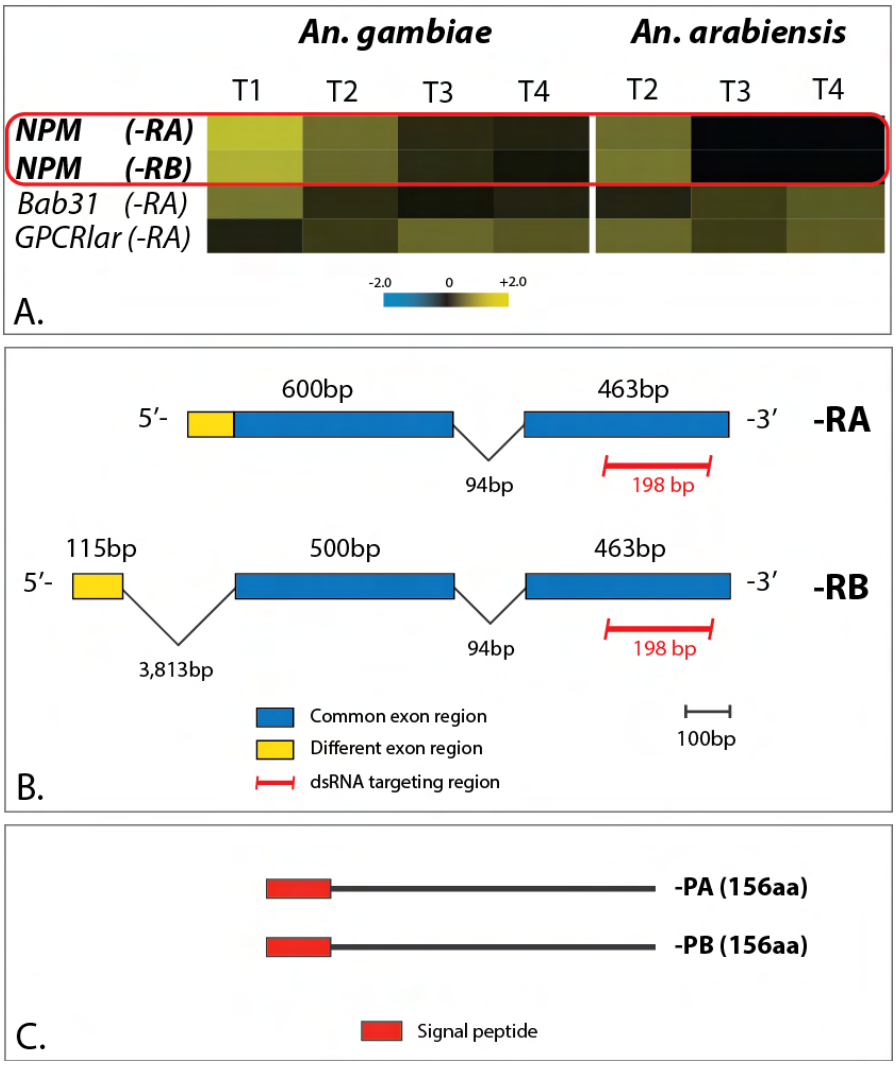


Figure 6.3.1 General features of candidate transcripts proceeded for functional characterization. A. Heatmap visualization of the candidates' transcriptional profiles resulted from the microarray analysis. B. Molecular architecture of the two NPM alternative transcripts. Blue colour indicates the common exon region; yellow colour indicates the different exon region. The red line highlights the 198bp-long fragment targeted by the dsRNA. C. The 156aa-long protein produced by the two transcripts. The red box illustrates the signal peptide motif that is included in both proteins.

In detail, NPM gene produces two alternative transcripts, "-RA" and "–RB", that are both highly upregulated 1h pi in *An. gambiae* (≈2.0 folds up) and also upregulated 6h pi in *An. gambiae* and *An. arabiensis* (≈1.5 folds up). Structurally, the two transcripts share a common 963bp-exon

region and a different, ≈100bp, region. Nevertheless, as the different region in their mRNAs is located in the 5'-UTR, both transcripts express the same Open Reading Frame (ORF) and, subsequently, are translated into the same protein ("-PA" = "-PB"). The protein product is only a 156aa-long peptide that includes a signal peptide motif (Figure 6.3.1). This feature indicates that this short peptide is probably exported from the cell, in order to find its target (putatively, a receptor).

Consequently, NPM's function is obstructed via disrupting the "–RA" and "–RB" transcripts' integrity. In detail, utilizing the endogenous RNAi mechanism of mosquitoes, long double-stranded RNA (dsRNA) is introduced into mosquitoes, inducing post-transcriptional/translational silencing. As there are two transcripts, the dsRNA targets a 198bp-fragment that is located in their common exon region and includes the 3'-UTR (Figure 6.3.1). In theory, the imported long dsRNA uses the internal RNAi mosquito mechanisms (Dicer, RISC complex etc.) and is partitioned into a plethora of small (19-22bp long) double-stranded RNA molecules, siRNAs. The siRNAs are denaturating into single strands and integrated into an active RISC complex, subsequently cleaving the targeted mRNA, blocking its translation. The knock-down efficacy is proportional to the extent of the targeting mRNA decay.

The process described is comprised of three major steps: dsRNA construction, the main RNAi experiments and the characterization of the outcoming phenotype (Figure 6.1.1).

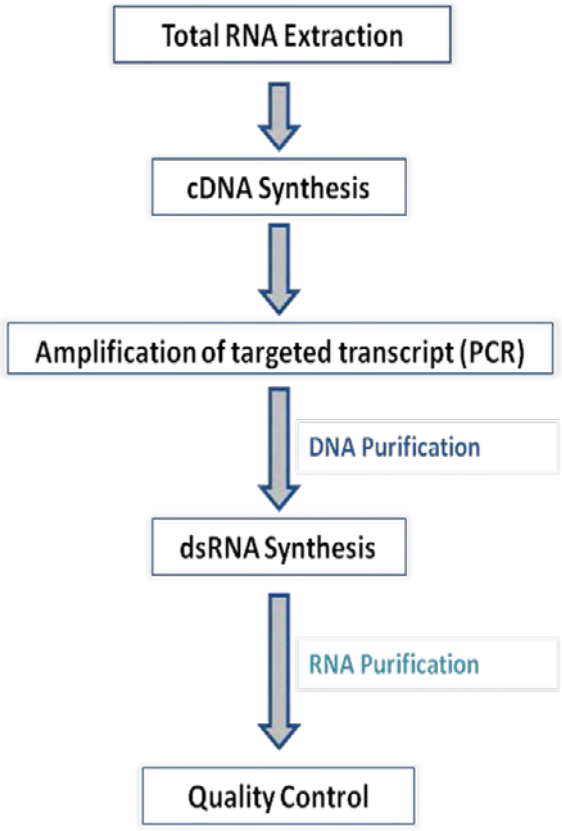


Figure 6.3.2 Workflow representation of the dsRNA construction and assessment basic steps. The succession of the experiments is indicated by the arrows' direction.

Initially, T7 primers should be in silico designed in order to designate the fragment that will be amplified later. This design can be accomplished via E-RNAi 3.2 online version software (the output is available in Appendix 1). The following process is divided in five major steps, as presented in Figure 6.3.2. Consequently, total RNA is extracted from 10-15 naïve mosquitoes, based on TRIzol® (Invitrogen) protocol, and then is used as a template for cDNA synthesis. Next, the designated fragment is amplified via Polymerase Chain Reaction (PCR), using the T7 primers designed before. Significantly, after amplification, the PCR product should be subjected to DNA purification process (using QIAquick PCR purification kit), so that the final solution is as clean as possible. Then, using the MEGAscript® T7 kit, the targeted fragment is converted into dsRNA. Again, due to the existence of enzymes, buffers etc. that "pollute" the final solution, RNA purification is a substantial step for obtaining a clean product. The purified dsRNA has to be assessed before use (Quality Control). For this reason, the final solution is electrophorized in 1% Standard Agaroze gel and spectrometerically quantified with the use of NanoDrop® ND-1000. The sample must have final concentration equal to 3 mg/μl and exhibit only one specific band in the electrophoresis (the dsRNA quality is described in Appendix 2).

The next stages describe the main RNAi experiments that are necessary to accomplish knock-down on mosquitoes (detailed description Ganver et al., *J Vis Exp.* 2007). According to the general overview (Figure 6.3.3), in DAY 0, naive female adult mosquitoes (*A. gambiae Ngousso*-strain individuals, laboratorial colony) are collected approximately one day post-emergence for micro-injections. In particular, three biological replicates must be accomplished, with each replicate containing at least two batches of 30-40 mosquitoes (one for the knock-down, and one as a control). Thus, long dsRNA is introduced in female mosquitoes via intrathoracic inoculations; the first batch is injected with the NPM-targeting dsRNA, and the second one with GFP-targeting dsRNA*. Logically, the first inoculation will provide the knocked-down mosquito line and the second one the control group.
Next, three days after injections, both mosquito groups are taking a parasite-infected blood meal. Due to several issues involved with *P. falciparum* manipulation (such as safety concerns, difficulties in cultivation, treatment etc.), the mosquito infections are accomplished using the rodent model *P. berghei.* Thereby, the mosquitoes are fed with mice, infected with the transgenic *PbGFPcon P. berghei* strain, which constitutively expresses the Green Fluorescent Protein (GFP) **.

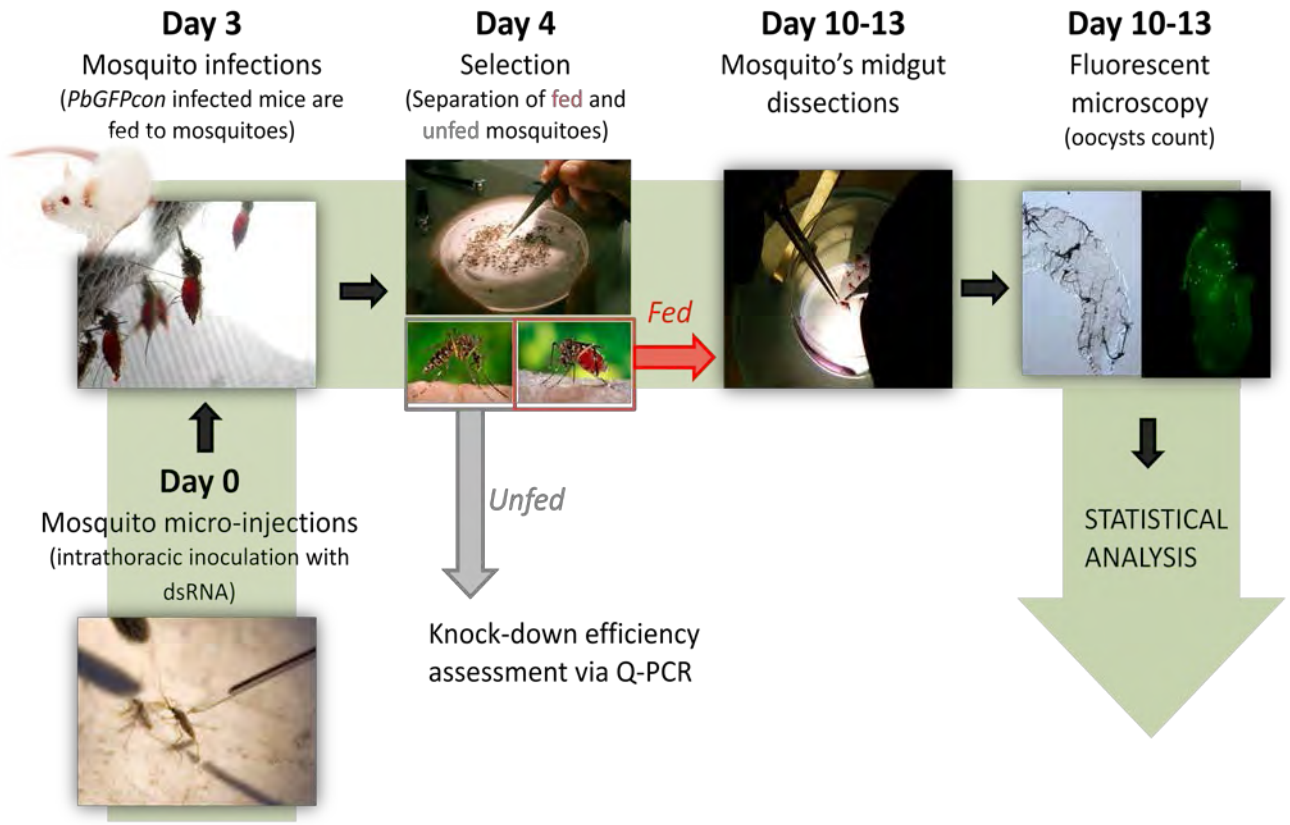


Figure 6.3.3 RNAi experimental procedures overview. The operations followed in order to accomplish dsRNA mediated RNAi silencing are demonstrated in chronological order.

* stock dsRNA solution that targets the GFP gene is introduced in naive mosquitoes causing no effect (there is no homology with the mosquito transcriptome).
** The expression of GFP in the *P. berghei* parasites is not compromised in day 10-13 from the GFP dsRNA, as the RNAi effect will be already attenuated.

Subsequently, 24-48h post-infections, the mosquitoes are divided in two groups: the blood fed and the unfed ones. Blood fed mosquitoes will proceed to the ensuing experimental stages, while 10-15 unfed mosquitoes will be stored for the Q-PCR knock-down efficiency assessment (the rest will be exterminated). Finally, in DAY 10-13, survived mosquitoes are anesthetized and their midguts go through dissection and fixation in microscope slides. The same day (or few days later), the slides are observed under UV light, with the assistance of a fluorescent microscope. The *P. berghei* parasites, some days earlier, were transformed into oocysts and, at this time point they should undergo maturation, constitutively expressing GFP (knocked-in transgene). Thereby, under UV light, on the midguts' surface there can be distinguished developing oocysts, that emit an intense green colour due to GFP production. The attached oocysts included in every midgut are enumerated, and the results are subjected to statistical analysis.

					Parasite intensity			**Mann Whitney Analysis**	
Mosquito	# Repl.	D.p.i.	# midguts	Infection Prevalence (%)	Arithmetic mean	Median	Parasite Range	P value	Fold difference
Control (GFP)	3	8	55	70	7.2	2	0-49		
NPM		8	66	67	25.3	10.5	0-150	**0.01***	+5.25

Table 6.3.1 RNAi experimental procedures results table. The table concentrates the data obtained from the knock-down assay. Oocyst enumeration data were subjected to Mann-Whitney analysis (U-test) revealing a significant difference between

The table above (Table 6.3.1) concentrates the results derived from this experimental process. In overview, three biological replicates were accomplished, during which, 55 midguts were dissected from the control group and 66 from the KD group in DAY 8. The infection prevalence (number of midguts that contained oocysts/number of vacant midguts x100(%)) was adequate. Interestingly, the arithmetic mean and the median of the values, such as the parasite range, were higher in the KD group in comparison with the control group. In order to identify the statistical difference between the two phenotypes, Mann Whitney t-test (U-test) was applied. With fold difference 5.25, the analysis presented P value = 0.011 (<0.05), which designates that the raise in the oocyst numbers in the KD group midguts is statistically significant.

The final results obtained from the RNAi experiments are synopsized in Figure 6.3.4. As illustrated, midguts included in the KD group present a higher abundance in oocysts in contrast to the control group (A). Statistical analysis implemented with U-test revealed that simultaneous silencing of both NPM transcripts induces a significant effect on the median oocyst numbers ($P < 0.05$) (C). The knock-down efficiency was assessed via quantitative RT-PCR, and revealed an average 50% reduction in the expression of both transcripts, relatively to the control.

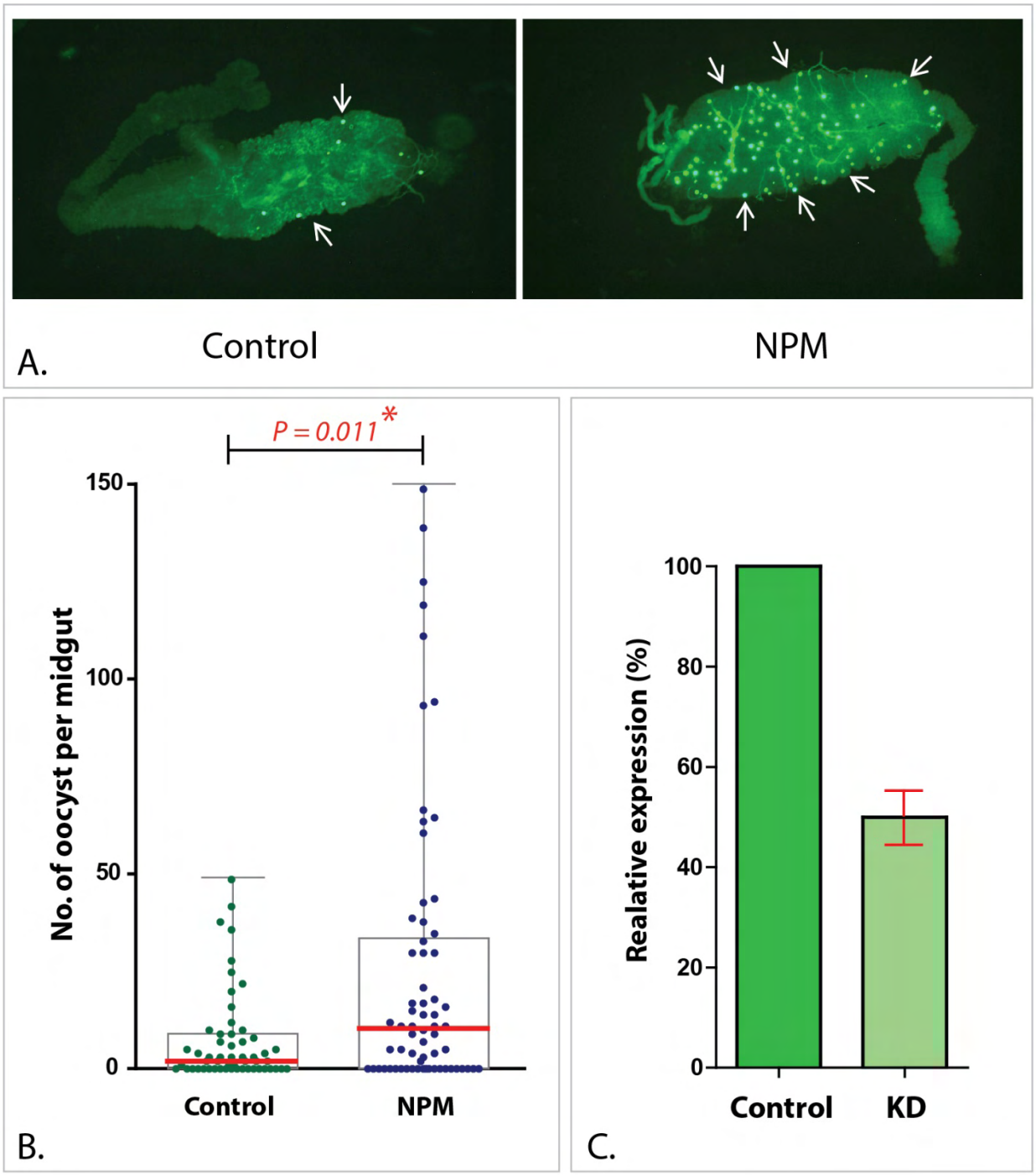


Figure 6.3.4 RNAi silencing results. A. Mosquito midguts dissected 8 days post infections, as visualized under UV light. The images reveal an evident increased number of attached oocysts in the KD mosquito midguts (Control: GFP, KD: NPM). B. Graphing illustration of oocysts distribution per midgut in KD and Control groups, enhanced with Mann-Whitney t-test (U-test) display the statistical significance of the difference between the means of the two groups ($P < 0.05$). Red line represents the values' mean and grey line (whiskers box plot) the values' range. C. Column graph depicts the expression of NMP transcripts exhibited four days post injections in KD mosquitoes in relation to the Control mosquitoes. Red bar represents the standard deviation among the three experimental replicates.

7 Discussion

7. Discussion

The Afrotropical species *Anopheles gambiae* and *Anopheles arabiensis* comprise two of the most efficient *Plasmodium* vectors that are responsible for malaria transmission. These organisms are included in the Anopheles gambiae species complex (along with *An. merus, An. melas* et al.), (Besansky et al., *PNAS* 1994), frequently mentioned as sister taxa. Moreover, genetic introgression, mediated by hybridizations, has also been reported between these mosquito species in nature (Besansky et al., *PNAS* 2003), although it yields sterile males (Neafsey DE et al., *Science* 2010). Despite the fact that these organisms are morphologically indistinguishable, they exhibit characteristically different behaviours; for instance, *An. arabiensis* exhibited higher average bites in humans than the sympatric *An. gambiae* in Barkedji region during the rainy season (Lemasson JJ., *J Med Entomol* 1997). However, until now, there is no functional discrimination reported between these two organisms, in terms of functional genomics.

In the current study there was attempted to trace the similarities and differences of two sympatric *An. gambiae* and *An. arabiensis* populations (Burkina Faso) when infected with indigenous *Plasmodium falciparum*. Previous studies have already accentuated the combination of a DNA microarrays and Reverse-Genetic analysis as a powerful heuristic approach to identifying genes implicated in vector-parasite interactions (Vlachou et al, *Curr Biol* 2005). For this reason, two main strategies were followed here: i) a high throughput approach via Genome-Wide Transcriptional Profiling and ii) a low throughput functional characterization approach (Figure 6.1.1).

The first strategy is based on the investigation of the expression profiles of both systems (*An. gambiae/ P. falciparum* and *An. arabiensis/P. falciparum*) via Transcriptomic Microarrays. These binary systems' profiles were interrogated in four distinct time points, in correspondence with the major alterations that *Plasmodium* undergoes during its sexual development. In order to focalize in these developmental events, only mosquito midguts (and not whole mosquitoes) were extracted and screened.

Thus, after analysis, the mosquito transcripts' profiles exhibited a complicated pattern (as demonstrated in Figure 6.2.4). Upregulated expression in *An. gambiae* appeared to be overrepresented in 1h (T1) and 24h (T4), while downregulated transcripts were arithmetically fading out, in relation with time. On the other hand, upregulated transcripts in *An. arabiensis* exhibited a time-depended increase, while downregulated did not. Although these data do not suggest a common expression pattern, the significant upregulation observed in both T1 and T4 timepoints do have a biological meaning. In 1h post infection, mosquito midgut is trying to digest the blood meal and maybe detect any invaders. Moreover, in 24h post infection, the ookinetes penetrate the midgut epithelium, killing cells, and traversing through the basal lamina. Thus, the major alterations in the mosquito expression profile are logical to mainly occur in these moments.

The additional exploration of these regulated transcripts' ontology, by categorizing them in distinct functional classes, explained how these transcripts are distributed in the timepoints (Figure 6.2.5). Initially, it is profound that a big number of transcripts are either of unknown or housekeeping function. However, the transcriptional, translational etc. events could possibly indicate the extent of the mosquito response (the more significant events are happening, the bigger energy is spent on these processes, and thus, the greater is the mosquito response).

Interestingly, the stimulation of signaling processes was highly represented in the early stages (1h and 6h) in both mosquito species. This intriguing result was further utilized in the second experimental approach (low throughput). Despite their commons, the two mosquitoes exhibited differences in the distribution of their transcripts' functions that may imply deeper molecular and functional differences; for instance, there is an important difference between *An. gambiae* and *An. arabiensis* in the distribution of Reduction-Oxidation, Apoptosis and Detoxification-associated transcripts in T4, proposing that these two organisms may recruit different molecular defenses, or the same defenses in a different scale (Oliveira et al., *Science* 2012).

Accordingly, the comparative analysis of their expression profiles (Figure 6.2.8) illuminated two groups of regulation: a common core (transcripts with similar regulation), suggesting conserved inter-species responses, and differential regulation between the two species (transcripts that presented regulation only in one mosquito), suggesting species-specific responses. Importantly, the identification of these two types of mechanisms (inter-species and species-specific) could reveal a) the molecular keys involved in the robust vectorial capacity of these two species in contrast with their less capable sister taxa, and b) the deeper biochemical and behavioral differences between these two species that have defined their evolutionary and ecological niches (e.g. why *Anopheles gambiae* occupies more humid areas, while *An. arabiensis* dominates in arid savannas and steppes). (Kamali M., *Plos Pathog* 2012)

On the other hand, *P. falciparum* exhibited a more conserved profile, when infected the two different mosquitoes. Specifically, *P. falciparum* transcriptional upregulation demonstrated a time-dependent increase in both mosquito species (Figure 6.2.6). Furthermore, SOM-based analysis upon these profiles revealed four clusters of transcripts, with the three of them displaying the same developmental trend in both datasets (C1, C2, and C4; Figure 6.2.7). Intriguingly, C2 and C4 demonstrated an expressional peak in 10h and 24h, respectively, and comprised an interesting candidate pool for further investigation of parasite stage-specific regulators. Finally, in Figure 6.2.9 the parasite's profile appeared to be similar in both datasets, and subsequently it is suggested that the parasite follows a strict developmental program, which does not change during infections of the two different mosquitoes.

The last figure (Figure 6.2.10) provides evidence upon this last theory. The statistical analysis of the two transcript groups, mosquito and parasite, compares the profiles displayed in the two datasets and demonstrates that there is a strong correlation ($P < 0.001$) between the *P. falciparum* regulated expression during *An. gambiae* and *An. arabiensis* infections. Unambiguously, the parasite's "tight developmental program" theory emerges as a possible scenario. In contrast, mosquito transcripts do not exhibit such a correlation, subsequently proposing that there are strong differences between their response patterns, and maybe different mechanisms are involved, as well. Although T3 does not seem to abide by this observation, it can be explained, as in 10h pi (ookinete traverses through midgut), mosquito does not interact with the parasite in the same extend as in 24h (ookinete invades the midgut epithelium), and thus the mechanisms are more likely to be homeostatic (not related to defense or response), ergo inter-species.

After the holistic approach of the microarray analysis, some candidate transcripts were further interrogated, in order to identify novel regulators of the mosquito response upon infection. The intriguing distribution pattern of the regulated transcripts involved in signaling processes (Figure 6.2.5), led this study to interrogate candidates based on this pattern. Specifically,

transcripts that displayed a significant upregulation in the early timepoints (T1 and T2) and were involved in signaling processes (actually or putatively), based on their GO and InterPro annotation, could be implicated in the mosquito's molecular defense mechanisms (Figure 6.3.1). It is important to mention that significant studies emerged in the last decade revealed a big part of the mosquito immune defense (Christophides GK et al., *Science* 2002, Povelones M et al., *Science* 2009, etc.) against *Plasmodium*. However, the exact mechanisms that orchestrate the initial recognition of *Plasmodium* parasites, the amplification and transduction of the recognition signal and, finally, the stimulation of the defense and the recruitment of the immunity components remain obscure. Thereby, the selected candidates were examined as potential regulators or participators of this early signal transmission.

Consequently, four transcripts were studied; however, due to issues of importance, only one gene is mentioned here. The Neuropeptide M gene (NPM), which transcribes "-RA" and "–RB" alternative mRNAs, was examined based on a Reverse Genetics approach. Specifically, post-transcriptional silencing was induced in both transcripts via dsRNA mediated RNAi; the impaired mosquitoes produced were infected with *P. berghei* parasites, in order to identify possible changes in their phenotype. The resulted significant ($P < 0.05$) increase in the oocysts number in the KD mosquito midguts suggests that NPM plays an important role in the mosquito's defense against the parasite invader. Given the fact that NPM is a signaling molecule (neuropeptide) overexpressed in the early infection stages, it can be proposed that it could possibly participate either in the activation/regulation of immune responses or in the activation/regulation of other processes that enhance the general mosquito defense. However, due to lack of further annotation details (such as GO function and InterPro domains) about NPM in *Anophelines* and their related genera, it is difficult to define the exact function of this peptide. Recently, though, a study followed the same silencing technique upon genes included in the rhodopsin-like GPCRs group (Famlily A), exhibited the same increase in the oocysts number in laboratorial and field *An. gambiae* individuals (Mendes et al., *Infection & Immunity* 2011). Subsequently, these findings suggest a putative scenario, according to which NPM interacts with one (or more) rhodopsin-like GPCRs, playing the role of their ligand (or activator/co-activator). Nevertheless, expletory experimental documentation is needed to accept this hypothesis.

8 Concluding remarks & Future perspectives

8. Concluding remarks and future perspectives

The current project attempted to compare the two major malaria vectors, *An. gambiae* and *An. arabiensis*, after infections with *P. falciparum* natural populations, in terms of functional genomics. For this reason, two different approaches were implemented, i) a Genome-Wide Transcriptional Profiling (high-throughput) approach and ii) a functional characterization (low-throughput) approach, from which there were educed several illative results.

Specifically, the transcriptional responses of *An. gambiae* and *An. arabiensis* exhibited in 1h, 6h, 10h and 24h post infections with *P. falciparum* parasites demonstrated significant similarities and differences between the two mosquito species. A common core of regulated transcripts occurred in the three comparable timepoints (6h, 10h, 24h), proposing inter-spieces responses; albeit, differentially regulated transcripts were also revealed in both species implying the presence of species-specific responses, as well. On the other hand, the transcriptional profile of *P. falciparum* displayed a robust correlation between *An. gambiae* and *An. arabiensis* infections in each defined timepoint (6h, 10h, 24h), unambiguously suggesting that the parasite follows a tight developmental program, during its sexual stages, that it is hardly affected by the mosquito vector (*An. gambiae* versus *An. arabiensis*).

The intriguing over-representation of upregulated signaling components in the initial stages of the infection in both mosquito species, subsequently led to the interrogation of transcripts that are potentially involved in the early signaling processes. Thus, the RNAi-induced knock down of Neuropeptide M (NPM), an uncharacterized neuropeptide, revealed its significant role as a component implicated in the early signaling processes that possibly activate defensive mechanisms against the rodent model *P. berghei*.

Furthermore, these findings structure a rudimental basis upon the study of the molecular interactions between the parasite invader and its mosquito vector, providing potential targets for further exploration. The detailed categorization and analysis of the transcripts that are similarly or differentially regulated in the two mosquito taxa would probably illuminate their intra- and inter- species responses, which maybe involve common or different defense mechanisms. Moreover, an expletory examination and functional characterization of the significantly regulated transcripts in both mosquito and parasite would reveal fundamental components implicated in their molecular interplay. NMP was revealed as one of these components that is involved in early signaling processes. However, further experiments that include *P. falciparum* parasites in laboratorial or field conditions are necessary to enhance this evidence. In addition, the potential role of the recently identified rhodopsin-like GPCRs as receptors of the NMP needs expletory examination. Finally, the revelation of the complete parasite-vector interactome during the midgut sexual stages still remains as the holy grail of Transmission Blocking Intervations (TBI).

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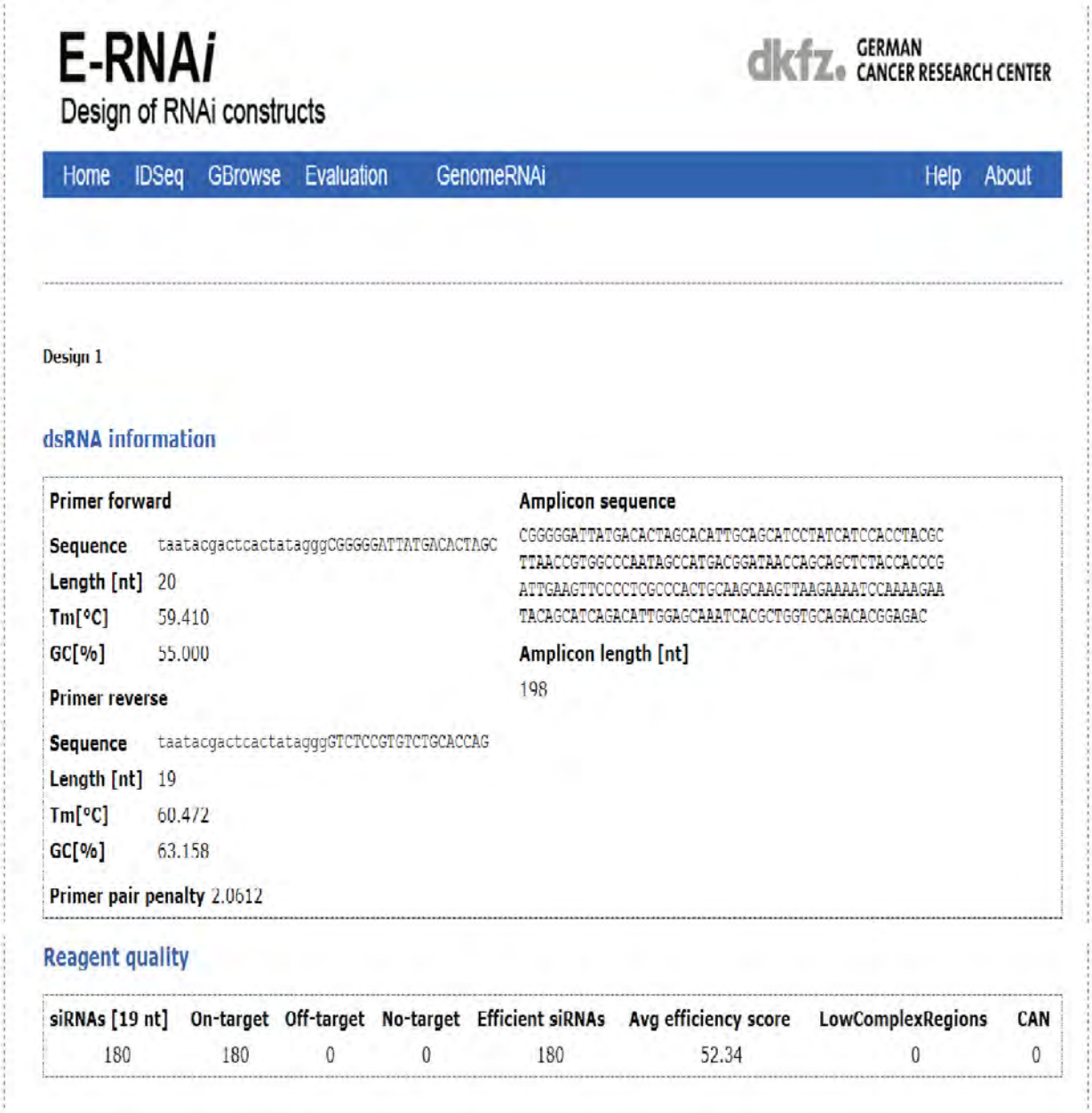
ABBREVIATIONS

aa	aminoacids
An. arabiensis	*Anopheles arabiensis*
An. gambiae	*Anopheles gambiae*
Arabiensis (dataset)	*Anopheles arabiensis / Plasmodium falciparum* set of data
Avg	Average
bp	base pairs
cRNA	labbeled RNA with cy3 or cy5 fluorescents
CV	Coefficient of variation
dpi	days post infection(s)
dsRNA	double stranded RNA
g	grams
Gambiae (dataset)	*Anopheles gambiae/ Plasmodium falciparum* set of data
GFP	Green Fluorescent Protein
h	hours
hpi	hours post infection(s)
KD	knock-down
Krpm	1000x rounds per minute
L	liters
mg	milligrams
min	minutes
ml (or mL)	milliliters
n	number
ng	nanograms
nl (or nL)	nanoliters
nm	nanometres
NPM	Neuropeptide M
^{o}C	temperature degrees in Celcius scale
ORF	Open Reading Frame
P. berghei	*Plasmodium berghei*
PA	Peptide No#1 (A)
PB	Peptide No#2 (B)
PCR	Polymerase Chain Reaction
pi	Post infection(s)
qRT-PCR (or Q-PCR)	Quantitative Real Time Polymerase Chain Reaction
R#	(biological) Replicate No#
RA	Transcript No#1 (A)
RB	Transcript No#2 (B)
RNAi	RNA interference
rpm	rounds per minute
SD	Standard deviation
sec	seconds
siRNA	small interfering RNA
SOM(s)	Self Organising Maps
ssDNA	single stranded DNA
U	units
UV	Ultraviolet
µg	micrograms
µl (or µL)	microliters
GO	Gene Ontology

Appendices



E-RNAi
Design of RNAi constructs

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Design 1

dsRNA information

Primer forward

Sequence taatacgactcactatagggCGGGGGATTATGACACTAGC
Length [nt] 20
Tm[°C] 59.410
GC[%] 55.000

Primer reverse

Sequence taatacgactcactatagggGTCTCCGTGTCTGCACCAG
Length [nt] 19
Tm[°C] 60.472
GC[%] 63.158

Primer pair penalty 2.0612

Amplicon sequence

CGGGGGATTATGACACTAGCACATTGCAGCATCCTATCATCCACCTACGC
TTAACCGTGGCCCAATAGCCATGACGGATAACCAGCAGCTCTACCACCCG
ATTGAAGTTCCCCTCGCCCACTGCAAGCAAGTTAAGAAAATCCAAAAGAA
TACAGCATCAGACATTGGAGCAAATCACGCTGGTGCAGACACGGAGAC

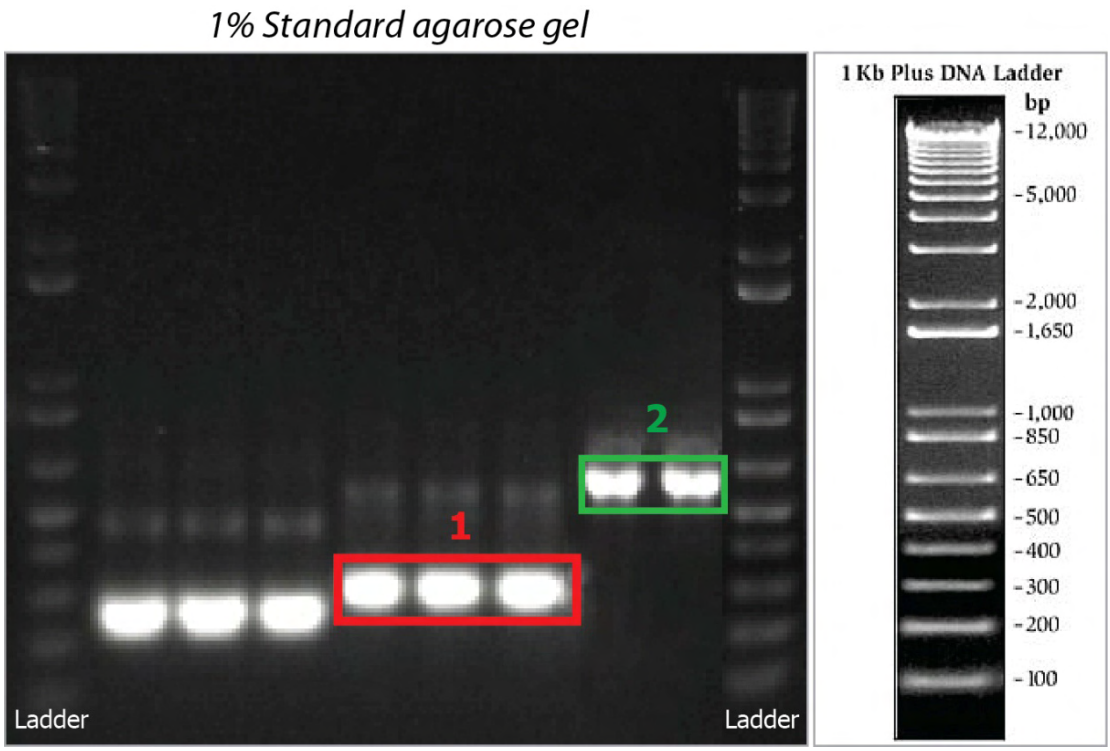
Amplicon length [nt]

198

Reagent quality

siRNAs [19 nt]	On-target	Off-target	No-target	Efficient siRNAs	Avg efficiency score	LowComplexRegions	CAN
180	180	0	0	180	52.34	0	0

Appendix 1. Output file resulted from T7 primers design via E-RNAi online software. There can be distinguished: the forward and reverse primers' sequences (small letters indicate the T7 free hang sequence) and general features; the amplicon length and sequence; the expecting number of different siRNAs to be produced after cleavage, their efficiency and potential targeting (on-target, off-target, no-target).



Appendix 2a. Quality assessment of the dsRNA product via electrophoresis. The image displays the bands created after electrophoresis of the NPM dsRNA (1) and the GFP dsRNA (2) in 1% standard agarose gel. The 1kb Plus DNA Ladder (Invitrogen, life technologies®) used is apposed in the right side.

Sample	Concentration (ng/µL)	260nm / 280nm ratio	260nm / 230nm ratio
NPM#1	3,042	2.13	2.15
NPM#2	2,975	2.13	2.07
NPM#3	2,965.6	2.12	2.12
GFP#1	3,050	2.13	2.06
GFP#2	2983.7	2.11	1.96

Appendix 2b. Quantity and quality control overview. The table concentrates the results occurred from quality and quantity control performed in the final dsRNA solutions via NanoDrop® ND-1000 Spectrophotometer. The five samples used, along with their respective concentrations (in ng/µL), 260/280 ratios and 260/230 ratios, are illustrated above.

Addendum

1. Περίληψη

Τα εγγενή στάδια της ανάπτυξης του πλασμωδίου (*Plasmodium*) μέσα στον ανωφελή (*Anopheles*) φορέα του είναι κρίσιμα για την διαδικασία μετάδοσης της ελονοσίας (malaria). Η παρούσα εργασία αποτελεί μια συγκριτική μελέτη των αποκρίσεων που εγείρονται από τους δυο κύριους φορείς της ελονοσίας, των κουνουπιών *An. gambiae* και *An. Arabiensis,* κατά την μόλυνσή τους με φυσικούς πληθυσμούς πλασμωδίων του είδους *P. falciparum.* Η συνδυαστική προσέγγιση που εφαρμόστηκε περιλαμβάνει: i) την ταυτοποίηση του τρανσκριπτομικού (transcriptomic) προφίλ των δυο βιολογικών συστημάτων (κουνούπι-παράσιτο), η οποία διεξάχθηκε σε συμπάτριους πληθυσμούς, σε συνδυασμό με ii) τον λειτουργικό χαρακτηρισμό πιθανών ρυθμιστικών στοιχείων της άμυνας του *An. gambiae* απέναντι στα στάδια της εγγενούς ανάπτυξης του πλασμωδίου-μοντέλου στα τρωκτικά, *P. berghei*. Ο παράλληλος προσδιορισμός των τρανσκριπτομικών προφίλ των *An. gambiae* και *An. Arabiensis* σε τέσσερα διακριτά χρονικά στάδια (1,6,10 και 24 ώρες μετά την μόλυνση) κατά τα στάδια της εγγενούς και σπορογονικής ανάπτυξης του παρασίτου κατέδειξε σημαντικές δια-ειδικές και συνακόλουθες ειδο-ειδικές μεταγραφικές αποκρίσεις που αφορούν την άμυνα του κουνουπιού. Παρόλα ταύτα, το *P. falciparum* παρουσίασε μια ισχυρή ρύθμιση της γονιδιακής του έκφρασης, το πρότυπο της οποίας δεν αλλάζει κατά την μόλυνση των δυο διαφορετικών κουνουπιών, προτείνοντας πως το παράσιτο ακολουθεί ένα αυστηρό αναπτυξιακό πρόγραμμα κατά τα εγγενή στάδια. Επιπρόσθετα, πραγματοποιήθηκε περαιτέρω διερεύνηση και πειραματική εξέταση σηματοδοτικών μορίων του κουνουπιού, τα οποία παρουσίασαν σημαντική υπερ-έκφραση στα αρχικά στάδια. Σημαντικά, πειράματα που περιέλαβαν γονιδιακή σίγηση επαγόμενη από RNAi κατέδειξαν τον σημαντικό ρόλο ενός νέου νευροπεπτιδίου, του NPM (Neuropeptide M), στον έλεγχο των αντι-παρασιτικών αποκρίσεων του κουνουπιού. Ολοκληρώνοντας, τα δεδομένα αυτά παρέχουν σημαντικές πληροφορίες ως προς τις πρόσφατες προσπάθειες για ολοκλήρωση της κατασκευής του ιντερακτώματος (interactome) του συστήματος κουνούπι-παράσιτο, το οποίο αποτελεί τον ακρογωνιαίο λίθο των παρεμβάσεων που σταματούν την μετάδοση της ελονοσίας (Transmission Blocking Interventions,TBI).