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UNIVERSIDADE Ð COIMBRA

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"Contribution of Sperm Ageing in Epigenetics Signature of *Anopheles* susceptibility to *Plasmodium* Malaria Parasite"

ANOSPERM

Dissertation in Biomedical Research – Specialization in Infection and Immunity under the supervision of Doctor Catherine Bourgouin from Institut Pasteur and Doctor Professor Joana Barbosa de Melo from Faculty of Medicine of University of Coimbra. Presented to the Faculty of Medicine of University of Coimbra.

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Declaration:

I attest that this report is entirely my authorship.

The sources and works consulted are duly referenced, as well as the citations of these works clearly identified. Therefore, this dissertation does not contain any type of plagiarism, either from published texts, whatever the means of publication, including electronic means, or from academic works.

Acknowledgment:

When it feels scary to jump, that is exactly when you should do it.

I think there is no better quote to describe my path that culminates in this document.

There was no time in my life when I was more afraid but at the same time when I risked everything than in this master's degree. It was all in, and it would not be possible without my mother's unconditional love and support. It is to her that I dedicate this work. For all the support, all the love and for every time that I obtained from her was: "The NO is always guaranteed so go for it and allow you to fly".

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Palavras-Chave: Malária; Efeito materno; Envelhecimento espérmico; Sobrevivência; 6mA.

Resumo:

A malária é uma patologia transmitida por vetores, onde o *Plasmodium* (parasita causador da malária) é transmitido ao humano pela picada de fêmeas *Anopheles* e representa um enorme fardo em mais de 90 países endémicos. Além do diagnóstico tardio, um dos maiores problemas no combate à malária é a disseminação de resistência ao tratamento pelo parasita e disseminação da resistência aos inseticidas pelo vetor, o mosquito.Ruiz et al mostrou que infeção por *Plasmodium* induz modificações nas histonas em mosquitos *Anopheles*. O laboratório de acolhimento mostrou a existência de um efeito materno na sobrevivência da descendência, o que poderá influenciar a epidemiologia da malária. Uma vez que as fêmeas *Anopheles* só acasalam uma única vez em todo o seu tempo de vida, poderá haver uma influência paterna por detrás do efeito materno já detetado. Os efeitos parentais estão associados a mecanismos epigenéticos como a metilação do DNA, onde 6mA (N6-metiladenina) é exemplo disso e intrinsecamente associada a DMAD (enzima responsável pela sua de-metilação).

O principal objetivo deste projeto é decifrar a base molecular do efeito materno-paternal em *Anopheles*, mais especificamente a influência do envelhecimento espérmico na sobrevivência e suscetibilidade ao *Plasmodium falciparum* na descendência de *Anopheles gambiae*.

Uma análise fenotípica foi realizada de modo a desvendar a existência de efeito paterno. Para atingir o objetivo foram desenhados três cruzamentos: DP1my (fêmea velha x esperma jovem), DP1o (fêmea velha x esperma velho) e DP1y (fêmea jovem x esperma jovem). Neles foi realizado um ensaio de sobrevivência até o último mosquito morrer, e paralelamente foram analisados os padrões de expressão de METTL3 (gene envolvido na metilação 6mA de RNA) e de DMAD (a demetilase) em embriões com 24h de desenvolvimento, e fêmeas e machos *Anopheles* de cada cruzamento planeado.

Confirmámos o efeito materno na sobrevivência da descendência de *Anopheles* fêmeas, onde fêmeas mais velhas conferem melhores probabilidades, porém não foi clara a ligação com efeito paterno. No entanto, na análise das marcas epigenéticas a existência de efeitos parentais (materno e paterno) foi detetada, onde fêmeas mais velhas produzem descendência com níveis mais baixo de DMAD e METTL3, enquanto esperma mais

velho mostra um efeito contrário no padrão de de-metilação. Foi também realizada a análise de conteúdo proteico das amostras revelando que a análise de fases embrionárias do desenvolvimento de *Anopheles* são cruciais para mais detalhes sobre efeitos parentais.

Keywords: Malaria; Maternal effect; Sperm Ageing; Survival; 6mA.

Abstract:

Malaria is a vector-borne disease where *Plasmodium* parasite is transmitted by the bite of female *Anopheles* and represents a huge burden in more than 90 endemic countries. Beside the late diagnosis, one of the biggest problems on tackling malaria is the dissemination of resistance to treatment by *Plasmodium* parasite and even dissemination of insecticide resistance by the vector, the mosquito. Whereas Ruiz et al showed that the infection by *Plasmodium* in *Anopheles* induces histone mark modifications, the hosting lab showed that there is a maternal influence on the survivorship of the offspring, that may influence the epidemiology of malaria. Since female *Anopheles* mosquitoes only mate once in their lifetime it may exist a paternal effect behind it as well. Parental effects are associated with epigenetic mechanisms as methylation of DNA, as example, 6mA mark intrinsically associated with DMAD (responsible for its demethylation).

The main goal of this project was to decipher the molecular basis of maternal-paternal effect in *Anopheles*, more specifically the influence of sperm ageing on survivorship and susceptibility to *Plasmodium falciparum* of *Anopheles gambiae* progeny.

A phenotypical analysis was performed in order to unravel the existence of paternal effect. To reach the purpose three crosses were designed: DP1my (old female x young sperm), DP1o (old female x old sperm) and DP1y (young female x young sperm). Therefore, was performed a survival assay until the last mosquito die and the expression patterns of METTL3 (a gene initially involved in 6mA RNA methylation) and of DMAD a 6mA demethylase, were analysed on embryos of 24h development and female and male *Anopheles* adults from each cross.

We confirmed the maternal influence on the survivorship of female progenies, where older mothers confer better chances, however the paternal effect was not that obvious. However, on the epigenetics marks the existence of both parental effects was evidenced, where older females produce offspring with diminished level of DMAD and METTL3, while the older sperm show an opposite effect on the demethylation pattern. The protein content was analysed as well, revealing that the embryogenic stages are relevant for further detailed studies on parental effects.

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List of Abbreviations:

5mC	5-methylcytosine
6mA	N6-methyladenine
ACT	Artemisinin-based Combination Therapy
cDNA	complementary DNA
CpG	Cytosine-Guanine islands
DAMT-1	DNA N^6-methyl methyltransferase
DMAD	DNA N^6-methyladenine demethylase
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferases
DP	Daughter Progeny
DP1my	Cross old female x young sperm
DP1o	Cross old female x old sperm
DP1y	Cross young female x young sperm
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FP	Female progeny
H_2O	Water
HCl	Hydrochloric Acid
METTL3	Methyltransferase 3, N^6-Adenosine-Methyltransferase Complex Catalitic
METTL4	Methyltransferase 3, N^6-Adenosine-Methyltransferase Complex Catalitic
MP	Male Progeny
MT-A70	N^6-Adenosine-Methyltransferase Complex Catalitic
METTL	Methyltransferase-Like proteins
ncRNA	non-coding RNA
PCR	Polymerase Chain Reaction
pd(N)6	Random hexamer
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
S7	Ribosomal protein
sncRNA	small non-coding RNA
SP	Sulfadoxine-pyrimethamine

sRNA	small RNA
t-RNA	transfer RNA
VC	Vectorial Capacity

A. General Introduction

1. A light overview of Malaria:

Malaria is a vector-borne disease caused by protozoan of the genus *Plasmodium* [1,2], which are transmitted to humans by the bite of an infected female mosquito of the genus *Anopheles* [3].

As many as 200 species of *Plasmodium* parasites exist, but only 5 of them are infectious to human (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi*)[4,5]. The main agents of disease are *P. falciparum* and *P. vivax*, where the first one affects mostly Africa and the second one Asia [5–7].

Malaria is endemic in more than 90 countries, affecting approximately 40% of the world's population [8].

1.1. *Plasmodium* malaria parasite:

Plasmodium is the genus of the unicellular protozoan parasites that are known to be the special causative agent of malaria [1,2]. To date, around 200 species of *Plasmodium* have been formally described, however only 5 infect humans and cause illness: *P. falciparum*, *P. vivax*, *P.malariae*, *P.ovale* and *P.knowlesi*, the first two being the ones which cause a more severe infection, as previous stated [6,7] .

P. knowlesi has long been considered a monkey parasite and causes zoonotic malaria with severe pathology in adults, widely in Southeast Asia [2].

P. ovale infections usually cause less severe illness [9]. However, as in *P. vivax* infections, the parasites can remain dormant in the liver for many months, causing symptoms to reappear months or even years later [9].

The geographic distribution of *P. malariae* coincides with that of *P. falciparum*, affecting then Africa, Southeast Asia, the Eastern Mediterranean and the Western Pacific [10,11]. But *P. falciparum* infections account for more than 80% of malaria mortality and that is why receive the most attention due to its extreme importance in public health on a global scale [7,12]. The severity of infection is due to the ability of *P. falciparum* to modify the surface of infected red blood cells, creating an adhesive phenotype able to sequester in micro vessels [13,14].

The transmission of *Plasmodium* species between vertebrate hosts depends on an insect vector, which is always the female *Anopheles* mosquito for *Plasmodium* species infecting mammals [2]. The vector is not just a carrier, but the definitive host, where sexual reproduction of *Plasmodium spp*. occurs, and the development of the parasite in the insect is essential for transmission to the next vertebrate host [2].

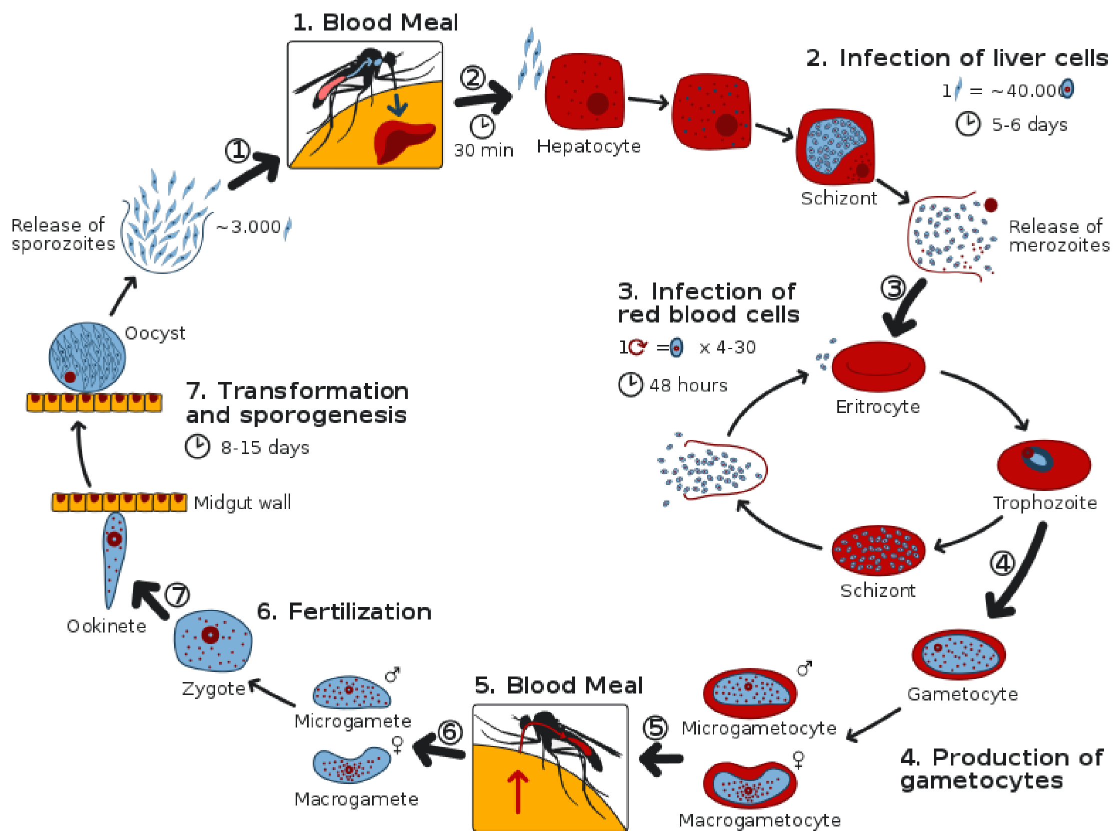

Figure 1 – Malaria life cycle. Life cycle of *Plasmodium* parasite in the *Anopheles* mosquito (left side) and in the human (right side). *Anopheles* mosquito infect the human host by bite (1). The sporozoite enter to the circulatory system and migrate to the liver, infecting the hepatocytes. There they multiply and develop into merozoites, damaging the hepatocytes and return to the bloodstream (2). Merozoites proceed to infecting RBCs, where they go through an evolution to produce more merozoites (3). Gametocytes are produced which the mosquito is going to take up with the blood meal (5). The gametocytes are going to mature in the mosquito's gut to gametes. The male and female ones are going to fuse (6). In this way, ookinete is formed and cross the midgut wall as oocyst, that will develop into new sporozoites and migrate to the mosquito's salivary glands (7). And now the new mosquito is ready to transmit the parasite to the next human host. (Adapted from: Own work, https://commons.wikimedia.org/wiki/File:Life_Cycle_of_the_Malaria_Parasite.svg)

Plasmodium parasites infecting humans complete their development from gametocytes to infectious sporozoites only in anopheline mosquitoes [15]. However, this does not mean that these parasites cannot differentiate from gametocytes in non-

anopheline mosquitoes, such as the *Culex* and *Aedes* mosquitoes, known for infecting birds [16,17].

Plasmodium spp. sporozoites in the salivary glands of the mosquito are delivered during the bite of the human by a mosquito (Figure 1 – 1)[18]. Sporozoites enter the circulatory system and colonialize the hepatocytes, where they go through an asexual pre-erythrocytic hepatic stage as hepatic schizonts enduring about fourteen days before the beginning of the blood stage (Figure 1 – 2) [19,20]. As they replicate inside hepatocytes, they structure motile merozoites that are subsequently delivered into the circulatory system, where they invade red blood cells (RBCs) (Figure 1 – 3) [19]. The interaction proceeds through sequential cycles of asexual replication of merozoites that pass through the ring, trophozoite, and schizont stages prior to framing and delivering new merozoites that therefore invade new erythrocytes, causing an increasing in parasite numbers (Figure 1 – 3) [21]. *P. falciparum* generates significant levels of blood stage parasites and is known to adjust the outer layer of infected RBCs, creating the adhesive phenotype associated to their high virulence, causing RBCs sequestration inside of small and medium-sized vessels, eliminating the parasite from the course for almost 50% of the asexual cycle [14]. A small part of intra-erythrocyte parasites change to sexual development, originating morphologically unmistakable male and female gametocytes (Figure 1 – 4) that reach at the host's dermis vessels and can be ingested by a mosquito, making it infectious to the next human (Figure 1 – 5) [20,21]. After ingestion by a female *Anopheles* mosquito, male microgametocytes go through an ex-flagellation process in the midgut of the mosquito, producing male microgametes that merge with female macrogametes to create a zygote (Figure 1 – 6) [22]. The zygote then reaches the ookinete stage which migrates through the mosquito midgut wall, matures into an oocyst, producing and upon rupturing, releasing numerous sporozoites which are scattered all through the mosquito's body, including the salivary glands, thus completing the life cycle (Figure 1 – 7) [22]. Gametocytes are therefore critically important to the malaria transmission cycle [20,22]. Clinical symptoms are, however, predominantly a result of the asexual stages of parasite replication in the human RBCs [21].

As we can attest that the malaria parasite develops in both humans and female *Anopheles* mosquitoes, the size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system, such as inducing complement-mediated death of gametocytes in the host's blood [8,23]. Complementarily in

the mosquito, it can prevent gamete fusion [24], induce gamete or ookinete complement death [8], prevent ookinete motility [8], and ultimately, penetration of the midgut wall and oocyst formation [8]. The parasite presents an incredible genetic flexibility to change over several life stages both in the human host and in the vector which makes it difficult to produce an effective vaccine, as it is difficult to aim a target that is in constant modification [8]. This same genetic flexibility allows as well adapting quick and so on developing resistance to antimalarial drugs [2].

1.2. The attempt of Prevention:

One of the possible ways to reduce malaria cases and consequently mortality in the world is to focus on prevention [25]. Despite this is being a very complex aspect associating high cost and an outcome not as favourable as desirable due to the countless variables to consider to an effective and efficient prevention [26].

Malaria prevention focuses on two pillars:

The first pillar is based on the appropriate use of chemoprophylaxis, where the use of antimalarial drugs must be balanced against the risk of acquiring malaria [27]. It balances numerous factors such as itinerary details: geographic area you will visit, whether it will be a more urban or rural visit, the type of accommodation you will use, the seasonal time of the trip and the duration of the trip, as well as the resistance patterns of the parasite itself [28].

Until nowadays there is any approved vaccination for malaria[29]. Then chemoprophylaxis is the only route of prevention used when traveling to continents with a high risk of contracting this disease (some countries in Asia and the African continent) [30].

The second pillar focuses on reducing vector-human contact. In a first instance, vector control of anophelines mosquitoes has countless variables to consider as species dependent, mosquito biology, epidemiological context, cost, and population acceptance [28]. The main basis of this second pillar is the adaptation of measures to reduce the contact between mosquitoes and humans. That can be reached by implementing the use of long-lasting insecticidal nets, control of oviposition sites, use of larvicides and indoor residual spraying [28,31] . These approaches are based on the behavioural tendency of the *Anopheles*

mosquitoes biting at night and often inside houses and resting inside the house after blood feeding [32,33].

Long-lasting insecticidal nets reduce malaria parasite transmission mainly by killing or blocking mosquitoes that attempt to feed upon humans under nets [34]. Indoor residual spraying kills mosquitoes and reduces longevity when they rest on insecticide-sprayed surfaces inside houses or other structures, usually they have already fed on humans [35].

The effectiveness of these approaches are based on the number of mosquitoes that are susceptible to the insecticides used, adequate coverage rates, quality and timely implementation, and user acceptance factors or compliance [34].

However, there are behaviours from the main vector that maintain residual transmission, like behavioural avoidance of house entry, diversion from contact with indoor treated surfaces or nets, and early exit from houses [36]. Such avoidance often occurs naturally but may also be due to insecticide-induced irritancy, repellence and/or toxicity [37]. As well avoid feeding upon humans when and where they are not protected or alternatively feeding upon animals in preference to humans [38]. Or having reduced contact with indoor treated surfaces or nets and resting outdoors away from indoor treated surfaces [38].

Long story short, malaria is a difficult disease to control due to the parasite and mosquito high adaptability to the barriers created [39].

1.3. Treatment, a way to help resistance:

The treatment of malaria has undergone some changes in recent decades in response to increasing resistance of the parasite, *P. falciparum*, to drugs and due to the increasing resistance and behavioural change by the vector, *Anopheles* mosquito [40].

For an effective treatment, the diagnosis must be effective as well [41]. One of the causes for the high mortality associated with malaria comes from late diagnosis, that fact leads to complication the treatment much more [9,41].

The clinical symptoms of malaria result from the replication of *Plasmodium* asexual stages in human red blood cells [42]. These include irregular fever, headache, and malaise, which are common symptoms to other pathologies also, so in the end complicate

a quick diagnosis of malaria [43]. Only 20% of patients develop vomiting, and less than 5% experience diarrhoea [43].

Therefore, for a quick and effective diagnosis it is very important to know the patient's history, more specifically in the last 2 months whether he whether travelled to an area with a high incidence of malaria cases, although the normal incubation time is only 2 weeks the symptoms may take longer time to develop [44].

The treatment of malaria, like of any other infection, depends on the severity of infection and the patient's characteristics (age, immunity, susceptibility to treatment, etc.) [45]. Even more important is to review the geographic region to see the prevalence of parasite resistance recorded [28].

So, there are two types of treatment, per say, one for endemic countries and another slightly different just for occasional travellers.

In endemic countries the treatment to malaria is based more on prevention (adaptation of prevention measures), sounds odd but it is believed that the exposure to the parasite and early detection of infection is the foundation of treatment, because will create immune responses to a frequent infection[46]. However, to pregnant women and infants it is administrated an intermittent preventive treatment, one dose of sulfadoxine-pyrimethamine (SP)[46]. To the seasonal malaria it is used a chemoprevention where children with less of 6 years receive a monthly dose of amodiaquine[46]. In case, of infection and early detection (by other words: uncomplicated malaria) is administrated an artemisinin-based combination therapy (ACT), for 3 days [46].

The treatment to a non-immune travellers is the same as the previous stated, if we are talking about uncomplicated *P. falciparum* infection[46].

One crucial key to the success in treating malaria is the early detection of it, however it does not always happen … Then, in case of severe malaria, the treatment is administering artesunate for 24h and after completing the treatment with 3 days of ACT[46].

Nowadays, the usual treatment to malaria infection are ACTs, but not always was this one. The first treatment to malaria was chloroquine, discovered 75 years ago [47]. However after 10 years of use chloroquine resistance started to emerged including in endemic areas (as showed in Figure 2) [47]. Therefore, it was needed to discover an alternative … So, it was around that time that appeared ACTs.

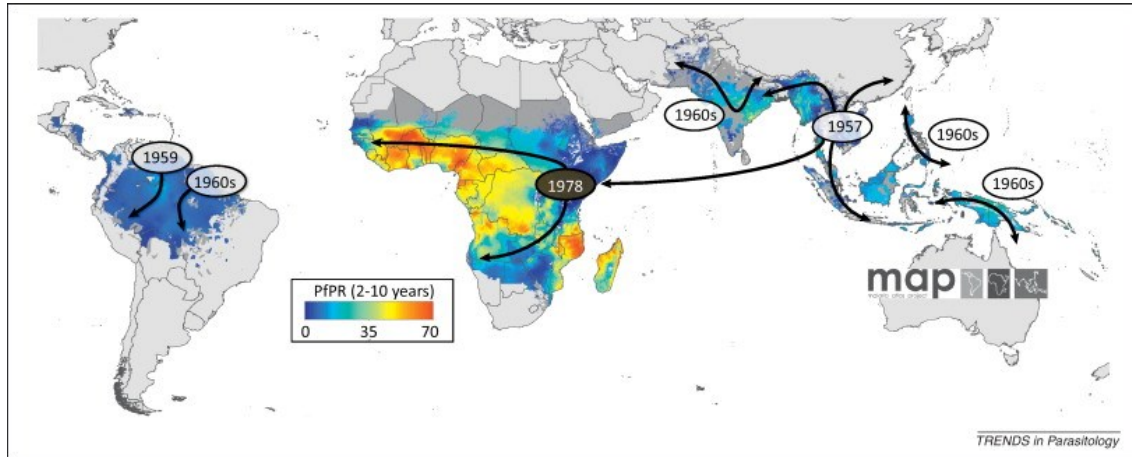

Figure 2 – Global spread of *P. falciparum* chloroquine resistant. Chloroquine was starting to be used as treatment around the 50s, however after 10 years of using the resistance by the *P. falciparum* started to arise. Began on the Southeast Asia, South America and the Western Pacific region and lastly progress to Africa [47]. (Adapted from Ecker et al [47])

Artemisin by itself it is not effective due to the rapidly elimination on the system and in the meanwhile the resistance to this drug is starting to emerge [48]. However, when conjugated with a partner drug, as amodiaquine, this elimination occurs more slowly, allowing a high effectiveness in the treatment [48]. Nevertheless, the possibility of resistance to one of the partner drugs is always an appalling possibility [48,49].

Although malaria affects about 241 million people and still killed around 627 thousands of children 2020 [12], it is still not in the interest of the pharmaceutical industry to develop new antimalarial drugs, as the risks are high, and the financial return is low [50]. It is not rewarding to develop a drug that is 90% effective but is only delivered to the 20% of the population that really need it [50]. The treatment and eradication of malaria is very dependent on the evolving health system and the operational component of access to treatment [51].

Frightening is if continuing the pace of increasing parasite resistance to malaria could become untreatable in some parts of the world by the beginning of the next millennium [49].

1.4.Social and Economic Burden of Malaria:

Malaria is a parasitic infection of global importance affecting 241 million people in the 2020 in 85 malaria endemic countries [12]. Most of the cases are centered in regions like sub-Sahara Africa, Southeast Asia, Western Pacific and Latin America, [4], so most of

the time associated with the concept of poverty, however due to the migratory currents became a problem in developed countries as well [52]. Some attempts have been made to define the economic cost of malaria, but it is difficult to come up with a number due to the innumerous variables such as personal expenditures on prevention, diagnosis, treatment and care of the disease [26]. But it is obvious that is unbearable to countries with lowest income [26].

This dissemination of malaria brings up a huge problem with it… Geographic expansion of drug resistance of *P. falciparum* followed by the perseverance of the mosquito due to increased resistance to insecticides and modification of the behavior [53,54].

Due to the late diagnosis of malaria, treatment and dissemination of resistance by the *Plasmodium* parasite and even by the vector, mosquitoes Anophelines, malaria still one of the infectious diseases with high morbidity and mortality associated [55].

Therefore, it is crucial to increase the knowledge of fundamental mechanisms involved in malaria resistance and transmission to have a more effective counterattack.

2. *Anopheles*, not only the mail carrier of disease:

Anopheles is a genus of mosquito, first described in 1818[56], belonging to the order *Diptera* [57], which contains more than 400 species[56]. However only 30-40 species can transmit the parasite that causes malaria to humans [58].

Drosophila and *Anopheles* mosquitoes diverged 260 million years ago [59]. As *Drosophila* it is an animal model very well-known [60], most of the times it is used as a reference to research on mosquitoes, due to the similarities between them [61].

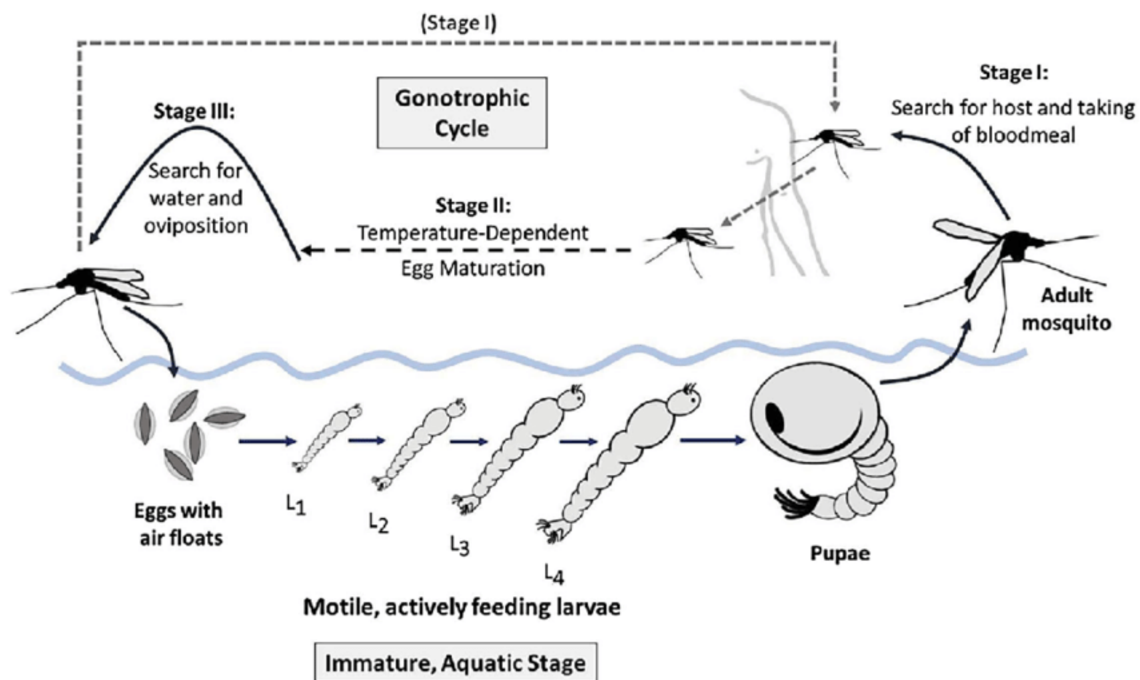

Figure 3 – Development of *Anopheles* mosquito through life. *Anopheles* mosquitoes pass through four stages in their life cycle. The first 3 stages (egg, larvae (L1 to L4) and pupae) are aquatic. The last one (adult) is outside water phase. (Adapted from K. Okuneye et al [62]).

Anopheles mosquitoes pass through four stages in their life cycle: egg, larvae, pupae and adult, as presented in figure 3 [63]. The first three stages are aquatic, and last between 7-14 days, this duration is strongly influenced by ambient temperature [63]. After emergence, it takes roughly 2-3 days to the male mosquitoes to be ready to mate[64], while the female ones are immediately ready after emergence[65]. After the female has been inseminated and has taken a blood meal it takes around 48-72 hours for her to lay eggs[65]. Each adult female can lay 50-200 eggs per oviposition, they are laid directly on water [63]. On the larvae stage the head with mouth brushes is well developed for feeding, presents as well a large thorax and a nine-segment abdomen, in this stage they breathe through a

non-well developed respiratory siphon[66], and they use the mouth brush as motor of propulsion to swim [63]. The pupae stage, well known for its coma-shape body [63], is the transitional phase between larva and adult, it is in this phase that undergoes radical metamorphosis, and the head and thorax merged into a cephalothorax with the abdomen curving underneath [63]. The pupae breathe from an non-well developed spiracle (denominated by trumpet) [66]. It is during the adult phase that the mosquito can turn on as malaria vector, but only female mosquitoes can transmit malaria to the host because they are the only ones that bite[67].

The anopheline mosquitoes' body have three sections: head, thorax and abdomen [63]. Head is specialized for acquiring sensory information and for feeding [63]. The thorax with three pairs of legs and a set of wings attached is specialized for locomotion [63]. The abdomen is specialized for digestion and egg development (in case of females) [63].

Anopheles female mosquitoes are distinguishable from the male mosquitoes due to the palps, which are longer as the proboscis (biting stiletto), and by presenting discrete blocks of black and white scales on the wings [63]. *Anopheles* are frequently identified by their typical resting position: abdomen sticking up in the air, rather than parallel to the surface (figure 1A) [63]. The male *Anopheles* are distinguished from female *Anopheles* by presenting a smaller size but the most stand out characteristic is the brushy, hairy antennae [68], as it showed by the figure 1B and C. However, these differences are common to other mosquitoes' species from *Diptera* order.

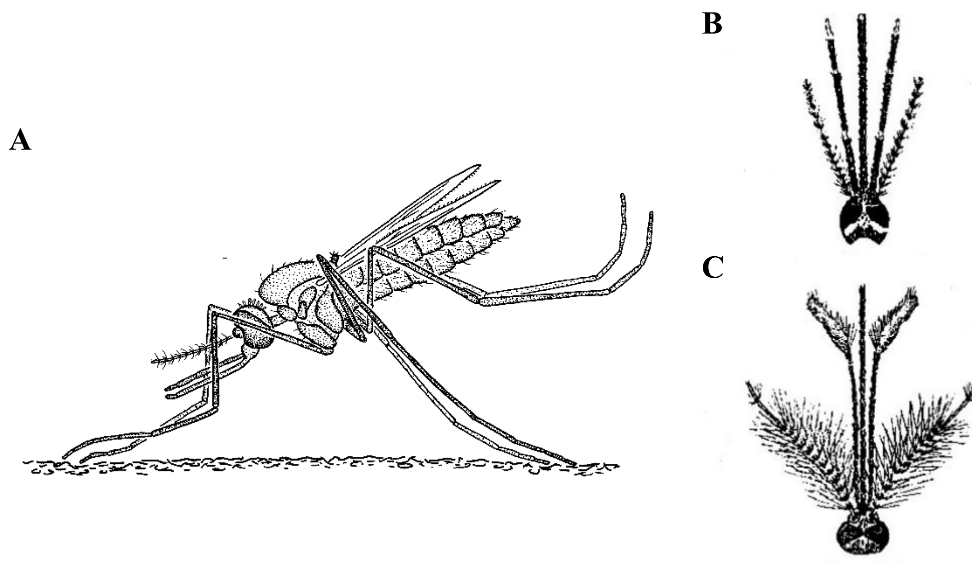

Figure 4 – Distinctive traits of *Anopheles gambiae* mosquitoes. A – Representation of the characteristically resting pose of anopheline mosquitoes[69] (Adapted from: https://www.publichealth.com.ng/types-of-

mosquitoes/anopheles-mosquito) . B – Representative image of the head of a female *Anopheles gambiae*, where the maxillary palps are the same size as the proboscis[68] (Adapted from Williams and Pinto[68]). C – Representative image of the head of a male *Anopheles gambiae*, where they present feathery antennae. The best trait to help to distinguish male and female anophelines mosquitoes are these characteristics of the head[68] (Adapted from Williams and Pinto[68]).

Males live for about a week and feed on nectar and other sources of sugar [70]. Males don't feed on blood because naturally they don't bite, unlike the females [70]. Females besides feeding on nectar and other sources of sugar have the ability to blood feed [71]. Typically, 48 – 72 hours after blood feeding the female mosquitoes lay eggs on water[63]. This cycle repeats itself until the female dies, therefore they can lay eggs more than once [72]. Since the act of searching for a host to blood feed, the ingestion of blood meal, digestion, and maturation of ovaries until the laying of mature eggs at the oviposition site is denominated the gonotrophic cycle (figure 3) [73]. Female *Anopheles* mosquitoes are known for mating only once in their lifetime [74].

Within the genus *Anopheles*, the best known and more investigated is *Anopheles gambiae* due to its dominant role in the transmission of the most fatal parasite when it comes to malaria, *Plasmodium falciparum* [75].

Anopheles gambiae complex or *Anopheles gambiae sensu lato* is composed of 8 species morphologically indistinguishable, recognized only in the 1960s [76,77]. This species is also capable to transmit other parasites as *Wuchereria bancrofti*, which causes lymphatic filariasis [78].

The complex consists of: *Anopheles arabiensis*, *A. bwambae*, *A. melas*, *A. merus*, *A. quadriannulatus*, *A. gambiae sensu stricto*, *A. coluzzi* and *A. amharicus*[76,77]. Despite of the species of the complex are morphologically similar, they exhibit different behavioural traits between them[77].

Anopheles mosquitoes are crepuscular, active at dusk or dawn, or they are nocturnal [79]. Some *Anopheles* mosquitoes feed indoors while other feed outdoors, and have the same behaviour after blood feeding, rest indoors or rest outdoors [32,33].

Anopheles gambiae sensus stricto, for example, prefers to feed on human instead on animals, so is considered anthropophilic[33], whereas *A. quadriannulatus* prefers animals, being zoophilic [80].

Understanding the biology and behaviour of *Anopheles* mosquitoes can aid in designing appropriate vector control strategies [81], for instance, affecting factors that disables the mosquito ability to transmit malaria (like messing with innate immunity to

Plasmodium parasite and/or longevity) [82]. Note that long-lived species that are anthropophilic are the most dangerous, as it is the case of *Anopheles gambiae* and so it is one of the most efficient malaria vector in the world [75].

Once the parasite is ingested by a mosquito, it develops in the mosquito for 9 days or longer, before it is transmitted to the vertebrate host [15]. Thus, if the female mosquito does not survive longer than the extrinsic incubation period (time that it is necessary to the parasite develop in the vector), she will not be able to transmit any malaria parasites [83]. The majority of mosquitoes do not live long enough to transmit the parasite, but some may live long as three weeks in nature [84]. So, any control measure that reduce the average lifespan of the mosquito population will reduce transmission potential [85].

However, bear in mind that vector control is a moving target with the globalization and demographic changes, so the infection patterns will change as well, and the current unprecedented shift of the environment on a global scale affect rates and patterns of vector-borne diseases in a totally unknown way [86].

2.1. *Anopheles* mating behaviour:

One of the critical behaviours that define the mosquito life strategy is mating, but this one is probably the least understood and the most many-sided behaviour [65].

Mosquitoes depend on sexual reproduction for species maintenance as every other species, so this event should receive attention when is to seeking new approaches for vector control and interventions for mosquito-borne disease [65].

In *Anopheles gambiae* the optimal mating moment occurs with 5–7-day-old males [64]. Females, otherwise, are ready to mate almost immediately after they emerge from the pupal stage [65]. In anopheline mosquitoes the mating encounter starts in flight [87,88].

Figure 5 – Mating of *Anopheles gambiae* mosquitoes. Mating position of *Anopheles gambia*e mosquitoes, engaged by the end of the abdomen (cercus). Female mosquito presented on the right, showing a bigger size comparative with the male on the left. (Source: Sam-Cotton, www.scienceupdate.com)

Most of the females deposit eggs when mated, however some virgin females are able to lay developed eggs meaning that mating in some instances is not required for egg deposition and oviposition[89]. Female mosquitoes mate before taking the first blood meal, and afterwards around 48-72 hours after lay eggs on still water[65]. The blood meal is essential for the development of a metabolic reservoir [90]. Note that anopheline mosquitoes only mate once in their lifetime [91].

The male body size in *Anopheles gambiae* has no effect on the mating choice by the female [92]. By contrast, female body size can give an advantage in mate selection where larger females are the preference [93] (aside, larger females has bigger probability of survival as well [94]). However, it appears that the age of the male might influence the mating success, where the younger ones are the winners [95].

Mating in mosquitoes remains an ongoing research field, because successful mating is critical for the success of strategies to vector-borne-disease [65]. A proper understanding of mosquito population biology and genetics, as well as the male feeding behaviour and fitness, location of mating, pre and post-mating behaviour can provide crucial cues to better tackle vector-borne-disease [65].

2.2.Light overview of immune defences in Mosquitoes:

About 60 of 422 known anophelines species worldwide are considered relevant malaria vector [96], due to the fact the variation between species and the ability to transmit

the parasite to the humans [97]. This variability is often attributed to the variety of physical and biochemical factors that could act on the mosquito [97,98]. The most dominant species are *Anopheles gambiae sensu stricto*, *Anopheles funestus sensu stricto* and *Anopheles arabiensis* (this last one only in Africa) [76,99].

Immune defences include physical barriers and internal molecular responses (immune defence). Mosquitoes' physical barriers include the cuticle covering the epidermis, synthesis of the peritrophic matrix in the midgut lumen and the chitin lining of the tracheal system [100]. As immune response, there are two principal mechanisms: the humoral and cellular components [101]. As mosquitoes possesses an open circulatory system and the organ tissues are exposed to a stream of hemolymph, where the immune responses occur [101].

The immune system of mosquito has an humoral answer, as opsonins or coagulation factors, and that triggers a cellular response like phagocytosis and encapsulation, melanisation and coagulation [97]. The mosquito can also produce antimicrobial peptides that are mostly produced by the body fat [100].

So, for a mosquito to become a vector of malaria the parasite must overcome the attacks that mosquito's immune system.

2.3. *Anopheles* and malaria parasite, the beautiful friendship:

The parasite-mosquito interactions are complex and look like war.

Beside the physical barriers, the innate immune system imposes a significant challenge for parasite development in the mosquito [102]. The mechanisms that operate in response to a *Plasmodium* infection are largely complex, yet the mosquito appear to sense the presence of the parasite in an infected blood meal [103]. The mosquito innate system plays an important role in controlling malaria infection and the strength of parasite clearance is under genetic control [104].

The microbiota of *Anopheles* mosquitoes interferes with mosquito infection by *Plasmodium* parasite and influences mosquito fitness, thus vectorial capacity can be compromised [83,105]. The gut, salivary glands and reproductive organs are colonised by a symbiotic dynamic microbial community known as microbiota [105]. The origin of mosquito's microbiota is from its mother's genitalia and from its larval and pupal breeding site [105]. It is known that the depletion of the gut microbes in *Anopheles gambiae*

hinders the larvae develop into adults, on the one hand, but on the other hand increases adult susceptibility to *Plasmodium* [106,107].

So, vectorial capacity (VC) relies on genetic and environmental factors, as it demonstrated on the formula of figure 4 [83].

$$VC = \frac{m \cdot a^2 \cdot b \cdot p^N}{-\ln p}$$

Figure 6 – Vectorial Capacity Equation. VC represents the ability of a population of vectors, in this case *Anopheles* mosquitoes, to transmit pathogens (*Plasmodium* parasite) to a host (human). Depends on: m (mosquito/human ratio, influenced by the fecundity, mating choice and lifespan); a (biting rate); b (probability of mosquito infection, influenced by immunity of itself); p (daily survival rate); and N (extrinsic incubation period)[83].

Only females *Anopheles* can transmit the parasite [3]. Although, if they ingest an infectious blood meal, they only become a vector if the parasite is capable to go through the bottlenecks in the gut and salivary glands (Figure 1 - 6 and 7) [105]. The microbiota can affect the ability of transmission of disease by interfering with *Plasmodium* colonisation in the gut, affecting different aspects of mosquito fitness like its lifespan [83].

The microbiota interferes with the parasite colonisation through at least two mechanisms: stimulation of the mosquito localized immune response and production of metabolites that impair the parasite survivorship [105].

The microbiota may also affect the parasite nutritionally in the mosquito gut [108]. The parasite to develop requires a high nutritional amount from the mosquito, so it can become a limiting factor in host colonisation [109].

On the parasite development, when the sporozoites are released from mature oocyst into the haemolymph, most of them are eliminated due to circulating immune components in the haemolymph [110]. In the end, only around 20% are able to reach the salivary gland lumen (Figure 7) [15].

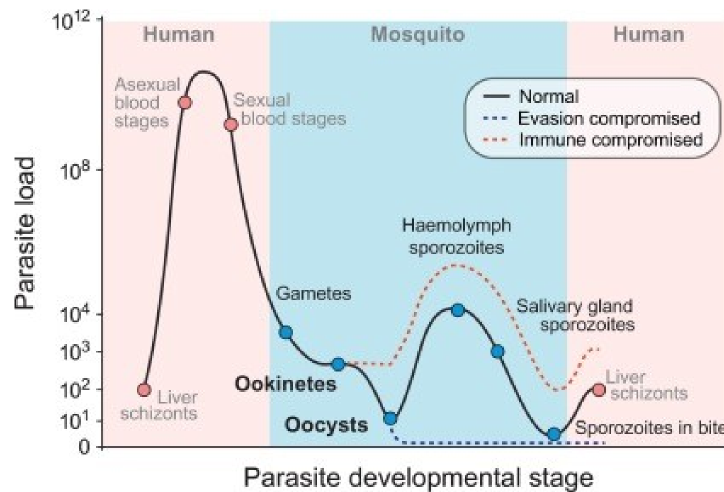

Figure 7 – Estimated parasite numbers during the different phases of its life cycle. Through the whole life cycle, *Plasmodium* parasite has to overcome several immune barriers imposed by the vector, *Anopheles* mosquito, and by the vertebrate host. It is observed a significant decrease in the parasite load [111].

The parasite-mosquito interactions are complex and look like war. Infection by the parasite is responsible for decreasing the mosquito fitness, as counterattack the mosquito's immune system provoke the same outcome on the parasite fitness[104]. *Plasmodium* infection affect the salivary enzyme, apyrase, demanding that mosquito bite more time to feed hence the transmission of sporozoites would increase but that allow the mosquito being detected and killed while feeding [104,112].

Unravelling the *Plasmodium-Anopheles* interaction contributes to a better knowledge of mosquito vector competence and promises to help onto the development of new malaria control strategies [104].

3. Epigenetics as a mechanism decoder:

Epigenetics, originally coined by Conrad Hal Waddington in 1942 [113], is a promising area in biology as a decoder of mechanisms [114] defined by stimuli-triggered change in gene expression without altering the genetic sequence, linking genotype and phenotype during development[113]. Epigenetic changes can occur through: DNA methylation; a broad spectrum of histone and chromatin modifications (methylation, acetylation, phosphorylation, and ubiquitination); small non-coding RNA (sncRNA); and even long ncRNA [115], which will give origin to the epigenome [114].

Epigenetic modifications can be transgenerational or inter-generational [113]. Transgenerational effects are described as non-sequence-based effects that can be transmitted from one generation to the next one [116]. Inter-generational effects refer to parental effects and are the result from stimuli on the parent that create an epigenetics change in the embryo [117].

Epigenetic mechanisms are considered "soft inheritance", allowing an easier adaptation to fluctuating environments and nutrition [118].

Therefore, the environment can influence gene expression, and can lead to disease [119]. Epigenetics has been showed a growing relevance on research of infectious diseases giving birth to a new research field: epigenetic epidemiology[114,120]. However these studies confront a causality problem, "chicken-and-egg" dilemma, where the association between the disease phenotype and the epigenome is difficult to establish [114,121].

Many of the advances in this field is owned by a simple model system, the fruit fly *Drosophila*, due to many epigenetic pathways that have been extensively described in this species, and the large number of tools that are available [122]. *Drosophila* is a model well conserved as well known, being this way a great foundation for research [123,124] .

Epigenetics is the foundation for a better understanding of parental effects and susceptibility to diseases and infection [113].

3.1.DNA methylation – the beginning of history:

DNA methylation is one of the mechanisms behind the epigenome, and is a potent way of transcriptionally silencing genes in eukaryotes [125].

The methylation of the genes can occur partially, and the degree of methylation is correlated with how active the genes remain[126]. DNA methylation could be responsible for heterochromatin formation and transposon silencing [127].

In many organisms methylation occurs at cytosine bases across the entire genome, but it is more frequently found at CpG (cytosine-guanine) islands[114]. The enzymatic mechanism behind DNA methylation is well understood now, with the recruitment of multiple DNMTs (DNA methyltransferase proteins) [128].

In eukaryotes the most common modified base is 5-methylcytosine (5mC) whereas in prokaryotes the most prevalent is N6-methyladenine (6mA) [129–131]. 6mA mark appears at extremely low levels in eukaryotes, and it is intrinsically associated with action of demethylases-mediated demethylation process (having that said, this mark can be reversed) [132].

6mA modification is ordinarily found in microbial genomes and it is linked to important regulatory functions in numerous biologic processes, as DNA replication or transcription, and host-pathogen interaction (as cellular defence mechanism) [135] .

Around 2015 there was a change of paradigm due to developments in *Drosophila* and *Caenorhabditis elegans* knowledge suggesting a small RNA (sRNA) as a component of the machinery that targets heritable silencing[113]. That modulate transcription mechanism which can be initiated and sustained in a more permanent form, being in that way heritable to the forward generation if this sRNA trigger is absent, being associated with DNA methylation [113].

And more recently, the 6mA mark has been identified in multicellular eukaryotes as *C. elegans* [133] and *D. melanogaster* [132].

6mA modification in *C. elegans* has raised questions about the paradigms of transgenerational inheritance [133]. Do 6mA mark carry any epigenetic information across generations[130]? Might 6mA mark communicate with other heritable epigenetic marks that reciprocally regulate the levels of 6mA[130]?

The presence and role of 6mA modification is well known in prokaryotes, however in higher-eukaryote cells remains a mystery [134], but shows promise as a new conserved layer of epigenetic regulation [131]

6mA modification is tightly associated with a family of DNA and RNA methyltransferase, MT-A70, which include members of the methyltransferase-like (MTTL) family in mammals, including the METTL3 (initially known as an N6-adenosine RNA methyltransferase) and METTL4 (a homolog of DAMT-1, a DNA and RNA methyltransferase found in *C. elegans*)[130,133,135]. If the exact same enzyme catalyses both RNA and DNA adenine methylation in different organisms remains an open question [130]. Another prevalent one is if whether N6-adenine methylation of DNA is co-ordinately regulated with N6-adenine methylation on RNA [130].

This modification, 6mA DNA, is present in *Drosophila* genome at a considerable level, 6mA modification is dynamic and tightly regulated by the *Drosophila* Tet homolog, DNA 6mA demethylase (DMAD), during embryogenesis and tissue homeostasis [132]. It has been proven that DMAD directly catalyses 6mA demethylation *in vitro* and *in vivo* [132] and DMAD-mediated 6mA methylation is correlated with transposon expression [131].

In *Anopheles* and *Drosophila* only one gene encodes a putative DNMT: DNMT2. This gene was shown in *Drosophila* that actually encodes a t-RNA methyltransferase [136].

The knowledge of this epigenetic mark in *Anopheles* is superficial and an active research field.

3.2.Epigenetics, the origin of parental effects:

Parental effect consists of any effect on progenies phenotype that is not determined by the progenies' DNA, but instead is acquired by the genotype or environmental experience of its parents, defined by epigenetic inheritance, that can be originated by DNA methylation, histone modification and/or sRNA transmission[137,138].

It is often assumed that parental effects are mediated solely by the mother, due to the fact of mothers invest more resources in production and care of offspring[139–141]. So, maternal effects are more under the scope than paternal effects. However, recent studies on transgenerational epigenetics effects prove otherwise, suggesting that on this field the paternal effect is the most viable candidate[142]. Nevertheless, the effect of the paternal

environment or the potential for joint effects of both parents' environments on offspring performance remain poorly understood in most the species[143].

Past environmental conditions, specially experienced by the mother, are considered relevant in shaping offspring phenotype and fitness, moreover, they show to have a role play in determining the way offspring respond to current environmental conditions[144–146].

In invertebrates, the influence of the age of the mother and nutritional conditions has been proven to have an epigenetic effect on the offspring[122,147,148].

Effects of transgenerational immunity on invertebrates has not been systemically investigated, however there is evidence in vertebrates that the offspring can inherit maternal immune function through antibodies[149,150].

Mosquitoes are well-suited for testing the hypothesis of parental influence on the offspring. The female mosquitoes usually mate only once in their lifetime and retain sperm in the spermatheca to fertilize the eggs[151]. The mosquitoes' dynamic population depends tightly on environmental factors, as temperature and its daily variability[152,153].

On the mosquitoes' population there is already proof of maternal environmental influence on the population dynamics and susceptibility to malaria, and consequently the epidemiology of malaria[154].

In *Anopheles stephensi*, the daughters of mother held on environment of starvation feed up more blood and lay more eggs, than the daughters of well-fed mothers, even if the daughters experience the same environment[144]. Usually, food deprived mothers are not able to compensate their poor environment, and therefore have offspring less fitness[155,156].

Nutrition is linked as well to the susceptibility of the vector to infection and its severity, where food stress can exacerbate the harmful effects of it [154]. The nutrition of the offspring and its mother's environment impact directly its immune response[154].

Besides the mother's nutritional effect, we are faced with the effect of the mother age on the offspring where, results from the hosting lab, show that progenies of older females exhibit: a higher survival rate and are more susceptible to the *Plasmodium* malaria parasite, possibly influencing this way the epidemiology of malaria. Ruiz et al. show that *P. falciparum* infection induces histone mark modifications on specific gene sets of *Anopheles gambiae*[157,158]. The hosting lab obtained preliminary results that there is indeed a 6mA modification on *Anopheles gambiae* embryos, and that mark increases

with the age of the embryos [157]. And importantly, the abundance of the 6mA mark in progeny embryos differ with the age of the mother.

While the host team proved the existence of maternal effect on progeny survival and susceptibility to *Plasmodium* infection, a potential paternal effect on the survival phenotype have not been addressed.

Since *Anopheles* mosquitoes only mate once in their lifetime[74], is there any paternal influence on the maternal effect already discovered? Progenies of old females result from fertilization events from aging sperm. This conjecture is supported by increasing evidence in mammals that sperm RNA can drive transgenerational stress inheritance[159].

3.3.Epigenetics on host-pathogen interactions: A story of two

Since there is a maternal effect due to diet and other environmental factors that can affect her offspring by shutting off or not the gene expression, why could not parasites influence it too[160]?

Host-pathogen interactions are plastic and dynamic, they must cope with the selective constraints imposed by their host, imposing that way evolution to the pathogen[121,161]. There is a usual assumption that parasitism can directly modulate host phenotype, and in some cases influence as well the offspring of the host indirectly[160]. So, pathogen-induced effects in host phenotype may have transgenerational consequences[121].

In this relationship, host-pathogen co-adaptations occurs frequently and quickly[162]. Epigenetic modifications can provide an accessory source for this fast adaptation, however reversible, but directly shaped by host and pathogen selection pressure[121,137,162].

One curious aspect of pathogens is the morphological and developmental plasticity which is tightly linked to their survival and transmission in the host[121]. The pathogen can affect the regulation of transcription of the host as well tackle the host's expression of genes associated with virulence processes, allowing to conquer the host system[163].

Epigenetic control of virulence factors is noticeable in several microbial pathogens[164]. DNA methylation is also an essential regulatory mechanism of virulence in pathogenic bacteria[165].

A surprisingly characteristic that many pathogens can manipulate on the host is their reproductive biology, then in case of vector-borne diseases as malaria can be a crucial missing piece for a better solution[166].

There is a lack of knowledge of epigenetic inheritance in host-pathogen interactions, like transgenerational immune priming in invertebrates or transgenerational infection effects on host behaviour (most known by maternal effects) is something that should be tackle [121,150].

What it is known is too little… *Plasmodium* parasite contains a rich repertoire of histone variants, chromatin, histone modifying enzymes and RNA-mediated silencing mechanisms[167]. *Plasmodium* infection on *Anopheles* mosquito induces significative changes in phenotypic traits that influence vector competence[158,168].

Adding to this, there is substantial variability in the response of the mosquito to the infection that depends on genetic and environmental context[169,170].

The mechanism behind the regulation of phenotypic responses to *Plasmodium* infection are little understood[158].

At the molecular level, *Plasmodium* infection induces changes in gene expression in mosquito tissues that has immunity and reproductive functions, but the molecular players behind these modifications are poorly known[158].

There is a considerable amount of work on the genomic basis of resistance to infection but a weak link to epigenomic studies[171].

Beyond the shadow of a doubt, unravel the causative relationship between the host epigenetics and the pathogen infection and the tiny player on these mechanisms will provide novel targets for drug development, like an epigenetic therapy[121].

4. Aims of dissertation:

As pointed out, the lack of knowledge of the model of transmission of malaria with insight to the vector, *Anopheles gambiae*, and with the recently open door to the maternal effect age-based on invertebrates on the survivorship and lastly the importance of epigenetics marks, this dissertation was born.

So, the main objective of this project is to decipher the molecular basis of the maternal-paternal effect in *Anopheles* mosquitoes, more specifically the influence of sperm ageing on survivorship and susceptibility to *Plasmodium falciparum* of *Anopheles gambiae* female progeny.

This will be addressed in three distinct approaches:

1. Comparing the survival rate of progenies of old females mated young (old sperm) to that of progenies of old females mated while old age (young sperm), to prove if there is any paternal effect associated with the previous maternal proven effect.
2. Establishing a purification pipeline of sperm from the spermatheca of young and old female *Anopheles* to facilitate future protocols with a similar aim.
3. Analysing the 6mA methylation on DNA of those samples of progenies from the different crosses, old and young sperm that fertilize females of same age. And a parallel follow up of the *Anopheles gambiae* DMAD demethylase ortholog.

B. Materials and Methods

1. Mosquito rearing conditions:

Anopheles gambiae Yaoundé strain (Tchuinkam et al., 1993) established at the Institut Pasteur in 1998 was produced under standardized laboratory conditions. All cages were medium (25x25x20 cm) or small (20x15x20 cm) and were maintained under the same conditions: in the greenhouse at 26°C ±1 with 70% humidity and a 12/12-hour light/dark cycle. For colony maintenance, females were blood fed twice a week on an anesthetized rabbit, following French regulation for animals (Agreement #A 75-15-01).

The larvae to generate new mosquitoes were reared at the same conditions of temperature, photoperiod, and humidity. However, larvae were fed on Tetramin® baby fish powder until reaching the L2 stage and then with cat food.

When adults, the mosquitoes were fed with 10% sucrose solution and a wet cotton on the top of the cage. These were replaced every 2 days.

2. Creation of Parent's population:

To analyse the paternal effect on the survivorship of the daughter progenies (DP), 3 different crosses were created where the only variables at play are the age of the mother and the sperm ageing (See figure 8). The initial mosquito population received 2 blood meals (BM) to be able to obtain old and young mosquitoes for establishing the different crosses. Each condition was duplicated.

In cage 1, we crossed 300 virgin females with 150 males added 11 days later, the males being younger than the females in this cross. In other words, this cage corresponds to a cross of old female mated with young males and therefore harbouring young sperm, giving rise to DP1my (**D**aughter **P**opulation **1**BM **m**ale **y**oung).

In cage 2, 300 virgin females and 300 males coming from the same emergence were let for free mating for 12 days. It is important to indicate that free mating with this *Anopheles* colony occurs during the first 3 to 4 days of mixing virgin females to virgin males. So, this cage corresponds to a cross of old female mated young and therefore harbouring old sperm, giving rise to DP1o (**D**aughter **P**opulation **1**BM **o**ld).

Lastly, in cage 3, 100 virgin females were crossed with 150 males that emerged at the same time from the second BM of the initial population. In other words, this cross corresponds to young female x young male, thus producing DP1y (**D**aughter **P**opulation **1** BM **y**oung).

All 3 cages received a single BM on the same day (see figure 8) and eggs were collected 72h after feeding, therefore cages 1 and 3 had 4 days of free mating, while cage 2 mated between 10 and 12 days earlier.

All the larval progenies were reared at the same density (300 larvae/basin).

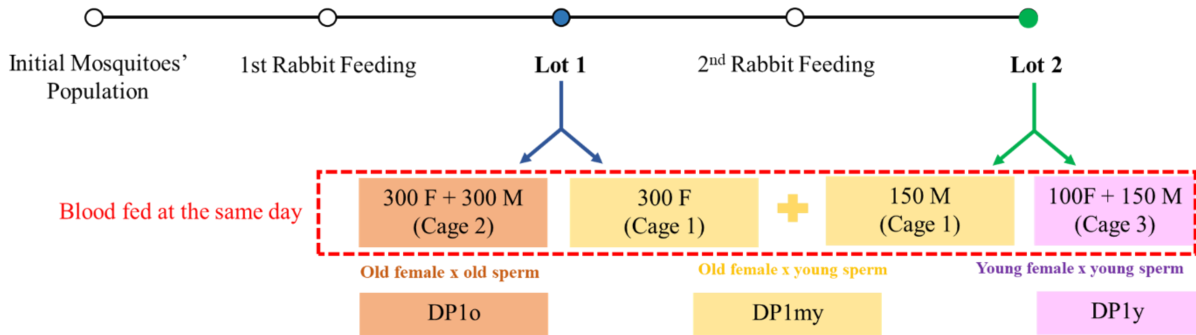

Figure 8 – Scheme of how the parent's population were created. The parent's population for the analysis were created from 2 lots of mosquitoes from an initial population. From the lot 1 we could create the cage 2 which will bear the cross old female x old sperm (DP1o), and the old females to cage 1. Afterwards, 1 week and so, from the lot 2 were isolated the males to cage 1, bearing that way the cross old female x young sperm (DP1my) and lastly the cage 3, young female x young sperm (DP1y). Finally, these cages were blood fed one time 72 hours after emerging.

3. *Egg collection to phenotypical analysis:*

To establish a connection of paternal effect on the DMAD and 6mA levels on embryo stages, eggs from the crosses previous explained were collected. One nest (half petri box with wet cotton and a filter paper on top) was placed per cage, leaving females to lay eggs for 1 hour. Eggs were allowed to mature for 24h after which time eggs were collected with the help of a tiny brush and transferred by 300 to tubes containing glass beads. All the eggs samples were stored at -80ºC until analysis.

4. *Development and assessment to the survivorship of Daughter Populations:*

To link the influence of paternal effect to survivorship of DPs was conducted a survival assay until the last mosquito stayed alive.

From each cross, the same rearing conditions as for the parents were repeated, even the larvae density (300 eggs per basin). 50 virgin females *Anopheles* from each basin were collected as they emerge and transferred to a small cage (20x15x20 cm). The

mortality was followed every day until the last survivor. No starving conditions were imposed to the DPs, meaning that there had free access to a 10% sucrose solution and wet cotton.

Aside, from each cross were collected additional adult progeny: 25 females and 25 males, that were frozen by 5 per tube and stored at -80ºC for further analysis.

5. Wings measurement:

In order to link the body size of the mosquito to the survivorship, wings were measured at the time of counting the number of dead mosquitoes during the survival assay. As they were dying, they were collected and placed on a slide under the dissecting microscope. With a scalpel one of the wings were cut and measured with graph paper, taking the measure as presented at figure 9.

Figure 9 – Scheme of how the wings of *Anopheles gambiae* were measured. As DPs were dying the measure of their wings were taken with a graph paper under the dissecting microscope.

6. RNA Extraction:

To produce cDNA to analyse DMAD expression patterns, first it was necessary to extract the RNA from each sample (eggs 24 hours development, male adults and female adults).

Primarily the samples were disrupted used the Precellys 24 tissue homogenizer (Bertin Instruments). Then, RNA was extracted by following the protocol provided with the Direct-zol Miniprep for the whole mosquitoes (Zymo Research, cat. no. R2051) and Direct-zol Microprep for the embryos samples (Zymo Research, cat. no. R2061), together with the TriReagent (Sigma). An additional DNAse treatment were made using Turbo DNA-free™ Kit (Invitrogen, cat. no. AM1907). RNA concentration was measured with a Nanodrop spectrophotometer, and the samples were kept at −80°C.

7. *RT-qPCR for METTL3 and DMAD expression analysis:*

With the aim of analysing the expression patterns of METTL3 (encoding a methyl transferase, sequence presented in Annex I) and DMAD (encoding a demethylase, AGAP007180, Forward: 5' - AGAAGGGCGAGAAGAAGGACAAGG -3'; Reverse: 5' - ATCGGTTCCTTCTTGACGCTT -3'), we conducted a RT-qPCR protocol, which is split in two parts. The first one, consists of the production of cDNA from RNA sample (reverse transcriptase). The second one is a quantitative PCR amplification to estimate the relative levels of METTL3 and DMAD RNAs quantification, using an RNA encoding the ribosomal protein S7 as reference.

cDNA was synthesized using 100 ng/µL of total RNA, previously treated with TURBO™ DNase (Invitrogen), with hexamer pd(N)6 and primers and the Thermoscript RT-PCR system kit (Invitrogen), following the manufacturer's instructions.

The SYBR Green-based qPCR assays were run in triplicates in 25 µL reactions, consisting of qPCR Master Mix, forward and reverse primers specific for each gene at a final concentration of 300 nM as well as 5 µL of cDNA template.

Table 1 – Composition of qPCR Master Mix.

Reagent	**Final Concentration**	**Volume on the Mix per reaction**
SYBRgreen	1X	12.5 µL
Primer Forward	300 nM	0.75 µL
Primer Reverse	300 nM	0.75 µL
H_2O DNA-RNA free		6 µL

qPCR detection was based on a thermal protocol consisting of a 15 minutes polymerase activation/initial denaturation step at 95 °C, 40 cycles of denaturation and annealing/extension steps at 95 °C for 15 s, 60 °C for 1 minute, followed by a melting curve analysis step. A cDNA template control (from 5 whole *Anopheles* mosquitoes, gender indifferent) was included in each qPCR run to allow the standard calibration curve method (method where the quantity of cDNA from each sample is first determined using a standard curve (determined from cDNA template control) and is then expressed relative to a calibrator sample (FP1y)).

8. Protein extraction and quantification:

To attempt linking the total quantity of protein to paternal effect embryos of 24h development as well as whole adults (product of each cross) were crushed in extraction buffer (25 mM Tris-HCl (pH=8); 1 mM EDTA; 1x Protease Cocktail) using a Precellys 24 tissue homogenizer (Bertin Instruments) and centrifuge at 4ºC for 15 minutes at 20000 G. The supernatants were collected.

The quantification of the total amount of protein we obtained through a Qubit™ Kit Assay (Invitrogen, cat. no. Q33211) to a classical range protocol following the manufacturer's instructions.

C. Results

The results presented here will be associated with only two of the three objectives initially proposed for the dissertation: establishing a link between paternal contribution and the survival of DPs, and expression analysis of genes associated with DNA methylation and demethylation in the same offspring samples.

The objective not mentioned in the results relates to the establishment of a purification pipeline of sperm from the spermatheca of female *Anopheles* with different ages that encountered technical obstacles. Despite this, the different attempts are presented in the supplementary information in this document (Annex II).

A paternal effect or a maternal effect on survivorship of the progeny can be deduced from the results obtained in the different crosses present on pages 26 and 27. As explained in Table 2, when we compare progeny from crosses 1 and 2 (DP1o vs DP1my) we could infer the existence of a paternal effect associated with sperm aging. When we compare progeny from crosses 1 and 3 (DP1y vs DP1my) we will be inferring the existence of a maternal effect associated with the mother's age.

Table 2 – Comparisons that allow an association of the results with paternal effect or mother effect.

Comparision		Mother Age	Sperm Age
DP1my vs DP1o	1 vs 2	-	X
DP1my vs DP1y	1 vs 3	X	-

In other words, when we compare the phenotype of the DP1o (resulting from crossing old female x old sperm) with DP1my (old female x young sperm) the only variable at play will be the aging of the sperm. By contrast when we compare DP1y (young female x young sperm) with DP1my (old female x young sperm) the only variable to consider in the result is the mother's age.

1. Survivorship of Anopheles daughter progenies:

Previous results from the hosting laboratory showed a correlation between the survival of DPs and the age of the mother, that is, there is a maternal influence on the survival of DPs, where older mothers showed a more epidemiological relevance [157] which may affect the epidemiology of malaria.

As *Anopheles* mosquitoes are only inseminated once in their entire lifetime, and the female stores the sperm in the spermatheca, the serial offspring of one female comes from sperm of different age [74]. It is unknown whether the age of the sperm, stored in female, might indeed contribute to the recently discovered maternal effect on daughter survival rate and susceptibility to *Plasmodium*[157], possibly contributing to a paternal effect associated with sperm aging.

Therefore, to address this question we followed the survival rate of female progeny coming from old female mosquitoes mated young (aged sperm) to old females mated old (young sperm), including a control with female just mated (young female, young sperm).

So, to obtain a phenotypic result of the paternal effect on the survival of DPs the survival assay was performed until the death of the last female in each cage. Note that DPs were not subject to starvation conditions, and all are virgin. We performed two biological replicates, as described in figure 8.

On the first biological replicate, each cage had 50 DPs (N = 50). On the second biological replicate, due to some complications of unknown origin, it was not possible to create cages with equal density population, then all the cages had different numbers of DPs in each cage (DP1my: N = 19; DP1o: N = 30; DP1y: N = 25).

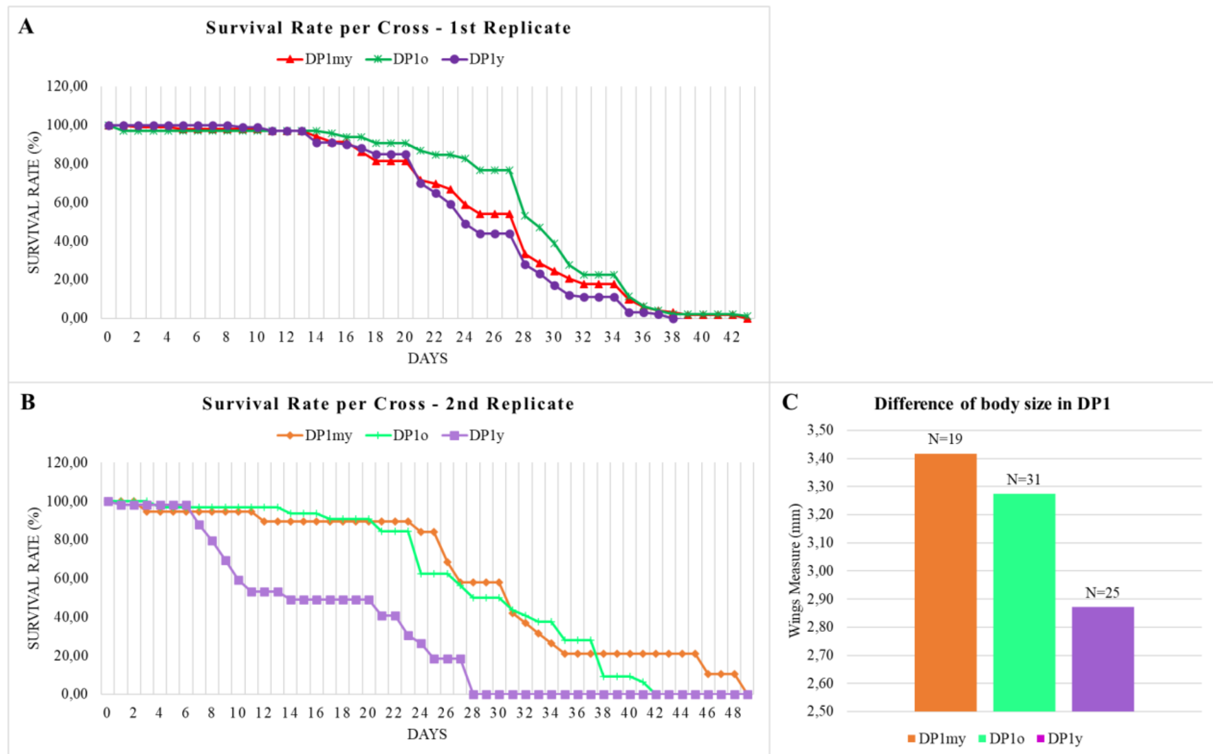

Figure 10 – Survival curves and wings measures data from DP1my, DP1o and DP1y on two replicates. A – Survival curves for the first biological replicate. Each cage had 50 virgin DPs per cross (DP1my (red ▲), DP1o (green ×) and DP1y (purple ●). The duration of the assay was 43 days; B – Survival curves for the second biological replicate. On this assay each cross had a different number of DP due to complication on the creation of DPs. DP1my (orange ♦) had 19 virgin females, DP1o (light green +) had 25 virgin females and DP1y (light purple ■) had 31 virgin females. The duration of the second assay was 49 days. C – Average of wings measures from each cross (DP1my (orange), DP1o (light green) and DP1y (light purple)) from the second biological replicate, as body size reference with the objective to link body size with survivorship of the DPs. The wing measures (average ± standard deviation) obtained are DP1my – 3.42 ± 0.266 mm, DP1o – 3.27 ± 0.240 and DP1y – 2.87 ± 0.214.

When we look up to the survival curves of the first replicate (figure 10A), we face up a constant trend across the cages of a non-death phase for 10 days, but at day 14 of the trial we started to see differences between the progeny from the 3 crosses. We observe a linear decline in two of the crosses, DP1y (young female x young sperm) and DP1my (old female x young sperm). By contrast, DP1o (old female x old sperm) exhibits a better survivorship throughout the entire survival assay.

The first cage to run out of survivors was DP1y on the 38th day, then DP1my on the 42nd and finally DP1o on the 43rd day of the trial.

We can also use the 50% survival mark as a reference, and if that is the case, we see the same outcome: first DP1y on the 24th day, then DP1my on the 27th day and finally DP1o on the 28th day.

The first survival trial lasts for 43 days.

As presented in Figure 10A, DP1o has better a better survival expectancy than DP1my. Therefore, according to Table 2, this indicates that there is a paternal effect on the survival of DPs where aged sperm at fecundation favours the survival of progeny. Conversely, DP1my exhibit a slightly better survival rate than DP1y which suggest that a maternal effect also contribute to differences in survival rate of progeny, with progeny from old females surviving longer, in agreement with the results previously obtained by the hosting laboratory [157]. However, the differences between the survival of DP1my and DP1y may not be robust enough, implying the needs for a second biological replicate.

According to Figure 10B, which refers to the second biological replicate, we observed a discrepancy on the survival curves when compared to the results obtained on the first replicate.

On the second replicate, the non-death phase only lasts for 6 days. And then we observe a huge difference in the survival curve then DP1y and the other two curves, DP1my and DP1o.

We confront the same outcome when we point to the cage with the worst survival tendency, DP1y (young female x young sperm), which in this replicate ends on day 28 of the assay. The second to finish is DP1o (old female x old sperm), unlike the first replicate in which it showed the best survival curve, lasting 42 days. The progeny that presents better survival on this second replicate is DP1my (old female x young sperm), which took the test until the 49th day.

On inspecting the 50% survival mark we verify that DP1y reaches this milestone on the 14th day of the trial, then DP1o on the 28th day and finally DP1my on the 31st day. In these last two crosses, in relation to the 50% survival mark, there is no significant difference with the first replicate.

Comparing DP1o with DP1my, we found a disagreement with the result obtained previously on the first biological replicate, as we verified the existence of a paternal effect, nonetheless in this replicate is the young sperm that confers better survival to its progeny. However, when comparing DP1my with DP1y, we found a full agreement with all the results obtained so far, where older mothers confer better survival to their offspring.

So, we verified the existence of a paternal effect on the survivorship of the progeny, however the true influence of sperm aging is not clear, requiring at least one

more biological replicate to confirm the tendency. Nevertheless, it is clear that there is a maternal effect on the survival of the progeny, and it is unanimous that older mothers provide better survivorship, proving their greater epidemiological relevance.

2. *Wing size measure of Anopheles daughter progeny as reference to body size:*

In 2018 Barreaux et al. attested that there would be a relationship between the size and the longevity of the vector, *Anopheles gambiae*, which could be relevant to the epidemiology of malaria, where larger mosquitoes showed better chances of survival [172].

In order to try to obtain a correlation between the size of the progeny with their survival, the wings were measured as a reference parameter to the body size of the mosquito.

Measurements were taken of each DPs from the 3 crosses of replicate 2. From cross 1, DP1my, 19 females were measured (N=19); Cross 2, DP1o, 31 females were measured (N=31); And finally cross 3, DP1y, 25 females were measured (N=25). The graphs were obtained through the average value of wings of each crossing (Figure 10C).

From the graph of Figure 10C, we conclude that the cross with the smallest progeny is DP1y (2.87 ± 0.214 mm) and the one with the bigger DPs is DP1my (3.42 ± 0.266 mm). DP1o presents DPs of 3.27 ± 0.240 mm, presenting values not so far from the values found in DP1my.

When we compare the wing size results (Figure 10C) with the survival results obtained in the second biological replicate (Figure 10B), we found that DP1y has the smallest size as well as the shorter survival curve. That is, the body size may be an indicator of the probability of survival where larger mosquitoes will have more chances, corresponding to the results obtained by Barreaux and his team [172].

As confronting Figure 10B and 10C, we can observe that there is a small difference between the progeny from cross DP1my and DP1o either on the size of the progeny as the survival curve, where young sperm is more relevant conferring better survivorship to the progeny. However, the difference between progeny from cross DP1my and DP1y is clear, stating that older mothers provide better expectancy to their progeny.

3. *Quantification of 6mA mark and DMAD expression on Anopheles adults, male and female, and on 24 hours development embryos:*

In 2015, Zhang et. al described in *Drosophila* the occurrence of relevant DNA methylation on Adenine residues (6mA or N6-Methyladenine) contributing to a paradigm shift in the field of epigenetics where epigenetic marks associated with DNA methylation involved almost exclusively methylation on cytosine (5mC) and was only marginalizing occurring in invertebrates[132]. Zhang et al. discovered the existence of the 6mA mark in *Drosophila melanogaster* DNA in embryonic stages, and that the rate of methylation was associated with the expression of a specific the demethylase, called DMAD (*Drosophila* MethylAdenine Demethylase) [132].

In 2019 Ruiz et al. discover histone mark modifications in *Anopheles* induced by the infection by *Plasmodium* parasite, opening in this way a new field of research in the story of malaria [158].

Following the work of Zhang et al in *Drosophila*, the host laboratory could also detect 6mA marks on the DNA of *Anopheles gambiae* embryos, by dot blot and ELISA assay using an antibody that recognize 6mA residues. It was also shown that the expression of the *Anopheles* DMAD ortholog negatively correlate with the abundance of 6mA marks in embryos.

We therefore decided to assess whether the expression of DMAD varies in adults of each cross as well as in embryos of 24h development. We extent our analysis to quantify the expression of the METTL3 ortholog gene, which might be involved in the N6 methylation of Adenine residues. The global objective is to determine whether there is a link between these epigenetic alterations with a possible paternal effect, associated with sperm aging, as well as a maternal effect related to mother's age.

The quantification was made through RT-qPCR from samples obtained from each cage: a pool of 200 eggs 24h of development, a mix of 5 adult males and a mix of 5 adult females. All results were normalized to the expression of S7, a gene encoding a ribosomal protein used as a reference gene in invertebrates. As a calibrator of the results we used the values obtained from the female adult progeny from cross 3, DP1y (young female x young sperm).

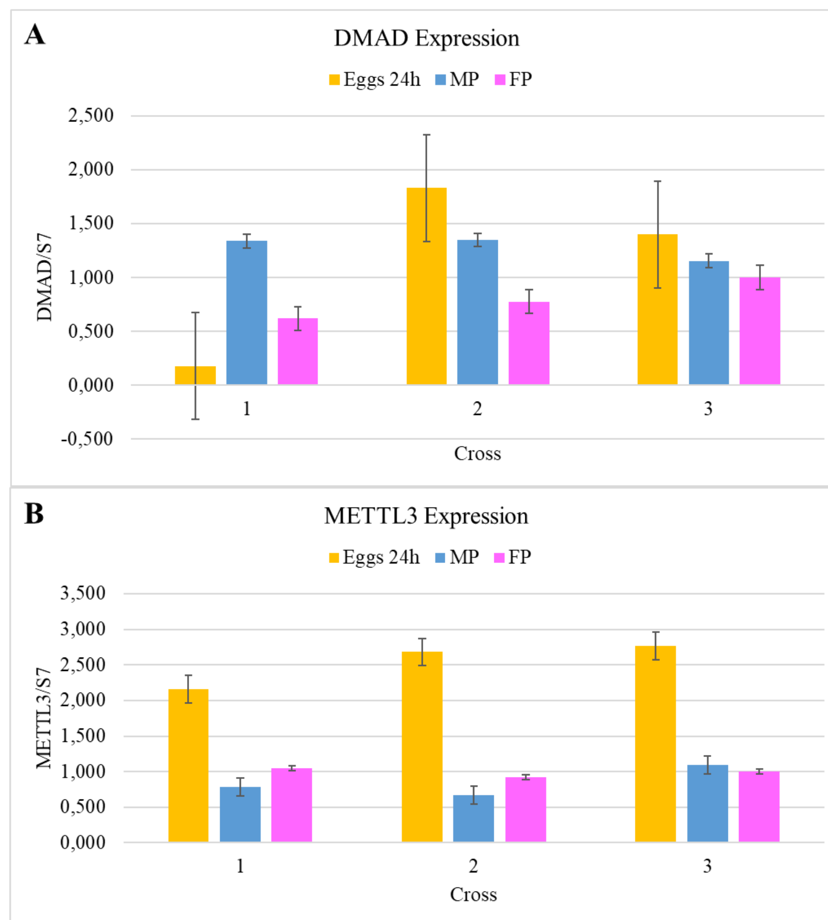

Figure 11 – Expression of DMAD and METTL3 as indicator of epigenetics mechanisms on *Anopheles* adults and 24 hours development embryos from DP1my, DP1o and DP1y. Cross number 1 as DP1my (old female x young sperm), cross number 2 as DP1o (old female x old sperm) and finally cross number 3 as DP1y (young female x young sperm). All the data were normalized using the expression of S7 gene and presented relative to a calibrator, females from DP1y (FP1y). A – Demethylase expression (DMAD) on *Anopheles* adults, male (blue, MP) and virgin female (pink, FP), and on 24h development embryos (yellow) from each cross. B – Expression of METTL3, an enzyme known for being involved on the epigenetic mark 6mA. The levels of METTL3 were evaluated on *Anopheles* adults, male (blue, MP) and virgin females (pink, FP) as well on 24 hours development embryos (yellow). The bars are error bars that represent the data variability towards standard deviation.

According to the Figure 11A, the overall picture shows us that the embryos of 24h development from cross 1 (DP1my) present notably less expression of DMAD.

The demethylation pattern (DMAD expression) on adults (figure 11 – A), we verify at cross 1, DP1my (old female x young sperm) we found that male *Anopheles* progenies (MPs) present higher expression when compared to female *Anopheles* progenies (FPs). At cross 2 (DP1o), MPs present higher DMAD expression than FPs. At cross 3, we still state the same outcome, MPs still present higher DMAD expression than

FPs. So, male progenies present higher values than female progenies in the same circumstances.

According to Table 2, when we compare cross 1 with cross 2, we can infer the existence of a paternal effect. FPs from cross 2 present more DMAD expression than the FPs from cross 1. On MPs the difference is not relevant to link to a paternal effect. So, through this, we clearly see an increase in the expression of DMAD with sperm ageing on FP. Then there is a paternal effect behind the expression of DMAD on female adult progenies.

And, when comparing cross 1 and cross 3, we state an increase of DMAD with the mother age on MPs, but on FPs we state the contrary, a decrease of DMAD with the mother age. So, there is a maternal effect on *Anopheles* adults, where older mothers influence oppositely male and female adults.

In relation to embryos of 24h development, embryos from cross 2 present the highest value followed by the ones from cross 3 and the cross 1 present the lowest value. We state the same outcome as the one present by FPs: an increase in the DMAD expression with sperm ageing and a decrease with the mother age.

According to Figure 11B, the methylation pattern is more balanced than the demethylation pattern, either on embryos of 24h development as on adult mosquitoes, pointing to a not so relevant maternal and paternal effect.

At cross 1, DP1my (old female x young sperm) we found that female *Anopheles* adult progenies (FPs) present higher expression of METTL3 when compared to male *Anopheles* progenies (MPs), presenting an inverted pattern that the one found on DMAD expression. At cross 2, DP1o (old female x old male), FPs present higher values than MPs. At cross 3, MPs present a slightly higher value than FPs.

Comparing between crosses: cross 1 vs cross 2, we infer the existence of a light paternal effect associated with sperm ageing where sperm ageing causes a decrease on METTL3 (levels of cross 2 are slightly lower than levels of cross 1) in adults. And cross 1 vs cross 3, we don’t see major differences between the METTL3 levels in the adult mosquitoes, which can lead to an inexistence of maternal effect on methylation levels.

However, when we look up to the levels of METTL3 on embryos of 24h development we don’t state the same tendency of the adults (opposite levels when compared to the DMAD levels). The embryos of 24 h development point to the same

outcome of DMAD levels. Embryos from cross 2 present the highest value followed by the cross 3 and the embryos of 24h development from cross 1 present the lowest value.

So, on embryos of 24h development there is a paternal and maternal influence on the METTL3 levels. The age of mother induces a decrease on METTL3 expression, and the ageing of the sperm induces an increase of METTL3 levels. Note that on embryos of 24h of development the paternal effect is more accented than the maternal effect.

4. *Quantification of total protein content on Anopheles adults and 24h hours development embryos:*

There is an extensive bibliography that associates protein content with immune response and this, in turn, may influence vector survival. To verify if there was an exploratory margin in relation to the protein content of *Anopheles gambiae* and if there was any paternal effect associated with this, the total amount of protein of progeny from each cross (DP1my, DP1o and DP1y) was quantified.

We determined the total amount of protein by the simple method of extracting protein under non-denaturing conditions and obtaining the value in Qubit™ by the classical interval method.

Then, the values of the total amount of protein were obtained from: a pool of 200 eggs 24h of development of each of the 3 crosses, a mix of 5 adult males and a mix of 5 adult females of each of the cross.

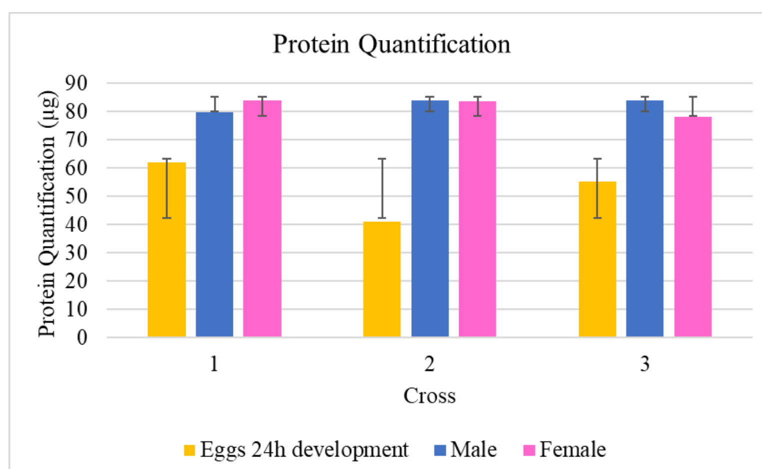

Figure 12 – Total amount of protein content on *Anopheles* adults and 24 hours development embryos from DP1my, DP1o and DP1y. Cross 1 as DP1my (old female x young sperm), cross 2 as DP1o (old female x old sperm) and lastly cross 3 as DP1y (young female x young sperm). Male progenies (MP) from each cross are represented in blue, FPs in pink and 24h development eggs in yellow. To assess the quantity of protein were collected eppendorfs

from each cross with 5 MPs, other with 5 FPs and to the eggs analysis were collected 200 eggs to a tube with beads. From cross 1 the value obtained are: MPs – 79.6 µg, FPs – 84 µg and eggs – 62 µg; From cross 2 the values are: MPs – 84 µg, FPs – 84 µg and eggs – 41.2 µg; Lastly, from cross 3 the results obtained are: MPs – 84 µg, FPs – 78 µg and eggs – 55.2 µg. The bars are error bars that represent the data variability towards standard deviation.

According to the figure 12, there are not big differences between *Anopheles* adults, female and male, and which cross they belong. However, on embryos 24h development there is significant differences between the different crosses, indicating an existence of a parental effect on the protein content in embryo stages.

Cross number 1 represents cage number 1, which is referred to in figure 12 as representation of the cross DP1my (old female x young sperm). In this cross we can state from the graph of figure y that there is a small difference between adult *Anopheles*, male and female (79.6 µg vs 84 µg, respectively).

In cross 2, representation of cage 2, DP1o (old female x old sperm), we verified the same trend, the inexistence of significant differences between adult *Anopheles*, male and female (84 µg both.

Finally, in cross 3, DP1y (young female x young sperm), we observed the trend shown by the other 2 crosses... Minimal difference between adult *Anopheles* male and female (84 µg vs 78 µg, respectively.

Then, it is possible to state that in adulthood there is any major difference between male and female adults as well does not seem to exist any paternal or maternal influence on the protein content in the adulthood.

However, in embryos of 24h development the outcome is totally different. There are differences on the results between the crosses. The embryos from cross 2 (DP1o) present the lowest value, 41.2 µg. Cross 1 (DP1my) present the highest value, 62 µg and cross 3 (DP1y) present 55.2 µg of protein content. So, compare the crosses as explained on table 2, we can state a possibility of a huge paternal effect on the protein content in embryonic stages (less provision of protein associated with old sperm) as well as a maternal effect (less provision of protein associated with young female, however the difference is marginal). In this case, young sperm confers higher values of protein content and older mothers as well.

D. Discussion

Two of the three objectives initially proposed were analysed in this project: investigating a possible link between paternal effect and survival of DPs, and analysis of expression patterns of DNA methylation and demethylation in these same offspring.

Regarding the first objective (existence of a paternal effect associated with sperm aging on the survival of DPs in addition to a maternal effect) the conclusions of the two biological replicates are somehow opposite. Indeed, in the first replicate, the survival of progeny from old females mated young (DP1o) was better than the survival of progeny from old females mated old (young sperm, DP1my). In other words, progeny from females of the same age do better when resulting from fecundation with old sperm, indicating a paternal effect on progeny survival. However, in the second biological replicate there was no clear paternal effect on the survival rate of the offspring. Conversely, when looking at the maternal effect driven by the age of the mothers, keeping the age of the sperm identical, replicate one does not evidence a clear difference between the progeny, while replicate 2 revealed a clear difference whereby the progeny of old females survive longer whether being produced with young or old sperm. This is fully in line with the results previously obtained by the hosting laboratory[157].

The difference in the results between the duplicates may be due to numerous variables. We believe that there was a problem in creating the initial population for the second replicate as the females did not lay as many eggs as expected, leading to slight changes in the larval density achieved, and in turn may have created some unexpected differences in the results. This all reinforces the need for at least two more replicates in order to strengthen the results obtained in relation to the maternal effect on the survival of DPs (where older mothers confer better survival on the offspring) and clarify the paternal effect that was detected with the first replicate.

In the second replicate, as additional data, one wing was collected from each offspring in order to be able to link body size with survival and we found that larger mosquitoes have a better expectation of surviving, in line with the results obtained by Barreaux and his team in 2018 [172]. This parameter should continue to be analysed in future replicates to confirm that these size differences are correctly linked to survival, as bigger progenies come from older females, providing in this way an extra criteria for the maternal effect previously detected. As well it is needed to confirm that the larval density did not influence the result obtained previously, since temperature and larval density are

closely associated with the development of the mosquito (such as its body size[173]) and survival[174].

Regarding the second objective to which we set out to respond, analysis of methylation and demethylation patterns, we observed that there is expression of METTL3 (associated with 6mA) in *Anopheles* both in the embryonic phase (embryos of 24h development) and in adults, as well as expression of DMAD, the 6mA demethylase. The DMAD data corroborate the one found by Zhang et. al in *Drosophila*, that this epigenetic mark can occur in invertebrates[132].

When looking at the results obtained in *Anopheles* embryos with 24h of development, we did not find an inverse relationship between the expression patterns of METTL3 and DMAD that would be expected after Zhang et al verified that in *Drosophila* embryonic stages the methylation of adenine residues (6mA) would be intimately and inversely associated with demethylation by DMAD[132]. However, expression of METTL3, as the putative DNA methyltransferase, has not been clearly shown in *Drosophila*, nor *Anopheles* yet. So, these results may point to another direction in the methylation mechanism of this epigenetic mark, in the way that METTL3 is not fully linked to 6mA DNA methylation in *Anopheles*.

We found the existence of a paternal and maternal effect on both METTL3 methylation patterns and DMAD demethylation patterns, in adult *Anopheles* samples as well as in 24h development embryos.

On the demethylation patterns, the results obtained in adult females (FPs) were in agreement with those obtained in embryos, where there is a negative influence on the levels of demethylation with the age of the mother (offspring from older mothers have lower levels of expression of DMAD) contrasting with the positive effect associated with sperm ageing (increase on the levels of expression of DMAD levels with sperm age).

On the METTL3 patterns, there is a probable paternal influence in the 24h development embryos, more pronounced than the maternal effect detected, indicating the potential of the study of the paternal effect on the epigenetic marks of the vector.

The analysis of methylation and demethylation patterns was performed only in the first replicate performed because it was not possible to obtain enough eggs for further analysis in the second replicate. Therefore, replication of the assay is strictly necessary in order to be able to clearly conclude this parental influence on epigenetic marks.

In the second replicate, the protein content of 24h embryos and adult progeny was also studied. No relevant differences were detected between male and female *Anopheles* adults from any cross. However, in embryos of 24h development was detected both paternal and maternal effects on protein content. We found a higher protein content with the age of the mother (older mothers give it to their embryos), opposing to sperm aging effect, where sperm ageing is related to a decrease in protein in the embryos.

The result obtained about the maternal effect does not aligned with the results obtained by Muller et al on other species of invertebrates, *Eupelmus vuilletti* (parasitic wasp), where the existence of maternal effect in the provisioning of the eggs is clear, however is reduced with the age of the mother [175].

Why are there protein differences in content levels in embryos but in adult *Anopheles* not? It is known that the embryonic phase is sensitive and crucial for the development of the organism, therefore, a greater provision of proteins and defences is necessary. But what drives this level to become normalized in adulthood? More replicates are necessary to clarify a paternal and maternal influence on the protein content of the offspring, at least in embryos. It would be interesting in future trials to integrate analysis at different time point of the embryo and even during the larval stages to verify the parent influence along development.

No statistical test was applied to the data presented, as there were not enough replicates to apply one that makes the results more reliable. In the analysis of survival data, we could apply analysis by Kaplan-Meier and Cox proportionally, but we would have to have at least 4 sets of results from each cross.

A constant statement throughout this project is the positive effect of the mother's age on her offspring, which older mothers provides to her offspring best expectation of survival and higher protein content in embryonic stages. We also constantly verify that embryonic is crucial in the development of *Anopheles* producing the most interesting and important results.

E. Conclusion

The main question that this project wanted to answer is whether there is a paternal effect on fitness of the offspring in different crosses, independent of the maternal effect previously detected by the hosting laboratory.

Several traits were evaluated in the process, from survival, body size (using the wing measurement as a reference) to the protein content of the offspring. At the same time, we tried to verify the existence of a paternal influence on epigenetic marks in the offspring, more specifically 6mA methylation and DMAD demethylation.

The answer obtained was not conclusive due to differences in the results obtained between the two biological replicates performed. Parental effect on offspring was detected. Regarding the paternal effect however, the true influence of sperm ageing on the offspring is not clear. In contrast to the maternal effect in which the results were unanimous showing that older mothers offer advantage to their offspring, in agreement with the results previously obtained by the hosting laboratory.

Zhang et al found that DMAD is involved in 6mA demethylation in *Drosophila*, and due to preliminary data from the hosting laboratory it is possible that is involved in the same process in *Anopheles*. However, the results obtained in this project pointing out to METTL3 (a known methyltransferase of RNA) suggest that METTL3 is possibly not implicated in the mechanism of DNA methylation in *Anopheles*.

Long story short, the maternal effect is evident where older mothers confer better odds of survival to their progeny however the paternal effect is not clear due to opposite results between the biological replicates. Further, we could state that a better odd of survival was associated with bigger *Anopheles* mosquitoes. Regarding to the epigenetic marks, METTL3 for 6mA DNA methylation and the 6mA demethylase DMAD, as well as to the protein content interesting results on embryos were obtained, showing us the necessity of more biological replicates at more time point (embryonic stages and larval stages). Nonetheless, the existence of paternal and maternal effect was detected.

One of the biggest problems that we encountered during the project was the impact of the variability among the biological replicates. Therefore, it is of upmost importance to master every step in the production of the replicates for the one that could be controlled, as larval density for producing the offspring. But as in many biological systems, undetermined parameters may affect some replicates. Such situation can be compensated by increasing the number of replicates in order to draw more concrete conclusions.

Nonetheless, the window of parental effects on survival and offspring epigenetics becomes an approach to take in order to gain a better understanding of host-vector dynamics.

References:

1. Talapko J, Škrlec I, Alebić T, Jukić M, Včev A. Malaria: The Past and the Present. *Microorganisms*. 2019;7(6):179. doi:10.3390/microorganisms7060179
2. Sato S. *Plasmodium*—a brief introduction to the parasites causing human malaria and their basic biology. *J Physiol Anthropol*. 2021;40(1):1. doi:10.1186/s40101-020-00251-9
3. (US) M, Arrow KJ, Panosian C, Gelband H. The Parasite, the Mosquito, and the Disease. *Nih.gov*. Published online 2022. https://www.ncbi.nlm.nih.gov/books/NBK215619/
4. Suh KN, Kain KC, Keystone JS. Malaria. *CMAJ*. 2004;170(11):1693-1702. doi:10.1503/cmaj.1030418
5. Baird JK. Evidence and implications of mortality associated with acute *Plasmodium vivax* malaria. *Clin Microbiol Rev*. 2013;26(1):36-57. doi:10.1128/CMR.00074-12
6. Garcia LS. Malaria. *Clin Lab Med*. 2010;30(1):93-129. doi:10.1016/j.cll.2009.10.001
7. Snow RW. Global malaria eradication and the importance of *Plasmodium falciparum* epidemiology in Africa. *BMC Med*. 2015;13:23. doi:10.1186/s12916-014-0254-7
8. Rénia L, Goh YS. Malaria Parasites: The Great Escape. *Front Immunol*. 2016;7:463. doi:10.3389/fimmu.2016.00463
9. Crutcher JM, Hoffman SL. Malaria. In: Baron S, ed. ; 1996.
10. Mace KE, Arguin PM, Tan KR. Malaria Surveillance - United States, 2015. *Morb Mortal Wkly report Surveill Summ (Washington, DC 2002)*. 2018;67(7):1-28. doi:10.15585/mmwr.ss6707a1
11. E. CW, M. JG. *Plasmodium malariae*: Parasite and Disease. *Clin Microbiol Rev*. 2007;20(4):579-592. doi:10.1128/CMR.00027-07
12. Organization WH. *World Malaria Report 2020: 20 Years of Global Progress and Challenges*. World Health Organization https://apps.who.int/iris/handle/10665/337660
13. Duffy F, Bernabeu M, Babar PH, et al. Meta-analysis of *Plasmodium falciparum*

var Signatures Contributing to Severe Malaria in African Children and Indian Adults. *MBio*. 2019;10(2). doi:10.1128/mBio.00217-19

14. Milner DAJ. Malaria Pathogenesis. *Cold Spring Harb Perspect Med*. 2018;8(1). doi:10.1101/cshperspect.a025569
15. Graumans W, Jacobs E, Bousema T, Sinnis P. When Is a *Plasmodium*-Infected Mosquito an Infectious Mosquito? *Trends Parasitol*. 2020;36(8):705-716. doi:10.1016/j.pt.2020.05.011
16. Molina-Cruz A, Lehmann T, Knöckel J. Could culicine mosquitoes transmit human malaria? *Trends Parasitol*. 2013;29(11):530-537. doi:https://doi.org/10.1016/j.pt.2013.09.003
17. Perkins SL. Malaria's Many Mates: Past, Present, and Future of the Systematics of the Order *Haemosporida*. *J Parasitol*. 2014;100(1):11-25. doi:10.1645/13-362.1
18. Frischknecht F, Matuschewski K. *Plasmodium* Sporozoite Biology. *Cold Spring Harb Perspect Med*. 2017;7(5). doi:10.1101/cshperspect.a025478
19. Breman JG. Eradicating malaria. *Sci Prog*. 2009;92(Pt 1):1-38. doi:10.3184/003685009X440290
20. Ashley EA, Pyae Phyo A, Woodrow CJ. Malaria. *Lancet (London, England)*. 2018;391(10130):1608-1621. doi:10.1016/S0140-6736(18)30324-6
21. Meibalan E, Marti M. Biology of Malaria Transmission. *Cold Spring Harb Perspect Med*. 2017;7(3):a025452. doi:10.1101/cshperspect.a025452
22. Drakeley C, Sutherland C, Bousema JT, Sauerwein RW, Targett GAT. The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends Parasitol*. 2006;22(9):424-430. doi:10.1016/j.pt.2006.07.001
23. Read D, Lensen AH, Begarnie S, Haley S, Raza A, Carter R. Transmission-blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing. *Parasite Immunol*. 1994;16(10):511-519. doi:10.1111/j.1365-3024.1994.tb00305.x
24. Blagborough AM, Sinden RE. *Plasmodium berghei* HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. *Vaccine*. 2009;27(38):5187-5194. doi:10.1016/j.vaccine.2009.06.069
25. Tizifa TA, Kabaghe AN, McCann RS, van den Berg H, Van Vugt M, Phiri KS.

Prevention Efforts for Malaria. *Curr Trop Med reports*. 2018;5(1):41-50. doi:10.1007/s40475-018-0133-y

26. Sachs J, Malaney P. The economic and social burden of malaria. *Nature*. 2002;415(6872):680-685. doi:10.1038/415680a
27. Prevention and control measures for malaria. *Eur Cent Dis Prev Control*. Published online 2019. https://www.ecdc.europa.eu/en/malaria/prevention-and-control#:~:text=The main current measures are,and insecticide-treated bed nets
28. Nothdurft HD, Kain KC. Chapter 6 - Malaria Prevention. In: Sanford CA, Pottinger PS, Jong ECBT-TT and TMM (Fifth E, eds. Elsevier; 2017:71-90. doi:https://doi.org/10.1016/B978-0-323-37506-1.00006-4
29. Wykes MN. Why haven't we made an efficacious vaccine for malaria? *EMBO Rep*. 2013;14(8):661. doi:10.1038/embor.2013.103
30. World. Malaria. *Who.int*. Published online 2021. https://www.who.int/news-room/fact-sheets/detail/malaria
31. Sougoufara S, Ottih EC, Tripet F. The need for new vector control approaches targeting outdoor biting anopheline malaria vector communities. *Parasit Vectors*. 2020;13(1):295. doi:10.1186/s13071-020-04170-7
32. Paaijmans KP, Thomas MB. The influence of mosquito resting behaviour and associated microclimate for malaria risk. *Malar J*. 2011;10(1):183. doi:10.1186/1475-2875-10-183
33. Costantini C, Sagnon N, della Torre A, Coluzzi M. Mosquito behavioural aspects of vector-human interactions in the *Anopheles gambiae* complex. *Parassitologia*. 1999;41(1-3):209-217.
34. Organization WHO-WH. *Control of Residual Malaria Parasite Transmission*.; 2014. https://www.who.int/malaria/publications/atoz/technical-note-control-of-residual-malaria-parasite-transmission-sep14.pdf?ua=1
35. Sherrard-Smith E, Griffin JT, Winskill P, et al. Systematic review of indoor residual spray efficacy and effectiveness against *Plasmodium falciparum* in Africa. *Nat Commun*. 2018;9(1):4982. doi:10.1038/s41467-018-07357-w
36. Carnevale P, Manguin S. Review of Issues on Residual Malaria Transmission. *J Infect Dis*. 2021;223(12 Suppl 2):S61-S80. doi:10.1093/infdis/jiab084
37. Govella NJ, Chaki PP, Killeen GF. Entomological surveillance of behavioural resilience and resistance in residual malaria vector populations. *Malar J*.

2013;12(1):124. doi:10.1186/1475-2875-12-124

38. Chareonviriyaphap T, Bangs MJ, Suwonkerd W, Kongmee M, Corbel V, Ngoen-Klan R. Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand. *Parasit Vectors*. 2013;6(1):280. doi:10.1186/1756-3305-6-280

39. Disease NI of A and I. Malaria Prevention, Treatment, and Control Strategies. *Nih.gov*. Published online 2011. https://www.niaid.nih.gov/diseases-conditions/malaria-strategies

40. Su X, Zhang C, Joy DA. Host-Malaria Parasite Interactions and Impacts on Mutual Evolution. *Front Cell Infect Microbiol*. 2020;10. doi:10.3389/fcimb.2020.587933

41. Bartoloni A, Zammarchi L. Clinical aspects of uncomplicated and severe malaria. *Mediterr J Hematol Infect Dis*. 2012;4(1):e2012026-e2012026. doi:10.4084/MJHID.2012.026

42. Paul AS, Egan ES, Duraisingh MT. Host-parasite interactions that guide red blood cell invasion by malaria parasites. *Curr Opin Hematol*. 2015;22(3):220-226. doi:10.1097/MOH.0000000000000135

43. White NJ. The Treatment of Malaria. *N Engl J Med*. 1996;335(11):800-806. doi:10.1056/NEJM199609123351107

44. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria diagnosis: a brief review. *Korean J Parasitol*. 2009;47(2):93-102. doi:10.3347/kjp.2009.47.2.93

45. Doolan DL, Dobaño C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev*. 2009;22(1):13-36. doi:10.1128/CMR.00025-08

46. Organization WH. *WHO Guidelines for Malaria, 31 March 2022*. World Health Organization https://apps.who.int/iris/handle/10665/352687

47. Ecker A, Lehane AM, Clain J, Fidock DA. PfCRT and its role in antimalarial drug resistance. *Trends Parasitol*. 2012;28(11):504-514. doi:https://doi.org/10.1016/j.pt.2012.08.002

48. Nosten F, White NJ. Artemisinin-Based Combination Treatment of *Falciparum* Malaria. *Nih.gov*. Published online 2007. https://www.ncbi.nlm.nih.gov/books/NBK1713/?report=classic

49. White NJ. Antimalarial drug resistance. *J Clin Invest*. 2004;113(8):1084-1092. doi:10.1172/JCI21682

50. Whitty CJM, Chandler C, Ansah E, Leslie T, Staedke SG. Deployment of ACT

antimalarials for treatment of malaria: challenges and opportunities. *Malar J*. 2008;7(1):S7. doi:10.1186/1475-2875-7-S1-S7

51. Tanner M, Greenwood B, Whitty CJM, et al. Malaria eradication and elimination: views on how to translate a vision into reality. *BMC Med*. 2015;13:167. doi:10.1186/s12916-015-0384-6
52. Monge-Maillo B, López-Vélez R. Migration and malaria in europe. *Mediterr J Hematol Infect Dis*. 2012;4(1):e2012014-e2012014. doi:10.4084/MJHID.2012.014
53. Thu AM, Phyo AP, Landier J, Parker DM, Nosten FH. Combating multidrug-resistant *Plasmodium falciparum* malaria. *FEBS J*. 2017;284(16):2569-2578. doi:10.1111/febs.14127
54. Greenwood B. Treatment of Malaria — A Continuing Challenge. *N Engl J Med*. 2014;371(5):474-475. doi:10.1056/NEJMe1407026
55. Phillips MA, Burrows JN, Manyando C, van Huijsduijnen RH, Van Voorhis WC, Wells TNC. Malaria. *Nat Rev Dis Prim*. 2017;3(1):17050. doi:10.1038/nrdp.2017.50
56. Meigen JW. *Systematische Beschreibung Der Bekannten Europäischen Zweiflügeligen Insekten* . Vol T.1 (1818). Bei Friedrich Wilhelm Forstmann : Gedrukt bei Beaufort Sohn,; 1818. https://www.biodiversitylibrary.org/item/45833
57. Harbach RE. The classification of genus *Anopheles* (*Diptera: Culicidae*): a working hypothesis of phylogenetic relationships. *Bull Entomol Res*. 2004;94(6):537-553. doi:10.1079/ber2004321
58. Prevention CDC-C for DC and. CDC - Malaria - About Malaria - Biology. *CDC - Centers Dis Control Prev*. Published online 2020. https://www.cdc.gov/malaria/about/biology/index.html
59. Zhou D, Zhang D, Ding G, et al. Genome sequence of *Anopheles* sinensis provides insight into genetics basis of mosquito competence for malaria parasites. *BMC Genomics*. 2014;15(1):42. doi:10.1186/1471-2164-15-42
60. Jennings BH. *Drosophila* – a versatile model in biology & medicine. *Mater Today*. 2011;14(5):190-195. doi:https://doi.org/10.1016/S1369-7021(11)70113-4
61. Behura SK, Haugen M, Flannery E, et al. Comparative genomic analysis of *Drosophila melanogaster* and vector mosquito developmental genes. *PLoS One*.

2011;6(7):e21504-e21504. doi:10.1371/journal.pone.0021504

62. Okuneye K, Eikenberry SE, Gumel AB. Weather-driven malaria transmission model with gonotrophic and sporogonic cycles. *J Biol Dyn*. 2019;13(sup1):288-324. doi:10.1080/17513758.2019.1570363
63. A.N. C. The Biology of Mosquitoes. *Biol Mosquitoes*. 1992;1.
64. Verhoek, B.A and Takken W. Age effects on the insemination rate of *Anopheles gambiae s.l. (Dipt., Culicidae*) in the laboratory. *Proc Sect Exp Appl Entomol Netherlands Entomol Soc*. 1990;1:99. https://eurekamag.com/research/002/294/002294302.php
65. Takken W, Costantini C, Dolo G, Hassanali A, Sagnon N, Osir E. Mosquito Mating Behaviour. In: *Bridging Laboratory and Field Research for Genetic Control of Disease Vectors*. Vol 11. ; 2006:183-188. doi:10.1007/1-4020-3799-6_17
66. Rutledge CR. Mosquitoes (8;Diptera: Culicidae(9; BT - Encyclopedia of Entomology. In: Springer Netherlands; 2005:1467-1468. doi:10.1007/0-306-48380-7_2740
67. Guidelines for Malaria Vector Control. *Nih.gov*. Published online 2019. https://www.ncbi.nlm.nih.gov/books/NBK538118/
68. Williams J, Pinto J. *Training Manual on Malaria Entomology*.; 2012.
69. Health P. *Anopheles* Mosquito | Public Health. *Public Health*. Published online 2020. https://www.publichealth.com.ng/types-of-mosquitoes/anopheles-mosquito/
70. Nikbakhtzadeh MR, Buss GK, Leal WS. Toxic Effect of Blood Feeding in Male Mosquitoes. *Front Physiol*. 2016;7:4. doi:10.3389/fphys.2016.00004
71. Gary RE, Cannon JW, Foster WA. Effect of sugar on male *Anopheles gambiae* mating performance, as modified by temperature, space, and body size. *Parasit Vectors*. 2009;2(1):19. doi:10.1186/1756-3305-2-19
72. Townson H. The biology of mosquitoes. Volume 1. Development, nutrition and reproduction. By A.N. Clements. (London: Chapman & Hall, 1992). viii + 509 pp. Hard cover £50. ISBN 0-412-40180-0. *Bull Entomol Res*. 1993;83(2):307-308. doi:DOI: 10.1017/S0007485300034830
73. Klowden MJ, Briegel H. Mosquito Gonotrophic Cycle and Multiple Feeding Potential: Contrasts Between Anopheles and Aedes (Diptera: Culicidae). *J Med*

Entomol. 1994;31(4):618-622. doi:10.1093/jmedent/31.4.618

74. Dahalan FA, Churcher TS, Windbichler N, Lawniczak MKN. The male mosquito contribution towards malaria transmission: Mating influences the *Anopheles* female midgut transcriptome and increases female susceptibility to human malaria parasites. *PLOS Pathog*. 2019;15(11):e1008063. https://doi.org/10.1371/journal.ppat.1008063

75. Sinka ME, Bangs MJ, Manguin S, et al. The dominant *Anopheles* vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic précis. *Parasit Vectors*. 2010;3:117. doi:10.1186/1756-3305-3-117

76. Coulibaly MB, Traoré SF, Touré YT. Chapter 3 - Considerations for Disrupting Malaria Transmission in Africa Using Genetically Modified Mosquitoes, Ecology of Anopheline Disease Vectors, and Current Methods of Control. In: Adelman ZNBT-GC of M and D, ed. Academic Press; 2016:55-67. doi:https://doi.org/10.1016/B978-0-12-800246-9.00003-X

77. Barrón MG, Paupy C, Rahola N, et al. A new species in the major malaria vector complex sheds light on reticulated species evolution. *Sci Rep*. 2019;9(1):14753. doi:10.1038/s41598-019-49065-5

78. Famakinde DO. Mosquitoes and the Lymphatic Filarial Parasites: Research Trends and Budding Roadmaps to Future Disease Eradication. *Trop Med Infect Dis*. 2018;3(1):4. doi:10.3390/tropicalmed3010004

79. Nava MR, Debboun M. Chapter 2 - Mosquito Species of Texas. In: Debboun M, Nava MR, Rueda Communities, and Public Health in Texas LMBT-M, eds. Academic Press; 2020:9-167. doi:https://doi.org/10.1016/B978-0-12-814545-6.00002-X

80. Athrey G, Cosme L V, Popkin-Hall Z, Pathikonda S, Takken W, Slotman MA. Chemosensory gene expression in olfactory organs of the anthropophilic *Anopheles coluzzii* and zoophilic *Anopheles quadriannulatus*. *BMC Genomics*. 2017;18(1):751. doi:10.1186/s12864-017-4122-7

81. Control T malERA CG on V. A Research Agenda for Malaria Eradication: Vector Control. *PLOS Med*. 2011;8(1):e1000401. https://doi.org/10.1371/journal.pmed.1000401

82. Clayton AM, Dong Y, Dimopoulos G. The *Anopheles* innate immune system in the defense against malaria infection. *J Innate Immun*. 2014;6(2):169-181.

doi:10.1159/000353602

83. Cansado-Utrilla C, Zhao SY, McCall PJ, Coon KL, Hughes GL. The microbiome and mosquito vectorial capacity: rich potential for discovery and translation. *Microbiome*. 2021;9(1):111. doi:10.1186/s40168-021-01073-2

84. Gravitz L. Vector control: The last bite. *Nature*. 2012;484(7395):S26-S27. doi:10.1038/484S26a

85. Ferguson NM. Challenges and opportunities in controlling mosquito-borne infections. *Nature*. 2018;559(7715):490-497. doi:10.1038/s41586-018-0318-5

86. Caminade C, McIntyre KM, Jones AE. Impact of recent and future climate change on vector-borne diseases. *Ann N Y Acad Sci*. 2019;1436(1):157-173. doi:10.1111/nyas.13950

87. Diabate A, Baldet T, Brengues C, et al. Natural swarming behaviour of the molecular M form of *Anopheles gambiae*. *Trans R Soc Trop Med Hyg*. 2003;97(6):713-716. doi:https://doi.org/10.1016/S0035-9203(03)80110-4

88. Garcia Castillo SS, Pritts KS, Krishnan RS, Harrington LC, League GP. Harmonic convergence coordinates swarm mating by enhancing mate detection in the malaria mosquito *Anopheles gambiae*. *Sci Rep*. 2021;11(1):24102. doi:10.1038/s41598-021-03236-5

89. Klowden MJ, Russell RC. Mating affects egg maturation in *Anopheles gambiae* Giles (*Diptera: Culicidae*). *J Vector Ecol*. 2004;29(1):135-139.

90. Dana AN, Hong YS, Kern MK, et al. Gene expression patterns associated with blood-feeding in the malaria mosquito *Anopheles gambiae*. *BMC Genomics*. 2005;6:5. doi:10.1186/1471-2164-6-5

91. Tripet F, Touré YT, Dolo G, Lanzaro GC. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. *Am J Trop Med Hyg*. 2003;68(1):1-5.

92. Charlwood JD, Pinto J, Sousa CA, Ferreira C, Do Rosário VE. Male size does not affect mating success (of *Anopheles gambiae* in São Tomé). *Med Vet Entomol*. 2002;16(1):109-111. doi:10.1046/j.0269-283x.2002.00342.x

93. Okanda FM, Dao A, Njiru BN, et al. Behavioural determinants of gene flow in malaria vector populations: *Anopheles gambiae* males select large females as mates. *Malar J*. 2002;1(1):10. doi:10.1186/1475-2875-1-10

94. Matthews J, Bethel A, Osei G. An overview of malarial *Anopheles* mosquito

survival estimates in relation to methodology. *Parasit Vectors*. 2020;13(1):233. doi:10.1186/s13071-020-04092-4

95. Charlwood JD, Thompson R, Madsen H. Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique. *Malar J*. 2003;2(1):2. doi:10.1186/1475-2875-2-2
96. Bertola M, Mazzucato M, Pombi M, Montarsi F. Updated occurrence and bionomics of potential malaria vectors in Europe: a systematic review (2000–2021). *Parasit Vectors*. 2022;15(1):88. doi:10.1186/s13071-022-05204-y
97. Levashina EA. Immune responses in *Anopheles gambiae*. *Insect Biochem Mol Biol*. 2004;34(7):673-678. doi:https://doi.org/10.1016/j.ibmb.2004.03.020
98. Shahabuddin M, Costero A. Spatial distribution of factors that determine sporogonic development of malaria parasites in mosquitoes. *Insect Biochem Mol Biol*. 2001;31(3):231-240. doi:10.1016/s0965-1748(00)00142-9
99. Sinka ME, Bangs MJ, Manguin S, et al. A global map of dominant malaria vectors. *Parasit Vectors*. 2012;5(1):69. doi:10.1186/1756-3305-5-69
100. Billingsley PF, Lehane MJ. Structure and ultrastructure of the insect midgut. *Biol Insect Midgut*. Published online 1996:3-30. doi:10.1007/978-94-009-1519-0_1
101. Wigglesworth VB. *The Principles of Insect Physiology*. Springer Netherlands PP - Dordrecht; 1972. doi:10.1007/978-94-009-5973-6
102. Smith RC, Jacobs-Lorena M. *Plasmodium*-Mosquito Interactions: A Tale of Roadblocks and Detours. *Adv In Insect Phys*. 2010;39:119-149. doi:10.1016/B978-0-12-381387-9.00004-X
103. Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog*. 2006;2(6):e52. doi:10.1371/journal.ppat.0020052
104. Boissière A, Tchioffo MT, Bachar D, et al. Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog*. 2012;8(5):e1002742. doi:10.1371/journal.ppat.1002742
105. Romoli O, Gendrin M. The tripartite interactions between the mosquito, its microbiota and *Plasmodium*. *Parasit Vectors*. 2018;11(1):200. doi:10.1186/s13071-018-2784-x
106. Coon KL, Vogel KJ, Brown MR, Strand MR. Mosquitoes rely on their gut

microbiota for development. *Mol Ecol*. 2014;23(11):2727-2739. doi:10.1111/mec.12771

107. Richman AM, Dimopoulos G, Seeley D, Kafatos FC. *Plasmodium* activates the innate immune response of *Anopheles gambiae* mosquitoes. *EMBO J*. 1997;16(20):6114-6119. doi:https://doi.org/10.1093/emboj/16.20.6114
108. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. *PLoS Pathog*. 2009;5(5):e1000423-e1000423. doi:10.1371/journal.ppat.1000423
109. Lavazec C, Boudin C, Lacroix R, et al. Carboxypeptidases B of *Anopheles gambiae* as targets for a *Plasmodium falciparum* transmission-blocking vaccine. *Infect Immun*. 2007;75(4):1635-1642. doi:10.1128/IAI.00864-06
110. Hillyer JF, Barreau C, Vernick KD. Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *Int J Parasitol*. 2007;37(6):673-681. doi:10.1016/j.ijpara.2006.12.007
111. Povelones M, Osta MA, Christophides GK. Chapter Eight - The Complement System of Malaria Vector Mosquitoes. In: Raikhel ASBT-A in IP, ed. *Progress in Mosquito Research*. Vol 51. Academic Press; 2016:223-242. doi:https://doi.org/10.1016/bs.aiip.2016.06.001
112. Thiévent K, Zilio G, Hauser G, Koella JC. Malaria load affects the activity of mosquito salivary apyrase. *J Insect Physiol*. 2019;116:10-16. doi:10.1016/j.jinsphys.2019.04.003
113. Heard E, Martienssen RA. Transgenerational Epigenetic Inheritance: Myths and Mechanisms. *Cell*. 2014;157(1):95-109. doi:https://doi.org/10.1016/j.cell.2014.02.045
114. Handel AE, Ebers GC, Ramagopalan S V. Epigenetics: molecular mechanisms and implications for disease. *Trends Mol Med*. 2010;16(1):7-16. doi:https://doi.org/10.1016/j.molmed.2009.11.003
115. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell*. 2007;128(4):669-681. doi:10.1016/j.cell.2007.01.033
116. Nelson VR, Nadeau JH. Transgenerational genetic effects. *Epigenomics*. 2010;2(6):797-806. doi:10.2217/epi.10.57
117. Curley JP, Mashoodh R, Champagne FA. Epigenetics and the origins of paternal effects. *Horm Behav*. 2011;59(3):306-314. doi:10.1016/j.yhbeh.2010.06.018

118. Richards EJ. Inherited epigenetic variation--revisiting soft inheritance. *Nat Rev Genet*. 2006;7(5):395-401. doi:10.1038/nrg1834
119. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet*. 2007;8(4):253-262. doi:10.1038/nrg2045
120. Relton CL, Davey Smith G. Epigenetic Epidemiology of Common Complex Disease: Prospects for Prediction, Prevention, and Treatment. *PLOS Med*. 2010;7(10):e1000356. https://doi.org/10.1371/journal.pmed.1000356
121. Gómez-Díaz E, Jordà M, Peinado MA, Rivero A. Epigenetics of Host–Pathogen Interactions: The Road Ahead and the Road Behind. *PLOS Pathog*. 2012;8(11):e1003007. https://doi.org/10.1371/journal.ppat.1003007
122. Somer RA, Thummel CS. Epigenetic inheritance of metabolic state. *Curr Opin Genet Dev*. 2014;27:43-47. doi:https://doi.org/10.1016/j.gde.2014.03.008
123. Matzkin LM, Johnson S, Paight C, Markow TA. Preadult parental diet affects offspring development and metabolism in *Drosophila melanogaster*. *PLoS One*. 2013;8(3):e59530. doi:10.1371/journal.pone.0059530
124. Buescher JL, Musselman LP, Wilson CA, et al. Evidence for transgenerational metabolic programming in *Drosophila*. *Dis Model Mech*. 2013;6(5):1123-1132. doi:10.1242/dmm.011924
125. Ptak C, Petronis A. Epigenetics and complex disease: from etiology to new therapeutics. *Annu Rev Pharmacol Toxicol*. 2008;48:257-276. doi:10.1146/annurev.pharmtox.48.113006.094731
126. Moore LD, Le T, Fan G. DNA Methylation and Its Basic Function. *Neuropsychopharmacology*. 2013;38(1):23-38. doi:10.1038/npp.2012.112
127. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet*. 2008;9(6):465-476. doi:10.1038/nrg2341
128. Rottach A, Leonhardt H, Spada F. DNA methylation-mediated epigenetic control. *J Cell Biochem*. 2009;108(1):43-51. doi:10.1002/jcb.22253
129. Wion D, Casadesús J. N6-methyl-adenine: an epigenetic signal for DNA–protein interactions. *Nat Rev Microbiol*. 2006;4(3):183-192. doi:10.1038/nrmicro1350
130. O'Brown ZK, Greer EL. N6-Methyladenine: A Conserved and Dynamic DNA Mark. *Adv Exp Med Biol*. 2016;945:213-246. doi:10.1007/978-3-319-43624-1_10
131. Luo G-Z, Blanco MA, Greer EL, He C, Shi Y. DNA N6-methyladenine: a new epigenetic mark in eukaryotes? *Nat Rev Mol Cell Biol*. 2015;16(12):705-710.

doi:10.1038/nrm4076

132. Zhang G, Huang H, Liu D, et al. N6-methyladenine DNA modification in Drosophila. *Cell*. 2015;161(4):893-906. doi:10.1016/j.cell.2015.04.018

133. Greer EL, Blanco MA, Gu L, et al. DNA Methylation on N6-Adenine in C. elegans. *Cell*. 2015;161(4):868-878. doi:10.1016/j.cell.2015.04.005

134. Ratel D, Ravanat J-L, Berger F, Wion D. N6-methyladenine: the other methylated base of DNA. *BioEssays*. 2006;28(3):309-315. doi:https://doi.org/10.1002/bies.20342

135. Liu J, Yue Y, Han D, et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol*. 2014;10(2):93-95. doi:10.1038/nchembio.1432

136. Schaefer M, Lyko F. Solving the Dnmt2 enigma. *Chromosoma*. 2010;119(1):35-40. doi:10.1007/s00412-009-0240-6

137. Bonduriansky R, Day T. Nongenetic Inheritance and Its Evolutionary Implications. *Annu Rev Ecol Evol Syst*. 2008;40(1):103-125. doi:10.1146/annurev.ecolsys.39.110707.173441

138. Lind MI, Spagopoulou F. Evolutionary consequences of epigenetic inheritance. *Heredity (Edinb)*. 2018;121(3):205-209. doi:10.1038/s41437-018-0113-y

139. Mousseau TA, Fox CW. The adaptive significance of maternal effects. *Trends Ecol Evol*. 1998;13(10):403-407. doi:https://doi.org/10.1016/S0169-5347(98)01472-4

140. Ridley M. Paternal care. *Anim Behav*. 1978;26:904-932. doi:https://doi.org/10.1016/0003-3472(78)90156-2

141. Tallamy DW. Insect Parental Care. *Bioscience*. 1984;34(1):20-24. doi:10.2307/1309421

142. Valtonen TM, Kangassalo K, Pölkki M, Rantala MJ. Transgenerational Effects of Parental Larval Diet on Offspring Development Time, Adult Body Size and Pathogen Resistance in *Drosophila melanogaster*. *PLoS One*. 2012;7(2):e31611. https://doi.org/10.1371/journal.pone.0031611

143. BONDURIANSKY R, HEAD M. Maternal and paternal condition effects on offspring phenotype in *Telostylinus angusticollis (Diptera: Neriidae)*. *J Evol Biol*. 2007;20(6):2379-2388. doi:https://doi.org/10.1111/j.1420-9101.2007.01419.x

144. Grech K, Maung LA, Read AF. The effect of parental rearing conditions on

offspring life history in *Anopheles stephensi*. *Malar J*. 2007;6(1):130. doi:10.1186/1475-2875-6-130

145. Donelson JM, Munday PL, McCormick MI. Parental effects on offspring life histories: when are they important? *Biol Lett*. 2009;5(2):262-265. doi:10.1098/rsbl.2008.0642

146. Mitchell SE, Read AF. Poor maternal environment enhances offspring disease resistance in an invertebrate. *Proc R Soc B Biol Sci*. 2005;272(1581):2601-2607. doi:10.1098/rspb.2005.3253

147. Greer EL, Maures TJ, Ucar D, et al. Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans. Nature*. 2011;479(7373):365-371. doi:10.1038/nature10572

148. Rechavi O, Houri-Ze'evi L, Anava S, et al. Starvation-induced transgenerational inheritance of small RNAs in *C. elegans*. *Cell*. 2014;158(2):277-287. doi:10.1016/j.cell.2014.06.020

149. Sadd BM, Schmid-Hempel P. Facultative but persistent trans-generational immunity via the mother's eggs in bumblebees. *Curr Biol*. 2007;17(24):R1046-R1047. doi:https://doi.org/10.1016/j.cub.2007.11.007

150. Little TJ, O'Connor B, Colegrave N, Watt K, Read AF. Maternal Transfer of Strain-Specific Immunity in an Invertebrate. *Curr Biol*. 2003;13(6):489-492. doi:https://doi.org/10.1016/S0960-9822(03)00163-5

151. Yanchula KZ, Alto BW. Paternal and maternal effects in a mosquito: A bridge for life history transition. *J Insect Physiol*. 2021;131:104243. doi:10.1016/j.jinsphys.2021.104243

152. Rogers DJ, Randolph SE. The global spread of malaria in a future, warmer world. *Science*. 2000;289(5485):1763-1766. doi:10.1126/science.289.5485.1763

153. Paaijmans KP, Blanford S, Bell AS, Blanford JI, Read AF, Thomas MB. Influence of climate on malaria transmission depends on daily temperature variation. *Proc Natl Acad Sci U S A*. 2010;107(34):15135-15139. doi:10.1073/pnas.1006422107

154. Lorenz LM, Koella JC. Maternal environment shapes the life history and susceptibility to malaria of *Anopheles gambiae* mosquitoes. *Malar J*. 2011;10(1):382. doi:10.1186/1475-2875-10-382

155. Frost PC, Ebert D, Larson JH, Marcus MA, Wagner ND, Zalewski A. Transgenerational effects of poor elemental food quality on *Daphnia magna*.

Oecologia. 2010;162(4):865-872. doi:10.1007/s00442-009-1517-4

156. Jones TM, Widemo F. Survival and reproduction when food is scarce: implications for a lekking Hawaiian *Drosophila*. *Ecol Entomol*. 2005;30(4):397-405. doi:https://doi.org/10.1111/j.0307-6946.2005.00705.x
157. Mitri C, Thiery I, Lecoq M-T, et al. *Anopheles gambiae* maternal age and parous state control offspring susceptibility to *Plasmodium falciparum*. *bioRxiv*. Published online 2020. doi:10.1101/2020.01.27.922070
158. Ruiz JL, Yerbanga RS, Lefèvre T, Ouedraogo JB, Corces VG, Gómez-Díaz E. Chromatin changes in *Anopheles gambiae* induced by *Plasmodium falciparum* infection. *Epigenetics Chromatin*. 2019;12(1):5. doi:10.1186/s13072-018-0250-9
159. Gapp K, Jawaid A, Sarkies P, et al. Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat Neurosci*. 2014;17(5):667-669. doi:10.1038/nn.3695
160. Poulin R, Thomas F. Epigenetic effects of infection on the phenotype of host offspring: Parasites reaching across host generations. *Oikos*. 2008;117:331-335. doi:10.1111/j.2007.0030-1299.16435.x
161. Reece SE, Ramiro RS, Nussey DH. SYNTHESIS: Plastic parasites: sophisticated strategies for survival and reproduction? *Evol Appl*. 2009;2(1):11-23. doi:https://doi.org/10.1111/j.1752-4571.2008.00060.x
162. Rando OJ, Verstrepen KJ. Timescales of Genetic and Epigenetic Inheritance. *Cell*. 2007;128(4):655-668. doi:https://doi.org/10.1016/j.cell.2007.01.023
163. Silmon de Monerri NC, Kim K. Pathogens hijack the epigenome: a new twist on host-pathogen interactions. *Am J Pathol*. 2014;184(4):897-911. doi:10.1016/j.ajpath.2013.12.022
164. Denzer L, Schroten H, Schwerk C. From Gene to Protein-How Bacterial Virulence Factors Manipulate Host Gene Expression During Infection. *Int J Mol Sci*. 2020;21(10):3730. doi:10.3390/ijms21103730
165. Løbner-Olesen A, Skovgaard O, Marinus MG. Dam methylation: coordinating cellular processes. *Curr Opin Microbiol*. 2005;8(2):154-160. doi:https://doi.org/10.1016/j.mib.2005.02.009
166. Richards EJ. Population epigenetics. *Curr Opin Genet Dev*. 2008;18(2):221-226. doi:https://doi.org/10.1016/j.gde.2008.01.014
167. Croken MM, Nardelli SC, Kim K. Chromatin modifications, epigenetics, and how

protozoan parasites regulate their lives. *Trends Parasitol*. 2012;28(5):202-213. doi:https://doi.org/10.1016/j.pt.2012.02.009

168. Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Curr Biol*. 2005;15(13):1185-1195. doi:10.1016/j.cub.2005.06.044
169. Lefèvre T, Vantaux A, Dabiré KR, Mouline K, Cohuet A. Non-Genetic Determinants of Mosquito Competence for Malaria Parasites. *PLOS Pathog*. 2013;9(6):e1003365. https://doi.org/10.1371/journal.ppat.1003365
170. Lambrechts L, Chavatte J-M, Snounou G, Koella JC. Environmental influence on the genetic basis of mosquito resistance to malaria parasites. *Proceedings Biol Sci*. 2006;273(1593):1501-1506. doi:10.1098/rspb.2006.3483
171. Rinker DC, Pitts RJ, Zwiebel LJ. Disease vectors in the era of next generation sequencing. *Genome Biol*. 2016;17(1):95. doi:10.1186/s13059-016-0966-4
172. Barreaux AMG, Stone CM, Barreaux P, Koella JC. The relationship between size and longevity of the malaria vector *Anopheles gambiae (s.s.)* depends on the larval environment. *Parasit Vectors*. 2018;11(1):485. doi:10.1186/s13071-018-3058-3
173. Gimnig JE, Ombok M, Otieno S, Kaufman MG, Vulule JM, Walker ED. Density-Dependent Development of *Anopheles gambiae (Diptera: Culicidae)* Larvae in Artificial Habitats . *J Med Entomol*. 2002;39(1):162-172. doi:10.1603/0022-2585-39.1.162
174. Lyimo EO, Takken W, Koella JC. Effect of rearing temperature and larval density on larval survival, age at pupation and adult size of *Anopheles gambiae*. *Entomol Exp Appl*. 1992;63(3):265-271. doi:https://doi.org/10.1111/j.1570-7458.1992.tb01583.x
175. Muller D, Giron D, Desouhant E, et al. Maternal age affects offspring nutrient dynamics. *J Insect Physiol*. 2017;101:123-131. doi:https://doi.org/10.1016/j.jinsphys.2017.07.011

F. Supplementary Data

Annex I: METTL3 Primer Sequence

<u>Without intron ACON002895 (Primer forward: 69.90; Primer reverse: 249.266):</u>

ATGTCTTCGTGGGAGGAAATACAAGCGGTCAAAGTGAAGCGCAACA
GCTTGCGTGAAAAGTTGGAGAAGCGCAAAAAAGAGAGACAAGACTTGCTG
GGAAACAGTAGTCCCGGTGGTCCAGGAGCGGTTGCCGGCTTAATCAAAATC
GAATCAGCCACCAACTTGAGCGAGGATAAGGGTAAACTTCTTCTGACTTCA
ATCAAATCGGATCAAACAGGAAGTGCGGATATTGATGCAGAAGTGGAAAA
ATGTTTGGTGCAGGTGCTTGCGGACAAAAGCTTAATACTGCCCTCCAACTCG
GCCCAGATAGCGGAGCTGGTAGAAAAACACGTACAGAAAGCAGTGCTGCG
TGACTCCATTGCCTATTACCTGCACAAGCTGGCCGGACAGAAATTAATCAAT
GTTAAAGAAGTCAGCATCGGTGGAACTGTGGGCTACGAGGTGATTTCAGCG
GAGCACATCAACCTGCAAGCGCTGCACGATGACATGGCTATGAACCATGGT
CCCGCTGGGCCCGGCAATCGAGACGGACACAAAAGAAAAGCAGATTGTTCC
AAAGATCTGCCCGACGGAGGTAGCAAAGTGGCCCGCGGTACCCTGGCGGGA
GCAAAGGATGAGGGAAGGAAGGGAAACTCGGTAGATGCTTCGCTTTCCTGT
AAAGCGTCCGATATTTTGTCGCTTCTCTCACTCCCATCGACGAGGGAAAAGC
AGAGCAAAAAGGTTGGGGAAGAAATTTTGGAACTGCTTTCGAAACCAACAG
CCAAAGAACGATCACTGGTAGAAAAGTTCAAATCTCAGGGCGGCGCTCAGG
TGATGGAATTCTGCCCGCATGGCACACGCATCGAATGTATGCGCTCTTCGGA
AGCGTCTCCCGAGAGCAAAGAGCCAAGCGACAGTAAAAAGGCCCACGAAA
CGGACGATGAATTCGAAAAGGATAAGGAAACCGCCGTACCAGCGGAAGAT
GTATCGAACAAAACGGACGCAGTGGGGCCTAATGAGATCAAGCTTGAGCCA
GAATCATCGGAAGCTGCGTCTGCCGAACCGCGGCTGGAAGCGGAAGACACT
AAAACGAAATATCAATGCAATAAGCTACATTTCAAGAAAATTATCCAAAAC
CACACGGATGAAACGTTGGGCGACTGTAGCTTCCTGAACACTTGCTTCCATA
TGGATACGTGCAAGTACGTGCACTACGAGGTGGACACGTATGTGGACCAGA
CACCGAATACTGTACCAGCCAAGTTTGAGACGACGGACGAACACGTTGCCG
GACCAAAGCGCCCCATTGCTGATGCGAGCGCTACTCTGTATCCTCCGCAATG
GATTCAGTGCGATTTGCGCTTCCTGGATATGACGGTGCTGGGGAAGTTTGCG
GTAGTAATGGCCGATCCACCGTGGGACATTCACATGGAGCTGCCCTACGGT
ACTATGTCCGATGATGAAATGCGTCAGCTCGGCGTTCCGGCCCTGCAGGAC
GATGGCCTAATTTTCCTGTGGGTTACTGGGCGAGCGATGGAGCTGGGTCGTG

AATGTTTGAAACTATGGGGCTACGAACGAGTGGACGAACTGATCTGGGTAA
AAACGAATCAATTGCAACGCATCATACGAACGGGACGCACCGGCCATTGGC
TAAACCATGGGAAGGAACATTGCCTGGTCGGCATGAAGGGCAATCCTCCGA
ATTTGAATCGTGGACTAGACTGCGATGTGATTGTTGCTGAGGTACGGGCCAC
CAGCCACAAGCCGGACGAAATTTATGGCATCATCGAGCGGCTAAGTCCAGG
CACACGAAAGATTGAGCTATTCGGTCGACCGCATAATGTGCAACCGAACTG
GATTACGCTTGGCAACCAGCTGGACGGCATTCGTTTGGTAGATCCTGAGTTG
ATTAATTCTTTTCAAAAGCGTTACCCGGATGGTAACTGTATGACACCTGGTA
AAATTCCTTAA

Annex II: Establishment of a purification pipeline of sperm from the Spermatheca of young and old *Anopheles* females

One of the three objectives we set to the project was not achieved, establishing a purification pipeline of sperm from the spermatheca of young and old females. The aim behind this objective was to have good quality samples to investigate the impact of sperm aging, while stored in the female spermatheca, on epigenetic marks both at the level of DNA and of RNA content.

Based on published data[1], the host laboratory showed that providing rhodamine in the sugar meal given to male mosquitoes has the capacity of labelling spermatozoa that could be detected in the sperm stored in the females after mating. Importantly, purified rhodamine labelled spermatozoa collected from the male testis could be sorted by (fluorescence activated cell sorting) FACS system.

Based on this data, our objective was to collect rhodamine spermatozoa from female spermatheca at different time after mating and to purified then away from any female cells (unlabelled) using the FACS protocol.

Initially we defined a protocol for labelling sperm with rhodamine B, where the first step was separate female *Anopheles* from male mosquitos, to guarantee the virginity of female mosquitoes, there is a need to separate males from females soon after an emergency.

So, from a general cage, with the help of a home-made small aspirating device, we start by catching the males. These are easier to detect due to the difference in the antennas, a more noticeable difference to a first throw. After verifying that there is no longer any male, in the female's cage a 10% sucrose solution was placed in the cage, so no starvation conditions were imposed to the mosquitoes.

[1] Aviles, E. I., R. D. Rotenberry, C. M. Collins, E. M. Dotson and M. Q. Benedict (2020). "Fluorescent markers rhodamine B and uranine for Anopheles gambiae adults and matings." Malaria Journal 19(1): 236.

Johnson, B. J., S. N. Mitchell, C. J. Paton, J. Stevenson, K. M. Staunton, N. Snoad, N. Beebe, B. J. White and S. A. Ritchie (2017). "Use of rhodamine B to mark the body and seminal fluid of male Aedes aegypti for mark-release-recapture experiments and estimating efficacy of sterile male releases." PLOS Neglected Tropical Diseases 11(9): e0005902.

The second step is marking the sperm of the males *Anopheles* with rhodamine B, so instead of the 10% sucrose solution placed at the female cage was placed in the male cage a solution of 10% sucrose and 0.2% rhodamine B for 48 hours.

After the 48 hours, the males' mosquitoes were added up to the female cage for 72 hours to mate. Then, the spermatheca of female *Anopheles* were dissected.

Female *Anopheles gambiae* were caught by the vacuum and placed in Eppendorf on ice, and the dissection of spermatheca was proceed on glass slide (with the mosquito placed with the wings down) and with the of two fine tweezers (one gripping the chest and the other on the last segment of the abdomen and pulling so as to break the mosquito into two parts, as showed in figure 13) under a glass coverslip with a drop of phosphate-buffered solution (PBS).

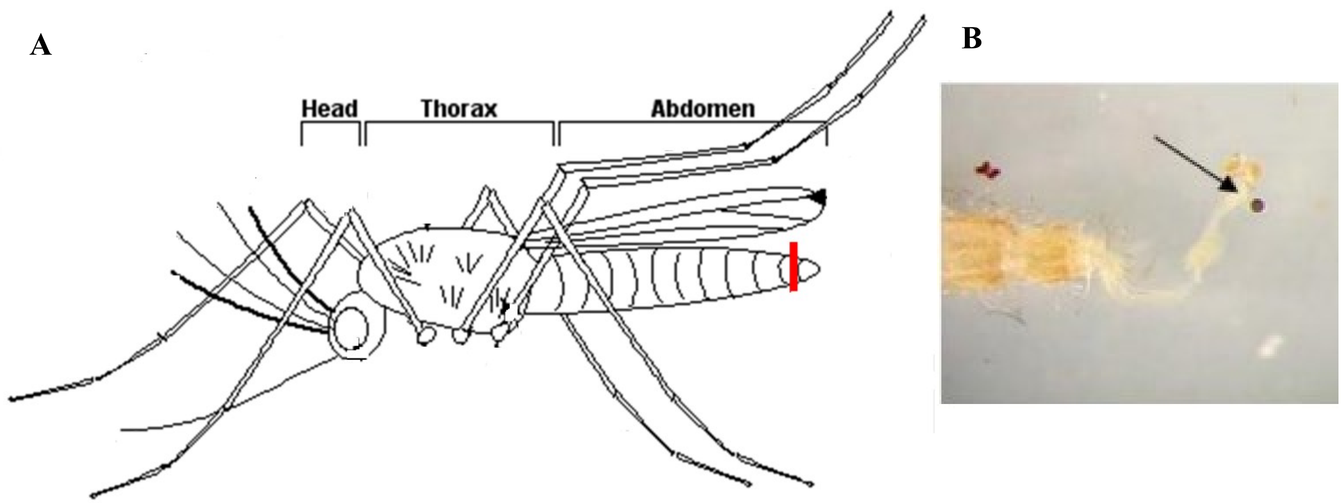

Figure 13 – Scheme of the place the female was tore apart and an intact spermatheca. A - To dissect the female *Anopheles* to obtain the spermatheca intact, the mosquito was ripped with two fine tweezers, one gripping the chest and the other on the last segment of the abdomen and pulling to break the mosquito into two parts (represented by the red line). B – Representation of an intact female *Anopheles* spermatheca as obtained by the dissection explained previously.

The spermatheca was gently cracked open with fine needles, and all the content was aspirated, with the micropipette with silicon tip, to an Eppendorf tube. All the procedure was done at KL1500 LC Zeiss binocular. At that stage a problem appeared, the sperm bundle clotted, which render the collection of large quantity of sperm difficult.

From this problem several questions arise... Why did the sperm clot? Is it from the buffer? Is it from being in direct contact with oxygen? Is it the normal state of this one inside the spermatheca? With the emergence of this problem, a hole was detected in the

bibliography where the theme of sperm motility in *Diptera* and more specifically in *Anopheles* is very poor. Through some articles that analysed sperm motility, some buffers recipes were taken so that it could be used as buffer in the dissection of the spermatheca and rupture to verify if it was the cause of the sperm bundle clotting.

On a first trial, we switch from PBS to an anticoagulation buffer described by Kwon H et al., (60% [vol/vol] Schneider's insect medium, 10% FBS, and 30% citrate buffer; 98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid, pH 4.5)[2]. The result was similar to that obtained previously, clotted sperm bundle.

On a second trial, we switch the dissection buffer to a buffer described by Pitts et al, (145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 1.3 mM CaCl2, 5 mM d-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4), with and without c-bromo-AMP [3]. We tried with c-bromo-AMP because bromo could be used as marked to sort by FACS system and the AMP co-linked to the bromo could be the activator of sperm motility[4]. But nothing worked, the result would still be the same.

The ideal would be to find out what causes this sperm clotting, such as if the dissection buffer, if contact with oxygen, or even rhodamine B... As a solution to this particular problem, sperm bundle clotting, a new dissection buffer could be included, reactivation of sperm movement allowing the "cloud" to de-coagulate or sonication of the spermatheca's contents to desegregate the "cloud". Another marker could be in mind, but additional experiments would have to be carried out to see if it would influence the Anopheles mosquito's mating behaviour or even its biology.

This question is highly relevant as the experiments performed during this project revealed that sperm age influence on some extent the fitness of the progeny, size and life expectancy.

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[2] Hyeogsun K, C. SR. Chemical depletion of phagocytic immune cells in Anopheles gambiae reveals dual roles of mosquito hemocytes in anti-Plasmodium immunity. *Proc Natl Acad Sci*. 2019;116(28):14119-14128. doi:10.1073/pnas.1900147116

[3] Pitts RJ, Liu C, Zhou X, Malpartida JC, Zwiebel LJ. Odorant receptor-mediated sperm activation in disease vector mosquitoes. Proc Natl Acad Sci U S A. 2014 Feb 18;111(7):2566-71. doi: 10.1073/pnas.1322923111. Epub 2014 Feb 3. PMID: 24550284; PMCID: PMC3932880.

[4] Wertheimer E, Krapf D, de la Vega-Beltran JL, Sánchez-Cárdenas C, Navarrete F, Haddad D, Escoffier J, Salicioni AM, Levin LR, Buck J, Mager J, Darszon A, Visconti PE. Compartmentalization of distinct cAMP signaling pathways in mammalian sperm. J Biol Chem. 2013 Dec 6;288(49):35307-20. doi: 10.1074/jbc.M113.489476. Epub 2013 Oct 15. PMID: 24129574; PMCID: PMC3853279.

Annex III: Survival Data Tables

First Biological Replicate – October 2021 to January 2022

Table 3 – Registration of individually daily deaths on each cage from the first biological replicate. Each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) had two replicates (A and B) with around 50 female *Anopheles* to perform the first survival assay until the last female died. The initial population start to be created by October 26th, the day 0 of the survival assay was at November 22nd and the survival assay finished at January 4th. At black are marked the weekends during the survival assay where the deaths were not counted.

Individually Daily Deaths						
	Cages					
Day	1A	1B	2A	2B	3A	3B
0	0	0	0	0	0	0
1	0	0	2	1	0	0
2	0	1	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	1	0	0	0	0
6						
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	1	0
10	0	0	0	0	0	0
11	1	0	0	0	2	0
12						
13						
14	3	0	0	0	4	2
15	3	0	0	1	0	0
16	0	0	1	1	1	0
17	5	0	0	0	0	2
18	3	2	0	3	1	2
19						
20						
21	7	3	2	2	9	6
22	2	0	0	2	2	3
23	0	3	0	0	4	2
24	5	3	1	1	6	4
25	3	2	1	5	2	3
26						
27						
28	11	10	10	13	11	5
29	3	2	3	3	2	3
30	1	3	5	3	3	3

31	1	3	8	3	2	3
32	1	2	2	3		1
33						
34						
35	1	7	3	8		8
36	1	3	5	0		0
37		2	2	0		1
38		1	1	1		2
39		1	0			1
40						
41						
42		0	0			
43		2	1			

Table 4 – Registration of individually sum of deaths on each cage from the first biological replicate. Sum of deaths on each day of the survival assay where each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) had two replicates (A and B) with around 50 females *Anopheles* (1A – 51, 1B – 51, 2A – 47, 2B – 50, 3A – 50 and 3B – 51) to perform the first survival assay until the last female died. The initial population start to be created by October 26th, the day 0 of the survival assay was at November 22nd and the survival assay finished at January 4th.

Individually Sum of Deaths						
	Cages					
Day	**1A**	**1B**	**2A**	**2B**	**3A**	**3B**
0	0	0	0	0	0	0
1	0	0	2	1	0	0
2	0	1	2	1	0	0
3	0	1	2	1	0	0
4	0	1	2	1	0	0
5	0	2	2	1	0	0
6	0	2	2	1	0	0
7	0	2	2	1	0	0
8	0	2	2	1	0	0
9	0	2	2	1	1	0
10	1	2	2	1	1	0
11	1	2	2	1	3	0
12	1	2	2	1	3	0
13	4	2	2	1	3	0
14	7	2	2	1	7	2
15	7	2	2	2	7	2
16	12	2	3	3	8	2
17	15	2	3	3	8	4
18	15	4	3	6	9	6
19	15	4	3	6	9	6
20	22	4	3	6	9	6
21	24	7	5	8	18	12
22	24	7	5	10	20	15
23	29	10	5	10	24	17
24	32	13	6	11	30	21
25	32	15	7	16	32	24
26	32	15	7	16	32	24
27	43	15	7	16	32	24
28	46	25	17	29	43	29
29	47	27	20	32	45	32
30	47	30	25	35	48	35
31	48	33	33	38	50	38
32	49	35	35	41	50	39
33	49	35	35	41	50	39
34	49	35	35	41	50	39
35	50	42	38	49	50	47

36	51	45	43	49	50	47
37	51	47	45	49	50	48
38	51	48	46	50	50	50
39	51	49	46	50	50	51
40	51	49	46	50	50	51
41	51	49	46	50	50	51
42	51	49	46	50	50	51
43	51	51	47	50	50	51

Table 5 – Registration of the survival rate of each cage from the first biological replicate. Survival rate on each day of the survival assay where each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) had two replicates (A and B) with around 50 females *Anopheles* (1A – 51, 1B – 51, 2A – 47, 2B – 50, 3A – 50 and 3B – 51) to perform the first survival assay until the last female died. The initial population start to be created by October 26th, the day 0 of the survival assay was on November 22nd and the survival assay finished on January 4th. At yellow is the closest mark to a 50% of survival of each cage and in light orange is the closest mark to a 85% survival of each cage, these two were choose as reference to infer some comparisons between the cages.

Individually Survival Rate						
	Cages					
Day	1A	1B	2A	2B	3A	3B
0	100,00	100,00	100,00	100,00	100,00	100,00
1	100,00	100,00	95,74	98,00	100,00	100,00
2	100,00	98,04	95,74	98,00	100,00	100,00
3	100,00	98,04	95,74	98,00	100,00	100,00
4	100,00	98,04	95,74	98,00	100,00	100,00
5	100,00	96,08	95,74	98,00	100,00	100,00
6	100,00	96,08	95,74	98,00	100,00	100,00
7	100,00	96,08	95,74	98,00	100,00	100,00
8	100,00	96,08	95,74	98,00	100,00	100,00
9	100,00	96,08	95,74	98,00	98,00	100,00
10	98,04	96,08	95,74	98,00	98,00	100,00
11	98,04	96,08	95,74	98,00	94,00	100,00
12	98,04	96,08	95,74	98,00	94,00	100,00
13	92,16	96,08	95,74	98,00	94,00	100,00
14	86,27	96,08	95,74	98,00	86,00	96,08
15	86,27	96,08	95,74	96,00	86,00	96,08
16	76,47	96,08	93,62	94,00	84,00	96,08
17	70,59	96,08	93,62	94,00	84,00	92,16
18	70,59	92,16	93,62	88,00	82,00	88,24
19	70,59	92,16	93,62	88,00	82,00	88,24
20	56,86	92,16	93,62	88,00	82,00	88,24
21	52,94	86,27	89,36	84,00	64,00	76,47
22	52,94	86,27	89,36	80,00	60,00	70,59
23	43,14	80,39	89,36	80,00	52,00	66,67
24	37,25	74,51	87,23	78,00	40,00	58,82
25	37,25	70,59	85,11	68,00	36,00	52,94
26	37,25	70,59	85,11	68,00	36,00	52,94
27	15,69	70,59	85,11	68,00	36,00	52,94
28	9,80	50,98	63,83	42,00	14,00	43,14
29	7,84	47,06	57,45	36,00	10,00	37,25
30	7,84	41,18	46,81	30,00	4,00	31,37
31	5,88	35,29	29,79	24,00	0,00	25,49
32	3,92	31,37	25,53	18,00	0,00	23,53
33	3,92	31,37	25,53	18,00	0,00	23,53

34	3,92	31,37	25,53	18,00	0,00	23,53
35	1,96	17,65	19,15	2,00	0,00	7,84
36	0,00	11,76	8,51	2,00	0,00	7,84
37	0,00	7,84	4,26	2,00	0,00	5,88
38	0,00	5,88	2,13	0,00	0,00	1,96
39	0,00	3,92	2,13	0,00	0,00	0,00
40	0,00	3,92	2,13	0,00	0,00	0,00
41	0,00	3,92	2,13	0,00	0,00	0,00
42	0,00	3,92	2,13	0,00	0,00	0,00
43	0,00	0,00	0,00	0,00	0,00	0,00

Table 6 – Registration of the survival rate of each cross (DP1my, DP1o and DP1y) from the first biological replicate. Survival rate on each day of the survival assay of each cross, where the first survival assay was performed until the last female died. The initial population start to be created by October 26th, the day 0 of the survival assay was on November 22nd and the survival assay finished on January 4th. At yellow is the closest mark to a 50% of survival of each cage and in light orange is the closest mark to an 85% survival of each cage, these two were choose as reference to infer some comparisons between the cages.

Survival Rate Crosses			
	Cross		
Day	**DP1my**	**DP1o**	**DP1y**
0	100,00	100,00	100,00
1	100,00	96,94	100,00
2	99,02	96,94	100,00
3	99,02	96,94	100,00
4	99,02	96,94	100,00
5	98,04	96,94	100,00
6	98,04	96,94	100,00
7	98,04	96,94	100,00
8	98,04	96,94	100,00
9	98,04	96,94	99,00
10	98,04	96,94	99,00
11	97,06	96,94	97,00
12	97,06	96,94	97,00
13	97,06	96,94	97,00
14	94,12	96,94	91,00
15	91,18	95,92	91,00
16	91,18	93,88	90,00
17	86,27	93,88	88,00
18	81,37	90,82	85,00
19	81,37	90,82	85,00
20	81,37	90,82	85,00
21	71,57	86,73	70,00
22	69,61	84,69	65,00
23	66,67	84,69	59,00
24	58,82	82,65	49,00
25	53,92	76,53	44,00
26	53,92	76,53	44,00
27	53,92	76,53	44,00
28	33,33	53,06	28,00
29	28,43	46,94	23,00
30	24,51	38,78	17,00
31	20,59	27,55	12,00
32	17,65	22,45	11,00
33	17,65	22,45	11,00
34	17,65	22,45	11,00

35	9,80	11,22	3,00
36	5,88	6,12	3,00
37	3,92	4,08	2,00
38	2,94	2,04	0,00
39	1,96	2,04	-1,00
40	1,96	2,04	-1,00
41	1,96	2,04	-1,00
42	1,96	2,04	-1,00
43	0,00	1,02	-1,00

Second Biological Replicate – February 2021 to April 2022

Table 7 – Registration of individually daily deaths on each cage from the second biological replicate. Each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) where crosses 1 and 2 had only one replicate, due to problems from the mother population in laying eggs and cross 3 had two replicates (A and B). On this replicate there was some differences on the density population with around 25 females *Anopheles*, the second survival assay was performed until the last female died. The initial population start to be created by February 5th, the day 0 of the survival assay was at March 5th to cross 1 and 2, and March 8th to cross 3 November 22nd and the survival assay finished at April 22nd. At blue where marked the weekends during the survival assay where the deaths were not counted.

Individually Daily Deaths				
	Cages			
Day	**1A**	**2A**	**3A**	**3B**
0	0	0	0	0
1			0	1
2			0	0
3	1	0	0	0
4	0	1	0	0
5	0	0		
6	0	0		
7	0	0	3	2
8			1	3
9			2	3
10	0	0	1	4
11	0	0	0	3
12	1	0		
13	0	0		
14	0	1	1	1
15			0	0
16			0	0
17	0	1	0	0
18	0	0	0	0
19	0	0		
20	0	0		
21	0	2	3	1
22			0	0
23			2	3
24	1	7	0	2
25	0	0	1	3
26	3	0		
27	2	2		
28	0	2	9	
29				

30				
31	3	2		
32	1	1		
33	1	1		
34	1	0		
35	1	3		
36				
37				
38	0	6		
39	0	0		
40	0	0		
41	0	1		
42	0	2		
43				
44				
45				
46	2			
47	0			
48	0			
49	2			

Table 8 – Registration of individually sum of daily deaths on each cage from the second biological replicate. Each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) where crosses 1 and 2 had only one replicate, due to problems from the mother population in laying eggs and cross 3 had two replicates (A and B). On this replicate there was some differences on the density population with around 25 females *Anopheles* (1A – 19; 2A – 32; 3A – 23 and 3B – 26), the second survival assay was performed until the last female died. The initial population start to be created by February 5th, the day 0 of the survival assay was on March 5th to cross 1 and 2, and March 8th to cross 3 November 22nd and the survival assay finished at April 22nd. At blue where marked the weekends during the survival assay where the deaths were not counted.

Individually Sum of Deaths				
	Cages			
Day	**1A**	**2A**	**3A**	**3B**
0	0	0	0	0
1	0	0	0	1
2	0	0	0	1
3	1	0	0	1
4	1	1	0	1
5	1	1	0	1
6	1	1	0	1
7	1	1	3	3
8	1	1	4	6
9	1	1	6	9
10	1	1	7	13
11	1	1	7	16
12	2	1	7	16
13	2	1	7	16
14	2	2	8	17
15	2	2	8	17
16	2	2	8	17
17	2	3	8	17
18	2	3	8	17
19	2	3	8	17
20	2	3	8	17
21	2	5	11	18
22	2	5	11	18
23	2	5	13	21
24	3	12	13	23
25	3	12	14	26
26	6	12	14	26
27	8	14	14	26
28	8	16	23	26
29	8	16	23	26
30	8	16	23	26

31	11	18	23	26
32	12	19	23	26
33	13	20	23	26
34	14	20	23	26
35	15	23	23	26
36	15	23	23	26
37	15	23	23	26
38	15	29	23	26
39	15	29	23	26
40	15	29	23	26
41	15	30	23	26
42	15	32	23	26
43	15	32	23	26
44	15	32	23	26
45	15	32	23	26
46	17	32	23	26
47	17	32	23	26
48	17	32	23	26
49	19	32	23	26

Table 9 – Survival Rate of cage (1A, 2A, 3A and 3B) from the second biological replicate. Each cross (1 – DP1my, 2 – DP1o and 3 – DP1y) where crosses 1 and 2 had only one replicate, due to problems from the mother population in laying eggs and cross 3 had two replicates (A and B). The second survival assay was performed until the last female died. The initial population start to be created by February 5th, the day 0 of the survival assay was on March 5th to cross 1 and 2, and March 8th to cross 3 November 22nd and the survival assay finished on April 22nd. At blue where marked the weekends during the survival assay where the deaths were not counted. At yellow is the closest mark to a 50% of survival of each cage, this mark was chosen as reference to infer some comparisons between the cages.

Individually Survival Rate				
	Cages			
Day	**1A**	**2A**	**3A**	**3B**
0	100,00	100,00	100,00	100,00
1	100,00	100,00	100,00	96,15
2	100,00	100,00	100,00	96,15
3	94,74	100,00	100,00	96,15
4	94,74	96,88	100,00	96,15
5	94,74	96,88	100,00	96,15
6	94,74	96,88	100,00	96,15
7	94,74	96,88	86,96	88,46
8	94,74	96,88	82,61	76,92
9	94,74	96,88	73,91	65,38
10	94,74	96,88	69,57	50,00
11	94,74	96,88	69,57	38,46
12	89,47	96,88	69,57	38,46
13	89,47	96,88	69,57	38,46
14	89,47	93,75	65,22	34,62
15	89,47	93,75	65,22	34,62
16	89,47	93,75	65,22	34,62
17	89,47	90,63	65,22	34,62
18	89,47	90,63	65,22	34,62
19	89,47	90,63	65,22	34,62
20	89,47	90,63	65,22	34,62
21	89,47	84,38	52,17	30,77
22	89,47	84,38	52,17	30,77
23	89,47	84,38	43,48	19,23
24	84,21	62,50	43,48	11,54
25	84,21	62,50	39,13	0,00
26	68,42	62,50	39,13	0,00
27	57,89	56,25	39,13	0,00
28	57,89	50,00	0,00	0,00
29	57,89	50,00	0,00	0,00
30	57,89	50,00	0,00	0,00
31	42,11	43,75	0,00	0,00

32	36,84	40,63	0,00	0,00
33	31,58	37,50	0,00	0,00
34	26,32	37,50	0,00	0,00
35	21,05	28,13	0,00	0,00
36	21,05	28,13	0,00	0,00
37	21,05	28,13	0,00	0,00
38	21,05	9,38	0,00	0,00
39	21,05	9,38	0,00	0,00
40	21,05	9,38	0,00	0,00
41	21,05	6,25	0,00	0,00
42	21,05	0,00	0,00	0,00
43	21,05	0,00	0,00	0,00
44	21,05	0,00	0,00	0,00
45	21,05	0,00	0,00	0,00
46	10,53	0,00	0,00	0,00
47	10,53	0,00	0,00	0,00
48	10,53	0,00	0,00	0,00
49	0,00	0,00	0,00	0,00

Table 10 – Survival Rate of each cross (DP1my, DP1o and DP1y) from the second biological replicate. Survival rate on each day of the survival assay of each cross, where the first survival assay was performed until the last female died. The initial population start to be created by February 5th, the day 0 of the survival assay was on March 5th to cross 1 and 2, and March 8th to cross 3 November 22nd and the survival assay finished on April 22nd. At blue where marked the weekends during the survival assay where the deaths were not counted. At yellow is the closest mark to a 50% of survival of each cage, this mark was chosen as reference to infer some comparisons between the cages.

Survival Rate of Crosses			
	Cross		
Day	**DP1my**	**DP1o**	**DP1y**
0	100,00	100,00	100,00
1	100,00	100,00	97,96
2	100,00	100,00	97,96
3	94,74	100,00	97,96
4	94,74	96,88	97,96
5	94,74	96,88	97,96
6	94,74	96,88	97,96
7	94,74	96,88	87,76
8	94,74	96,88	79,59
9	94,74	96,88	69,39
10	94,74	96,88	59,18
11	94,74	96,88	53,06
12	89,47	96,88	53,06
13	89,47	96,88	53,06
14	89,47	93,75	48,98
15	89,47	93,75	48,98
16	89,47	93,75	48,98
17	89,47	90,63	48,98
18	89,47	90,63	48,98
19	89,47	90,63	48,98
20	89,47	90,63	48,98
21	89,47	84,38	40,82
22	89,47	84,38	40,82
23	89,47	84,38	30,61
24	84,21	62,50	26,53
25	84,21	62,50	18,37
26	68,42	62,50	18,37
27	57,89	56,25	18,37
28	57,89	50,00	0,00
29	57,89	50,00	0,00
30	57,89	50,00	0,00
31	42,11	43,75	0,00

32	36,84	40,63	0,00
33	31,58	37,50	0,00
34	26,32	37,50	0,00
35	21,05	28,13	0,00
36	21,05	28,13	0,00
37	21,05	28,13	0,00
38	21,05	9,38	0,00
39	21,05	9,38	0,00
40	21,05	9,38	0,00
41	21,05	6,25	0,00
42	21,05	0,00	0,00
43	21,05	0,00	0,00
44	21,05	0,00	0,00
45	21,05	0,00	0,00
46	10,53	0,00	0,00
47	10,53	0,00	0,00
48	10,53	0,00	0,00
49	0,00	0,00	0,00

Annex IV: A small history from the second biological replicate

Female Anopheles body Size vs Male body Size:

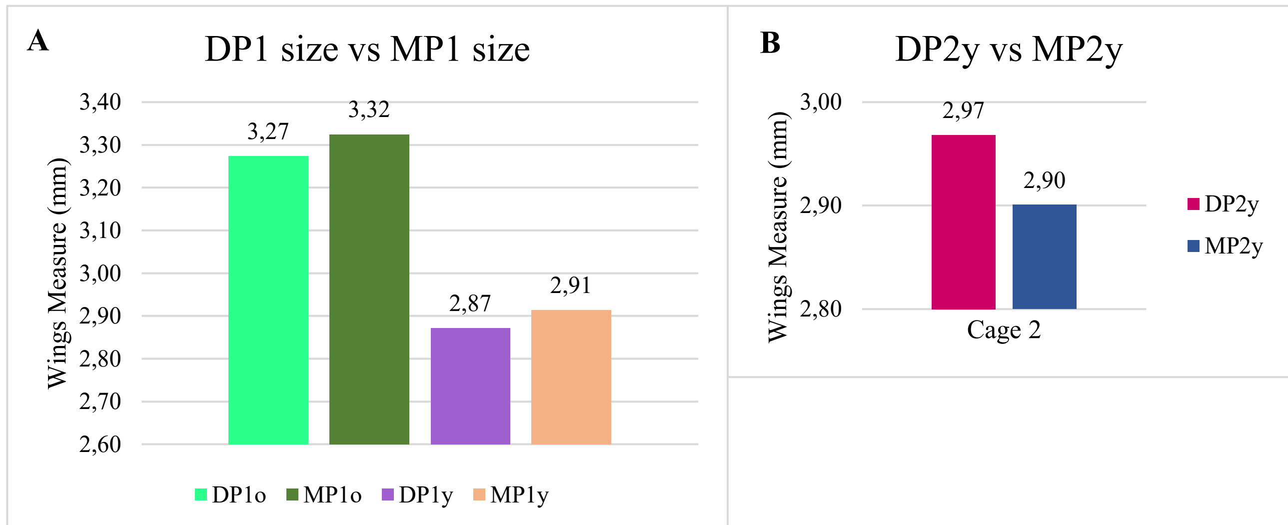

Figure 14 – Wings measure of male and female *Anopheles* from DP1o, DP1y and DP2y. A - Average of wings measures of daughter progenies (DP1o – light green, and DP1y – light purple) and male progenies (MP1o – dark green, and MP1y – light orange) from crosses as previously described after only one blood meal to each parental population of the second biological replicate. The wings measures were used as body size reference with the objective to observe size differences between male and female mosquitoes. The wing measures (average ± standard deviation) obtained are DP1o – 3.27 ± 0.240 mm (N=31), MP1o – 3.32 ± 0.183 (N=19), DP1y – 2.87 ± 0.214 (N=25) mm and MP1y – 2.91 ± 0.198 (N=25). B - Average of wings measures of daughter progenies (DP2y – hot pink) and male progenies (MP2y – dark blue) from DP2y (cross of young female with young sperm after a second blood meal to each parental population of the second biological replicate) as body size reference with the objective to observe size differences between male and female mosquitoes. The wing measures (average ± standard deviation) obtained are DP2y – 2.97 ± 0.132 mm (N=21), MP2y – 2.90 ± 0.190 (N=14).

In the second biological replicate, as mentioned previously, wing size was measured (as explained in the Materials and Methods section on page 28) in male and female *Anopheles* to use as a reference for mosquito body size.

Due to the difficulties encountered in the production of this biological replicate, it was only possible to obtain measurements of DP1o (daughter progenies of the old mother x old sperm cross), MP1o (male progenies of the old mother x old sperm cross), DP1y (daughter progenies of the young mother x young sperm) and MP1y (male progenies from the cross between young mother and young sperm). And the following graph of figure 13A was obtained. Analysing the graph, we can see that males have a bigger size compared to females from the same cross, which is odd because anatomically female *Anopheles* are bigger than male *Anopheles*. And that the progenies of the crossing of

young mother with young sperm are smaller than the progenies of the crossing of old mother with old sperm.

In order to corroborate the results obtained, it was decided to give a second blood meal to the parental population, but this continued to present difficulties in egg laying, so only data on the crossing of young mother x young sperm (DP2y) could be obtained. In this one, the size of the female wings are slightly larger than the male wings (0.07 mm of difference) (figure 13B).

No statistical tests were applied to the data presented and future data from additional biological replicates are required for a conclusion to be drawn.

However, it is strange that DP1o and DP1y are smaller than MP1o and MP1y, respectively, as *Anopheles* females are generally larger than males. We also must take into account that due to the problems encountered in the formation of this biological replica and the differences in larval density it may have created this distinction.

There is no literature regarding the number of blood meals influencing the size of offspring, and our data do not show significant differences in order to raise this possibility (2.87 vs 2.97 and 2.91 vs 2.90).

Fight of DP1y vs DP2y:

Female Story: Body Size vs Number of Blood Meals vs Survival:

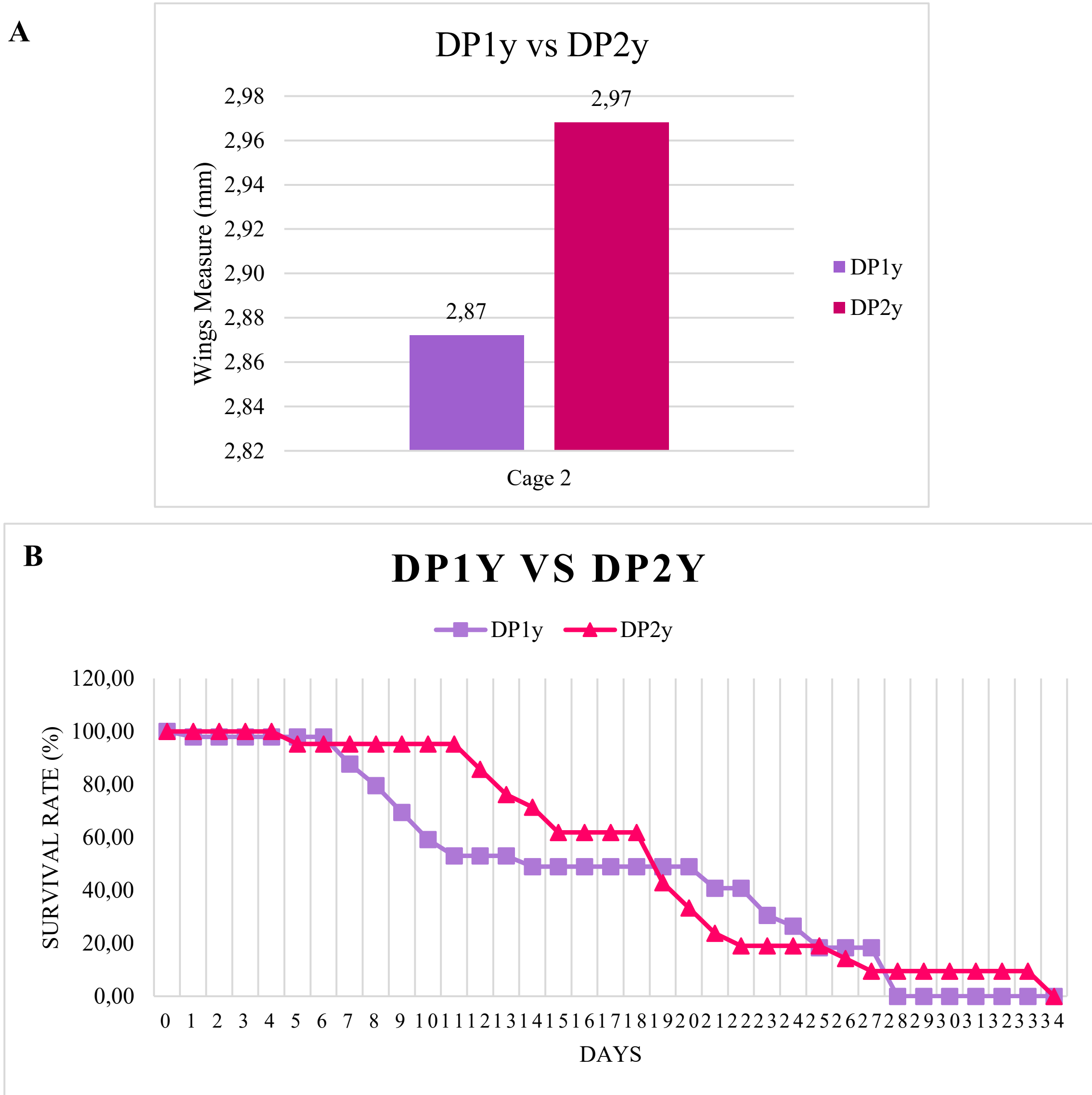

Figure 15 – Wings measure data and Survival curves of female progeny after one or two blood meals. A – Average of wings measures of daughter progenies (DPs) from crosses young female with young sperm where the only differences is the number of blood meals of the initial population who give rise both of them, DP1y (light purple) after only one blood meal and DP2y (hot pink) after two blood meals. The wing measures (average ± standard deviation) obtained are DP1y – 2.87 ± 0.214 (N=25) and DP2y - 2.97 ± 0.132 (N=21). B – Survival curves of DP1y and DP2y. On this assay each cross had a different number of DP due to complication on the creation of DPs. DP1y (light purple ■) had 31 virgin females and DP2y (hot pink ▲) had 21 virgin females. The duration of the second assay was 34 days.

Despite the technical difficulties, it was possible to obtain data from the crossing of young mother x young sperm only differing in the number of blood meals of the

parental population. The one that originated in the first blood meal, DP1y and the one that originated after a second blood meal, DP2y.

Differences in size were found in females, as presented in figure 14A, where female wings of DP2y are larger than female wings of DP1y. These differences may simply be due to differences in larval density; however, the difference is not significant, being only 0.10 mm.

In addition to the size of the wings, a survival test with DP2y females was also performed in order to compare with the survival of DP1y. The survival assay was performed under the same conditions as for DP1y, as described in the materials and methods on pages 27 and 28. In the survival assay, the curves shown in the graph of Figure 14B were obtained. We found that DP2y has better survivorship than DP1y, in line with the results explained on page 35, where we associate better survival with larger mosquitoes.

It is important to keep in mind that at the time of producing the DP2y cross, the mother and the sperm were older compared to the time of producing DP1y. Therefore, the data obtained and presented at figure 15B are in full agreement with the data presented previously at figure 10B and 10C, where older mother harbouring older sperm produce females with bigger size that do survive better.

It would be interesting to replicate this same test in order to strengthen the results obtained.

Annex V: qPCR data

Analysis tables of DMAD levels:

Table 11 – Data of qPCR of amplification of S7 gene from biological samples of the progenies. Data of amplification of S7 gene from male and female *Anopheles* as well from 24h development embryos from each cross. The data include cycle thresholds (CT) and its mean and standard deviation (St. Dev), the calibration curve obtained from the qPCR and the log of cDNA (from where we can calculate the concentration of S7 gene in the cDNA of each sample).

S7										
Cage	Sample	Real Name	CT	CT	CT	CT (mean)	ST. Dev	Calibration curve associated	log(cDNA)	cDNA
1	1B	MP1my	23,005	22,860	22,819	22,895	0,08003	y = -3.5184x + 16.97	-1,684	0,021
	1:10 1B	MP1my dil	26,141	26,046	26,090	26,093	0,03875	y = -3.5184x + 16.97	-2,593	0,003
	1C	FP1my	19,053	19,157	19,052	19,087	0,04943	y = -3.5184x + 16.97	-0,602	0,250
	1:10 1C	FP1my dil	20,656	20,983	21,255	20,965	0,24508	y = -3.5184x + 16.97	-1,135	0,073
	1A	DP1my eggs	21,148	21,136	21,186	21,157	0,02136	y= -3.2025x + 17.436	-1,162	0,069
	1:10 1A	DP1my eggs	24,308	24,449	24,387	24,381	0,05761	y= -3.2025x + 17.436	-2,169	0,007
2	2B	MP1o	21,004	20,977	20,917	20,966	0,0362	y = -3.5184x + 16.97	-1,136	0,073
	1:10 2B	MP1o dil	23,168	23,708	23,687	23,521	0,24982	y = -3.5184x + 16.97	-1,862	0,014
	2C	FP1o	18,438	18,498	18,457	18,464	0,02527	y = -3.5184x + 16.97	-0,425	0,376
	1:10 2C	FP1o dil	20,576	20,766	21,003	20,782	0,1747	y = -3.5184x + 16.97	-1,083	0,083
	2A	DP1o eggs	20,863	20,874	20,921	20,886	0,02512	y= -3.1782x + 17.624	-1,026	0,094
	1:10 2A	DP1o eggs	24,188	24,100	24,104	24,131	0,04027	y= -3.1782x + 17.624	-2,047	0,009
3	3B	MP1y	20,948	20,985	20,970	20,968	0,01521	y = -3.2179x + 17.019	-1,227	0,059
	1:10 3B	MP1y dil	24,141	24,209	24,089	24,146	0,04927	y = -3.2179x + 17.019	-2,215	0,006
	3C	FP1y	19,872	19,866	19,914	19,884	0,02131	y = -3.2179x + 17.019	-0,890	0,129
	1:10 3C	FP1y dil	22,927	23,071	23,131	23,043	0,08565	y = -3.2179x + 17.019	-1,872	0,013
	3A	DP1y eggs	19,656	19,639	19,743	19,679	0,04551	y= -3.2025x + 17.436	-0,700	0,199
	1:10 3A	DP1y eggs	22,933	23,035	22,888	22,952	0,0614	y= -3.2025x + 17.436	-1,722	0,019

Table 12 – Data of qPCR of amplification of DMAD gene from biological samples of the progenies. Data of amplification of DMAD gene from male and female *Anopheles* as well from 24h development embryos from each cross. The data include cycle thresholds (CT) and its mean and standard deviation (St. Dev), the calibration curve obtained from the qPCR and the log of cDNA (from where we can calculate the concentration of S7 gene in the cDNA of each sample). The normalization was calculated in relation to the expression of S7 gene. And the value of calibration was obtained in relation to the value obtained on the FP1y sample (adult female from young female x young sperm cross).

DMAD												
Cage	Sample	Real Name	CT	CT	CT	CT (mean)	ST. Dev	Calibration curve associated	log(cDNA)	cDNA	Nomalization	Calibrator
1 - my	1B	MP1my	28,217	28,276	28,142	28,212	0,05493	y = -3.2427x + 23.986	-1,303	0,050	2,403	1,338
	1:10 1B	MP1my dil	31,891	30,670	30,788	31,116	0,54994	y = -3.2427x + 23.986	-2,199	0,006	2,477	1,379
	1C	FP1my	25,646	25,888	25,832	25,789	0,10327	y = -3.2427x + 23.986	-0,556	0,278	1,111	0,619
	1:10 1C	FP1my dil	27,313	27,627	28,148	27,696	0,34426	y = -3.2427x + 23.986	-1,144	0,072	0,980	0,546
	1A	DP1my eggs	28,384	28,386	28,868	28,546	0,22746	y = - 3.3387 + 23.007	-1,659	0,022	0,318	0,177
	1:10 1A	DP1my eggs	24,308	24,449	24,387	24,381	0,05761	y = - 3.3387 + 23.007	-0,412	0,388	57,159	31,815
2 - O	2B	MP1o	26,453	26,421	26,392	26,422	0,02494	y = -3.2427x + 23.986	-0,751	0,177	2,424	1,349
	1:10 2B	MP1o dil	30,566	30,274	29,465	30,102	0,4656	y = -3.2427x + 23.986	-1,886	0,013	0,946	0,527
	2C	FP1o	24,970	24,914	24,803	24,896	0,06928	y = -3.2427x + 23.986	-0,280	0,524	1,394	0,776
	1:10 2C	FP1o dil	26,900	27,527	27,571	27,333	0,30669	y = -3.2427x + 23.986	-1,032	0,093	1,125	0,626
	2A	DP1o eggs	24,763	25,237	24,615	24,871	0,26542	y = - 3.4313x + 23.125	-0,509	0,310	3,292	1,832
	1:10 2A	DP1o eggs	27,958	27,669	27,697	27,775	0,13005	y = - 3.4313x + 23.125	-1,355	0,044	4,923	2,740
3 - Y	3B	MP1y	26,440	26,527	26,570	26,512	0,05435	y = - 3.0453x + 23.74	-0,910	0,123	2,074	1,154
	1:10 3B	MP1y dil	28,624	28,802	29,103	28,843	0,19743	y = - 3.0453x + 23.74	-1,676	0,021	3,461	1,927
	3C	FP1y	25,577	25,518	25,934	25,676	0,18367	y = - 3.0453x + 23.74	-0,636	0,231	1,797	1,000
	1:10 3C	FP1y dil	28,103	28,202	28,292	28,199	0,07718	y = - 3.0453x + 23.74	-1,464	0,034	2,558	1,424
	3A	DP1y eggs	23,936	23,853	24,241	24,010	0,16716	y = - 3.3387 + 23.007	-0,300	0,501	2,512	1,398
	1:10 3A	DP1y eggs	26,984	25,044	32,548	28,192	3,18038	y = - 3.3387 + 23.007	-1,553	0,028	1,477	0,822

Analysis tables of METTL3 levels:

Table 13 – Data of qPCR of amplification of S7 gene from biological samples of the progenies. Data of amplification of S7 gene from male and female *Anopheles* as well from 24h development embryos from each cross. The data include cycle thresholds (CT) and its mean and standard deviation (St. Dev), the calibration curve obtained from the qPCR and the log of cDNA (from where we can calculate the concentration of S7 gene in the cDNA of each sample).

S7										
Cage	Sample	Real Name	CT	CT	CT	CT (mean)	ST. Dev	Calibration curve associated	log(cDNA)	cDNA
1	1B	MP1my	22,707	22,721	22,836	22,755	0,0578979	y = -3.3245x + 17.02	-1,725	0,019
	1:10 1B	MP1my dil	25,851	26,087	26,219	26,053	0,1523071	y = -3.3245x + 17.02	-2,717	0,002
	1C	FP1my	18,815	18,877	18,911	18,868	0,0396361	y = -3.3245x + 17.02	-0,556	0,278
	1:10 1C	FP1my dil	20,552	21,232	22,641	21,475	0,8699252	y = -3.3245x + 17.02	-1,340	0,046
	1A	DP1my eggs	21,220	21,043	21,220	21,161	0,0834684	y = -3.2801x + 17.453	-1,130	0,074
	1:10 1A	DP1my eggs	24,329	24,573	24,453	24,452	0,0993977	y = -3.2801x + 17.453	-2,134	0,007
2	2B	MP1o	20,703	20,717	20,739	20,719	0,0148177	y = -3.3245x + 17.02	-1,113	0,077
	1:10 2B	MP1o dil	23,675	23,771	23,728	23,725	0,0391319	y = -3.3245x + 17.02	-2,017	0,010
	2C	FP1o	18,109	18,176	18,287	18,191	0,0730515	y = -3.3245x + 17.02	-0,352	0,445
	1:10 2C	FP1o dil	20,953	20,953	21,003	20,970	0,0233761	y = -3.3245x + 17.02	-1,188	0,065
	2A	DP1o eggs	-	20,911	20,785	20,848	0,062842	y = -3.2801x + 17.453	-1,035	0,092
	1:10 2A	DP1o eggs	23,973	24,083	23,989	24,015	0,0484638	y = -3.2801x + 17.453	-2,001	0,010
3	3B	MP1y	21,469	21,313	21,338	21,373	0,0682955	y = -3.3201x + 17.139	-1,275	0,053
	1:10 3B	MP1y dil	24,289	24,318	24,362	24,323	0,0302172	y = -3.3201x + 17.139	-2,164	0,007
	3C	FP1y	20,268	20,308	20,241	20,272	0,0277903	y = -3.3201x + 17.139	-0,944	0,114
	1:10 3C	FP1y dil	23,151	23,112	23,036	23,100	0,0476103	y = -3.3201x + 17.139	-1,795	0,016
	3A	DP1y eggs	19,805	19,791	19,821	19,805	0,0124524	y = -3.2801x + 17.453	-0,717	0,192
	1:10 3A	DP1y eggs	22,386	22,919	22,801	22,702	0,2283606	y = -3.2801x + 17.453	-1,600	0,025

Table 14 – Data of qPCR of amplification of METTL3 gene from biological samples of the progenies. Data of amplification of METTL3 gene from male and female *Anopheles* as well from 24h development embryos from each cross. The data include cycle thresholds (CT) and its mean and standard deviation (St. Dev), the calibration curve obtained from the qPCR and the log of cDNA (from where we can calculate the concentration of S7 gene in the cDNA of each sample). The normalization was calculated in relation to the expression of S7 gene. And the value of calibration was obtained in relation to the value obtained on the FP1y sample (adult female from young female x young sperm cross).

METTL3												
Cage	Sample	Real Name	CT	CT	CT	CT (mean)	ST. Dev	Calibration curve associated	log(cDNA)	cDNA	Nomalization	Calibrator
1 - my	1B	MP1my	31,149	31,303	31,205	31,219	0,0633533	y = -3.1053x + 25.322	-1,899	0,013	0,670	0,783
	1:10 1B	MP1my dil	36,517	-	35,770	36,144	0,37339	y = -3.1053x + 25.322	-3,485	0,000	0,171	0,199
	1C	FP1my	27,103	27,081	27,405	27,196	0,1475605	y = -3.1053x + 25.322	-0,604	0,249	0,896	1,047
	1:10 1C	FP1my dil	30,438	31,306	31,204	30,983	0,387188	y = -3.1053x + 25.322	-1,823	0,015	0,329	0,384
	1A	DP1my eggs	28,272	28,571	28,031	28,291	0,2206979	y = -3.4897x + 25.275	-0,864	0,137	1,845	2,158
	1:10 1A	DP1my eggs	31,524	32,154	31,942	31,873	0,2618047	y = -3.4897x + 25.275	-1,891	0,013	1,749	2,045
2 - O	2B	MP1o	29,019	28,748	30,796	29,521	0,9084076	y = -3.1053x + 25.322	-1,352	0,044	0,576	0,674
	1:10 2B	MP1o dil	32,486	33,644	32,917	33,016	0,4779788	y = -3.1053x + 25.322	-2,478	0,003	0,346	0,405
	2C	FP1o	26,807	26,737	26,649	26,731	0,0646183	y = -3.1053x + 25.322	-0,454	0,352	0,791	0,925
	1:10 2C	FP1o dil	30,240	30,121	30,031	30,131	0,0852242	y = -3.1053x + 25.322	-1,549	0,028	0,436	0,510
	2A	DP1o eggs	27,658	27,450	27,775	27,628	0,1342493	y = -3.4897x + 25.275	-0,674	0,212	2,295	2,683
	1:10 2A	DP1o eggs	23,973	24,083	23,989	24,015	0,0484638	y = -3.4897x + 25.275	0,361	2,296	229,939	268,833
3 - Y	3B	MP1y	29,806	29,746	29,623	29,725	0,0764494	y = -3.1843x + 25.573	-1,304	0,050	0,936	1,095
	1:10 3B	MP1y dil	32,997	33,409	33,934	33,447	0,3835951	y = -3.1843x + 25.573	-2,473	0,003	0,491	0,574
	3C	FP1y	28,760	28,657	28,965	28,794	0,1282525	y = -3.1843x + 25.573	-1,012	0,097	0,855	1,000
	1:10 3C	FP1y dil	32,342	31,672	32,274	32,096	0,3012256	y = -3.1843x + 25.573	-2,048	0,009	0,558	0,653
	3A	DP1y eggs	26,309	26,132	26,976	26,473	0,3633415	y = -3.4897x + 25.275	-0,343	0,454	2,366	2,766
	1:10 3A	DP1y eggs	30,051	30,911	31,481	30,814	0,5876749	y = -3.4897x + 25.275	-1,587	0,026	1,030	1,205