The genetics and ecology of male reproductive investments in the malaria mosquito

Anopheles gambiae s.s

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Abstract

Malaria continues to be a major global health problem due to high mortality and morbidity rate in endemic regions. *An. gambiae s.s* is the major vector in endemic African countries. About 198 million cases of malaria were recorded globally in 2013 and this have led to over 584 000 deaths. Different measures have been implemented in order to reduce and control the transmission rate. However, the drug resistant parasites and insecticide resistant mosquitoes have created problems towards achieving this goal. The use of the sterile insect technique and genetically modified mosquitoes as a control measure seems very promising but requires massive releases of males that are vigorous and competitive for the strategy to be realistic. Thus, there is need to understand the genetics and ecology of male mosquitoes with reference to their reproductive investments particularly for the laboratory reared *An. gambiae* due to inbreeding effects in colonized strains. Here we developed a qPCR technique based on TaqMan assay to quantify male sperm investment and used the assay to examine the effects of hydric stress on sperm investment by males and sperm maintenance in mated females. From two inbred strains of *An. gambiae s.s*, we generated heterotic supermales and tested for inbreeding and heterosis effects on sex peptide and sperm investments by males in large and individual male mating cages to determine male reproductive success. No evidence of heterosis was found in the large group mating cages except in sperm activity. However, in the individual male mating cages, the heterotic supermales achieved higher reproductive success than the inbred strains. They produced more eggs and fathered numerous larvae. Furthermore, inbreeding affects the size of the sex peptide deposited and survival in inbred males. Conclusively, heterosis could be the quickest method to produce vigorous and competitive laboratory reared males for vector control projects where male reproductive success is required.

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cheers we are now PhD holders. To my mother, brothers and sisters, thanks for all your encouragement.

Finally, I would like to acknowledge all those who through their words and actions encouraged me in this journey, especially Dr. and Mrs Victor Chilaka aka 'aunty and uncle Derby' for all the babysitting job. You guys made life easy for me. To my colleagues in Zoology department UNN particularly Dr. Felicia Ekeh and Prof. P. U. Ubachukwu I express my gratitude.

Dedication

In loving memory of my father Robinson Chinweuba Nwokolo

Chapter 1

1.1 Introduction

One of the serious health problems threatening Africa, and sub Saharan African regions is the malaria disease. It is caused by the apicomplexan *Plasmodium* parasite. About 198 million cases of malaria occurred globally in 2013 and the disease led to 584 000 deaths (WHO 2014) (Fig 1.1). Well over 40% of the world's population live in these malaria endemic regions (Aultman *et al*. 2002). Approximately, 90% of the people affected are the poor and less privileged (Saeed and Ahmed 2003). Major victims are pregnant women with high anaemia cases and young children between the ages of 0-5 years (Onifade *et al*. 2007).   In Africa, every 45 seconds a child dies of malaria, this accounts for 90% of child deaths (WHO 2014).
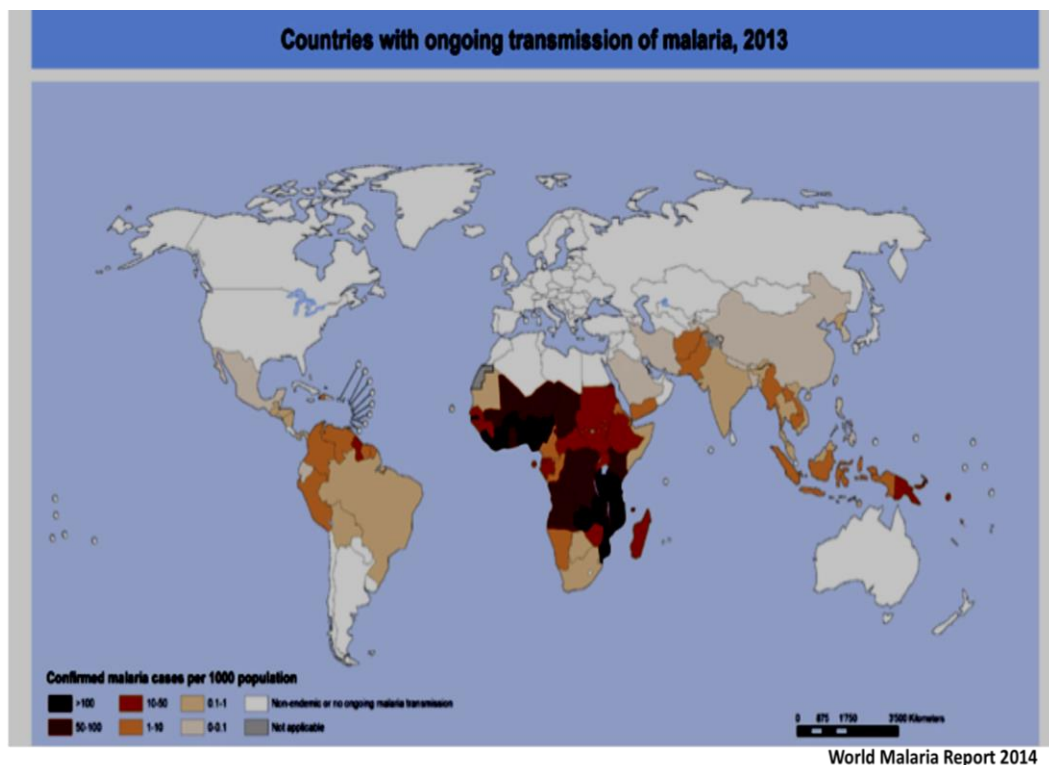
Fig 1.1: Countries with ongoing transmission of malaria (WHO 2014).

Malaria together with acquired immune deficiency syndrome (AIDS) is part of the principal causes of high mortality and continued impoverishment in places like Latin America, Southeast Asia, and Africa, where more than 1.5 million deaths are recorded each year (WHO/TDR 2008). *Anopheles* mosquitoes are the sole vectors of *Plasmodium* parasites that cause human malaria. In Africa, *Anopheles gambiae sensu stricto* is a sibling species within the *Anopheles gambiae* complex (Coluzzi *et al.* 1979) and is known as the primary vector of human malaria because of its abundance and vector competence (Budiansky 2002; Koutsos *et al.* 2007). Desperate efforts have been made towards malaria control by international bodies such as the United Nations/World Health Organization through the special programme for Research and Training in Tropical Diseases (TDR) and Roll Back Malaria (RBM) Programme (TDR 2005; RBM 2005). Environmental change (elimination of breeding and resting sites), Indoor Residual Spraying (IRS) of insecticides like pyrethroids and carbamates, and the utilization of insecticide treated nets (ITNs) are various methods currently in use to control malaria transmission. Insecticide treated nets (ITNs) could achieve up 90% reduction in transmission rate if used by entire populations in these areas (WHO 2011). The use of novel techniques like sterile insect technique (SIT) and the Genetically Modified Mosquito (GMM), as part of the Integrated Vector Management (IVM) tool are promising prospect, particularly now that strong population reduction is required. While the SIT programme targets population reduction by mating without production of viable offspring, GMM focuses on population replacement by the introduction of *Plasmodium* resistant genes into the malaria vector (Tabachnick 2003). Recently, a new technique to generate flightless females has been designed to prevent swarming thereby avoiding mating contact (Alphey 2013).

Ongoing research on the mating biology of the malaria mosquito tries to understand male mating behaviour. Of particular interest in male mating behaviour is the consequence

of variations in the male reproductive potential. The requirement to consistently produce and release millions of healthy, 'sexually vibrant' and ready to mate competitive male mosquitoes into the natural environment is of paramount interest in the success of the vector control programme. However, the inability for the laboratory- reared male *Anopheles gambiae* to compete favourably during mating with the wild strain has been a major limiting factor encountered in the SIT vector control programme (Benedict and Robinson 2003). The reduced competitiveness of the sterilized males is also an issue that needs to be addressed in the GMM programme (Manzava *et al.* 2006). It has now, become crucial that this knowledge gap is bridged for the progression of control measure targeting high population reduction.

1.1.1 Morphology and life cycle

In epidemiological studies, the life cycle and external morphology of the vector are of great importance. The information it gives provides relevant criteria for recognizing genus and species of the vector. The *Anopheles* mosquito passes through four stages in their life cycle (Fig1.2). The duration of these cycles is species and temperature dependent (Floore 2000). While in tropical condition, it takes between 10 to 14 days. In some species, it takes as little as 5 days. Adult female *Anopheles* can live up to one month in rearing conditions, but usually not more than 2-3 weeks in nature. Female *Anopheles* lay eggs in batches of 70-200 on the surface of water (Cross 2004; CDC 2010). *Anopheles gambiae* eggs are usually boat-shaped with unique lateral floats on either side not resistant to desiccation (Service 2004). They hatch within 2-3 days, although hatching may take up to 2-3 weeks in colder climate.
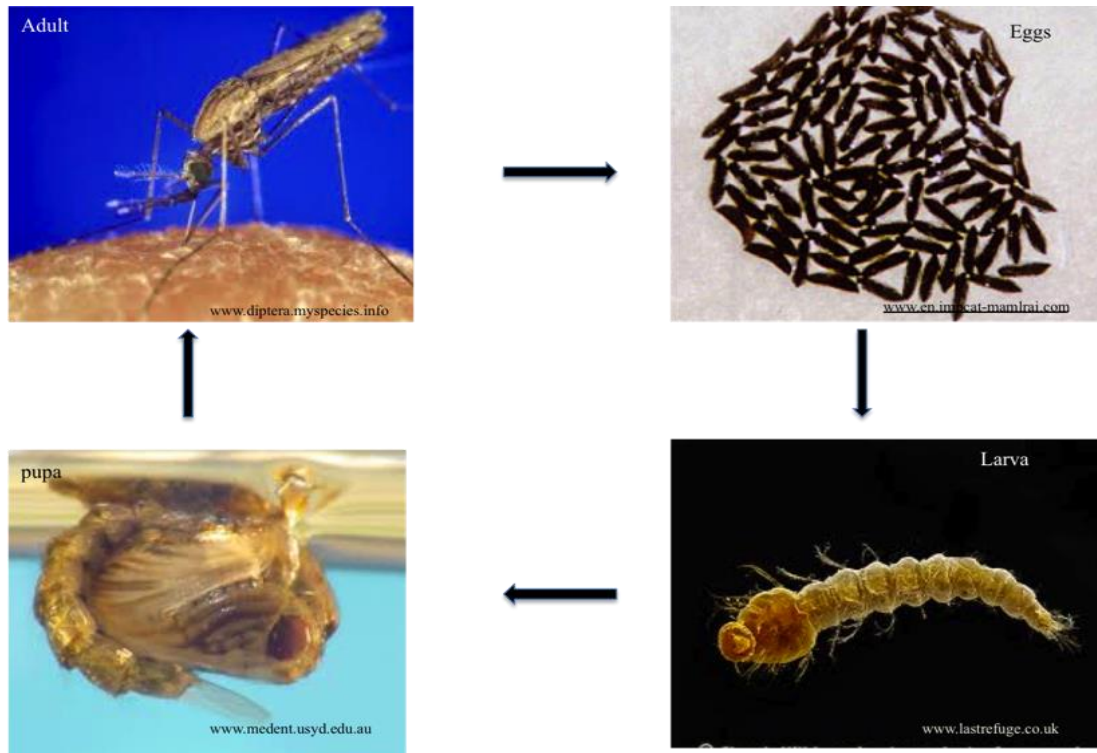
Fig1.2: The life cycle of *Anopheles gambiae* mosquito

The *An. gambiae* larva is an active feeder and feeds on algae, bacteria, and other microorganisms directly on the surface of the water by tilting the head 180° with the mouthpart facing directly on the water surface film (Robert and Collins 1996). The pupae are active and comma-shaped when viewed from the side. The head and thorax are joined into a cephalothorax with the abdomen curving around underneath. They breathe through a thoracic "horn" connected to the thoracic spiracles. The palps of adult *Anopheles gambiae* are as long as the proboscis, which distinguishes them from other mosquitoes (CDC 2010). The presences of discrete blocks of black and white scales on their wings together with the adult resting position are characteristic feature of the *Anopheles* (Metapathogen 2011). The male and female rest with their abdomen sticking up in the air, rather than parallel to the surface on which they are resting. The *An. gambiae* adults are morphologically indistinguishable from the other members of the *An. gambiae* complex (Fontenille and Lochouarn 1999).

1.1.2 Systematics

Current classification of the Anopheline has 459 formally named subfamily species and more than 50 unnamed members. The recognized named species are now classified within seven subgenera. They are *Anopheles* 182, Baimaia 1, Cellia 217, Kerteszia 12, Lophopodomyia 6, Nyssorhynchus 35 and Sethomyia 5 species (WRBU 2011). The three largest subgenera (*Anopheles, Cellia* and *Nyssorhynchus*) are further divided into sections, series, groups and complexes based on disease transmission, overt morphological similarities and female cibarial armature (Harbach 2011; Harbach 1994). The *Nyssorhynchus* subgenera are Neotropical in distribution and comprise of three sections based on the hind tarsus morphology (Harbach 1994). Albimanus has 2 series. Argyritarsis also has 2 series and Myzorhynchella with just one series and 4 species. Anopheles subgenera are cosmopolitan in distribution, divided into two sections, based on the shape of pupae trumpet. Those with a wide funnel-shaped trumpet having the longest axis transverse to the stem are known as Laticorn sections. The Angusticorn sections include those with a semi-tubular trumpet having the long axis vertical more or less in line with the stem. Within these two sections are 6 series. *Anopheles, Cycloleppteron* and *Lophoscelomyia* series are Angusticorn while *Arribalzagia, Christya* and *Myzorhynchus* are Laticorn section (Harbach 2004). Only the females of *Myzorhynchus* and *Anopheles* series contain vector species. *An. freeborni, An. sinensis, An. atroparvus,* and *An. pseudopunctipennis* are primary vectors in malaria transmission. Some other members of this subgenus are also vectors of microfilaria and are associated with the transmission of encephalitis viruses (WRBU 2011). Subgenera Cellia found in the Old World comprises of 239 species is segregated into six series based on the cibarial teeth (Harbach 1994). Celia series is Afro-tropical in distribution (8 species). Myzomyia series contains 69 species, and are found mainly in Afro-tropical regions, the Mediterranean and Oriental. Neocellia series

have 33 species and are mainly Oriental and Afro-tropical. Neomyzomyia series is Afro-tropical, Oriental, and Australasian in distribution with 99 species. The Paramyzomyia series are found mainly in the Mediterranean. The East Africa and North India species contains only 6 species. Finally, the Pyretophorus series (*christyi, daudi, indefinitus, limosus, litoralis, ludlowae, parangensis,* and *vagus*) are Oriental and Afro-tropical, with 24 species (Harbach 2004) of which 22 are recognized species representing 11 morphologically distinct taxa. Fourteen (14) species are part of sibling species complexes (Anthony *et al.* 1999). They include -Subpictus Complex; *Subpictus* A, B, C, and D. Sundaicus Complex; *Sundaicus* A, B and C (Sukowati and Baimai 1996), -Gambiae Complex (seven sibling species) *arabiensis, bwambae, gambiae, melas, merus, quadriannulatus* A and B (White 1985; Coluzzi *et al.* 2002). *An gambiae s.s* belongs to the Gambiae complex. Further classification of the *An. gambiae s.s* was done based on molecular analysis of their rDNA to show the two molecular forms M and S (Favia *et al.* 2001).

Recently, the *An. gambiae* molecular "M form" was upgraded to a specie status, and is named *Anopheles coluzzii* while the "S form" maintains the nominotypical name *Anopheles gambiae* Giles (Coetzee *et al.* 2013). However, in this study, the *An. gambiae s.s* still refers to the two molecular forms M and S.

1.1.3 Distribution

Geographically, the Anopheline are found worldwide except in Antarctica and depending on the species and the region, they transmit malaria (CDC 2010). *Anopheles gambiae* plays an important role in malaria transmission in Africa and sub Saharan Africa. Strategic planning of malaria control programme requires comprehensive information on the distribution of those *Anopheles* species and complexes that transmit malaria (Fig1.3).
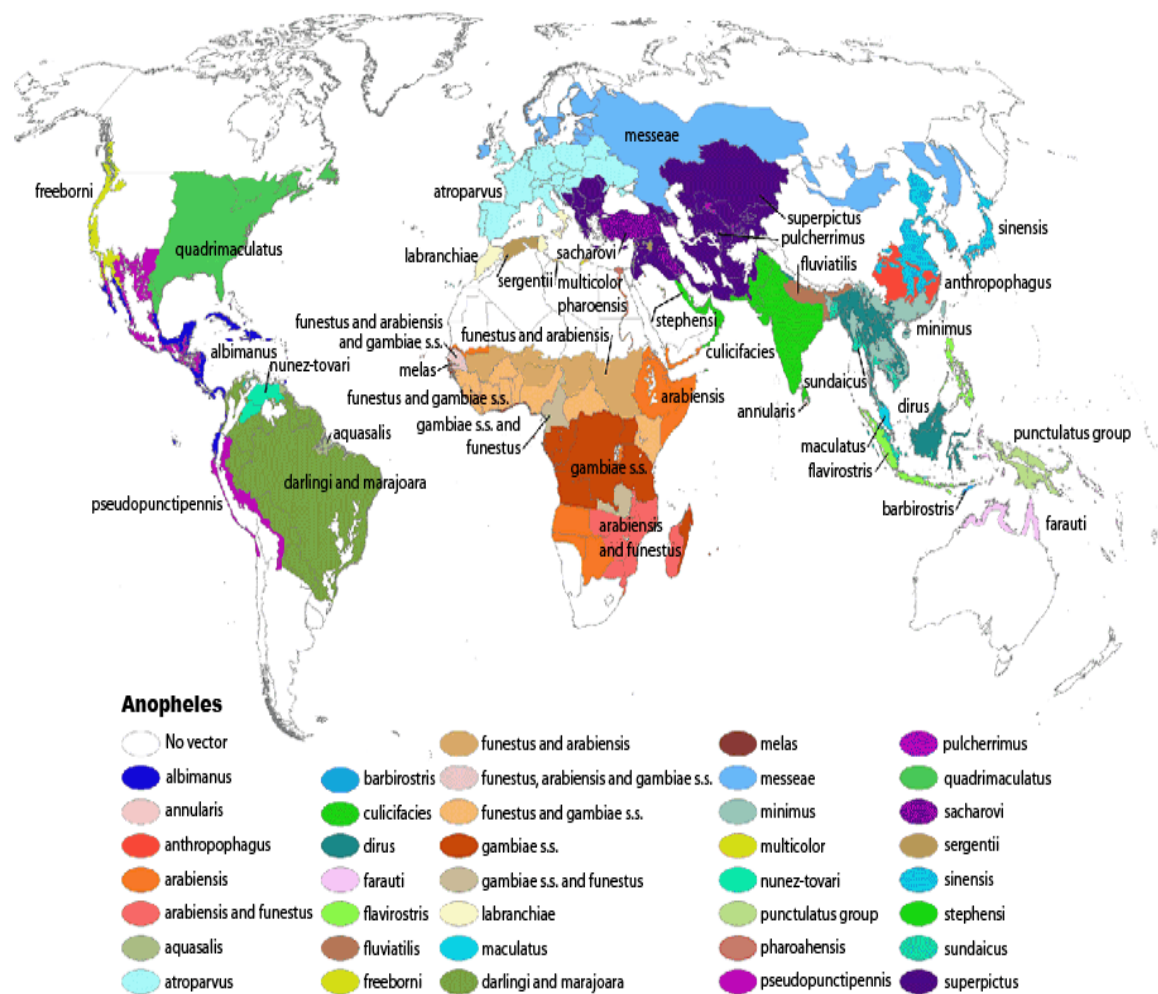
Fig1.3: Global distribution of dominant or potentially important malaria vectors(CDC 2010).

1.1.4 Population structure and chromosomal forms

The *An. gambiae* population structure is affected by fixed inversions and post mating reproductive barriers that may produce infertile or inviable crosses. In Africa, the two molecular forms of *An. gambiae* exist and thrive well in different environmental conditions (Lanzaro and Tripet 2003) (Fig.1.5). Forest M form is abundant in the humid tropical areas, whereas the Bissau S forms, enjoys the West African coast (della Torr *et al.* 2002). In Mali, Ghana, and Burkina-faso, the Mopti, (M form) and Savanna/Bamako (S form) forms exist as sympatric population (Favia *et al.* 1997; Favia and Louis, 1999), as well as in Cameroon (Turner and Hahn 2007). In Angola, the M forms of *An. gambiae s.s* is found in tropical dry sites, but the S forms appeared to be restricted to the humid area (Calzetta *et al.* 2008). The larvae of M forms are semi permanent in habitat, whereas the S form habitats are temporary (Caputo *et al.* 2008). In the *An. gambiae* population, geographic distance is one of the main factors affecting reproductive isolation. Population of the same species may exhibit distinct mating behaviour due to a different geographical location and divergence in the ecological niches (Boake 2000). Studies in West Africa have demonstrated that *An. gambiae* population reproduces in isolation, whereas in East Africa there is no reproductive isolation amongst the population (Kamau *et al.* 1998). Mating observations in three species of *An. stephensi* by Black and Lanzaro (2001) noted that the population was affected by geographic location however, in *An. arabiensis* geographic barriers did not interfere with mating because populations from La Reunion mate freely with those from continental Africa (Howell and Knols 2009). Across the Rift Valley in Africa, there is high differentiation amongst the *An. gambiae* population dwelling there but down the West and East Africa, population differentiation is low (Besansky *et al.* 1997; Lehmann *et al.* 1996, 2000). Good knowledge of the population structure of the malaria mosquito will help in the development of analytical tools in epidemiological studies on

vector control (Ng'habi *et al.* 2011). This understanding is very crucial for interpreting genetic variation, which has remained a limiting factor in several studies (Lehmann et al. 2003). The Afro-tropical, malaria vector *An. gambiae s.l* complex consists of 7 sibling species that are morphologically identical. They are *An. arabiensis, An. melas, An. merus, An. bwambae, An. gambiae s.s* (African malaria mosquito), *An. quadriannulatus* A, and B (Coluzzi *et al.* 2002; Touré *et al.* 1994) The *An. gambiae s.s* is extremely versatile tolerating a wide range of micro and macro-environmental conditions and seasonal changes in climate (Lanzaro and Tripet 2003). This versatility is ascribed to genetic polymorphism in the form of paracentric chromosomal inversions (*Touré et al.* 1998). The inversions occur on chromosome 2, with just one inversion on the left arm of the chromosome (2La), and the remaining ones (2Rb, c, d, j and u) on the right arm (Coluzzi *et al.* 2002; della Torre *et al.* 2005; Lanzaro and Tripet 2003) (Fig 1.4).
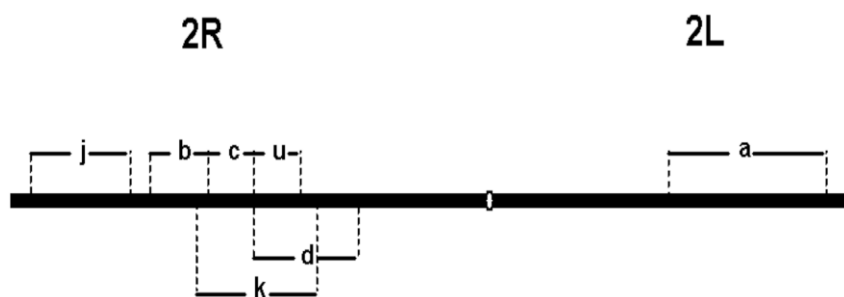
Fig1.4: Position of the inversions on the second chromosome of An. gambiae s.l. Five chromosomal forms have been described based on the arrangements of such inversions described from the banding patterns of polyethene chromosome (Lanzaro and Tripet 2003).

Five chromosomal forms have been described based on the arrangement of these paracentric inversions. They are Forest, Savannah, Mopti, Bamako, and Bissau (della Torre *et al.* 2001; della Torre 2002). These chromosomal forms are considered partially reproductively isolated and are in continuous speciation (Coluzzi *et al.* 2002; Touré *et al.* 1998). The Forest form is believed to be the ancestral stock. It displays a standard

arrangement on chromosome 2, with 2Ru, 2Rb, and 2Rd present at low frequencies (Table1.1). The Savannah form occurs in southern Mali and shows 2Rb and 2La. In some cases, it displays 2Rcu and 2Rbcu inversions. In Nigeria, the 2Rcu/bcd polymorphic inversion also occurs. Another southern Mali form is the Bamako, which displays 2Rj inversion. The Sahel savannah Mopti form displays 2Rbc polymorphic inversion and the West African coastal- dwelling Bissau form displays 2Rd inversion in high occurrence and are usually found in agricultural areas. In West Africa, certain inversion arrangements are abundant in dry periods, but deplete in the rainy periods (Coluzzi *et al.* 1985).

Table1.1:Typical and less frequent chromosomal arrangement on the 2R and 2L arms of second chromosomes in the five chromosomal forms described by Coluzzi and DI-Deco in 1985. (Lanzaro and Tripet 2003).

Chromosomal forms	Typical arrangement		Less frequent arrangement	
Forest	2R+	2L+	2Rb/d/+	2La
Bissau	2Rd	2L+	2R+	2La
Savanna	2Rb+	2La/+	2Rcu/bcu/bd/bcd/d/j/jb/jbd /jbcu/jcu/bk/+	
Bamako	2Rjcu/jbcu			
Mopti	2Rbc/u		2R+	2L

The spread of a gene of sub cryptic taxa into the gene pool of another by hybridization and back crossing is sometimes limiting (Lanzaro and Tripet 2003). Good evidence is the kdr allele that is resistant to pyrethroids observed in Mali. Burkina-faso and Ghana M-form populations have also been passed to their sympatry S-form population. Gene flow amongst forms is continuous and its cessation period is yet to be determined (Turner *et al.* 2005). Based on the fixed difference in the ribosomal DNA (rDNA) locus, two molecular forms were identified, "M and S" (Favia *et al.* 2001). Using restriction digests of a 1.3

kilobyte (kb), Polymer chain reaction (PCR) was conducted on the X-linked 28s rDNA. The downstream intergenic spacer (IGS) region produced a banding pattern that was in a position to differentiate between Mopti, Savannah, and Bamako chromosomal forms from Burkina Faso and Mali (Favia *et al.* 2001). The study on these sequence regions identified a single nucleotide polymorphism within the IGS region of the rDNA as the cause of the differing restriction digest-banding pattern. These molecular forms were designated M-forms which correspond to the Mopti chromosomal form in Mali and S forms corresponding to the Savannah and Bamako chromosomal form also in Mali. Based on X-linked marker as described by Weetman *et al.* (2011), a site within the internal transcribed sequence (ITS) of the same rDNA sequence containing three fixed nucleotide differences in complete linkage disequilibrium with the M and S form was further identified. They were Type I, and Type II, corresponding to S Form and M form linkage respectively (Gentile *et al.* 2002).

Fig 1. 5: Molecular and chromosomal forms of Anopheles gambiae in sub-Saharan African countries (della Torre *et al.* 2002).

1.1.5 Malaria disease and Transmission

In Africa, about two billion of its poor population are at risk of malaria disease (Snow *et al.* 2005). For years now, through vector control strategies, efforts have been made to eradicate malaria from these endemic regions (WHO 2008). Improved surveillance methods coupled with new drugs and vaccines have become an area of priority for researchers due to drug resistant parasites and pesticide resistant mosquitoes (Greenwood *et al.* 2008). Four *Plasmodium* parasites are known to cause malaria, *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum*. The *Plasmodium* knowlesi, which was originally described as a malaria parasite of the long tail-monkeys, now naturally infects humans in certain regions like Malaysia (Singh *et al.* 2004). This disease-causing parasite is transmitted to humans by the *Anopheles* mosquitoes. The females need a blood meal from a vertebrate host to obtain the nutrients to sustain

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Oogenesis and reproduce. The *An. gambiae* species are highly anthropophilic therefore, are very effective vectors of human malaria (Barillas-Mury and Kumar 2005). Of the four *Plasmodium* parasites, *Plasmodium falciparum* causes the most fatalities. It is also distinguished by its ability to bind to the brain endothelium during the blood stage of infection (Greenwood *et al.* 2008).

Malaria is the most prevalent parasitic and most dreaded insect borne disease in the African disease endemic regions (Steven 2007). Environmental status (temperature and relative humidity), availability of larval breeding sites (water), human host, and duration of suitable conditions are some of the factors that favour the abundance and survival of the mosquito (WHO 2010; Shiff 2006). The exposure of human host to the bite of a female *Anopheles* mosquito infected with *Plasmodium* results in infection with malaria (Yeshiwondim *et al.* 2009). In Africa, two members of the Gambiae complex (*An. gambiae s.s* and *An. arabiensis*) cause over 240 million transmission cases resulting in about 280,000 deaths annually (Toure *et al.* 1994; Ng'habi *et al.* 2010). Malaria transmission differs from country to country, with both stable and unstable transmission (Fig1.6). Stable malaria can be holoendemic when almost the entire population is affected, and transmission occurs all year round, or hyperendemic which is intense but with seasonal breaks. Unstable transmission on the other hand can be mesoendemic that is, periodic (leaving some areas without transmission), highly symptomatic and quick to diagnose. Other unstable transmission type is hypoendemic, which occurs after an unusual rainfall with severe clinical outbreaks in children and adults. Overall, high mortality is experienced in all populations (Shiff 2006). Stable malaria transmission (holoendemic) is transmitted by vectors with regular human biting behaviour. These vectors have a relatively long life span and live in environmental conditions that favour rapid completion of their extrinsic cycle (Molineaux and Gramiccia 1980; Enayati and Hemingway 2010). In few places with

unstable and mesoendemic transmission, malaria occurs as epidemic striking now and then. It is not age dependent, but in children, it is the commonest cause of hospital outpatient cases (Molineaux *et al.* 1980).
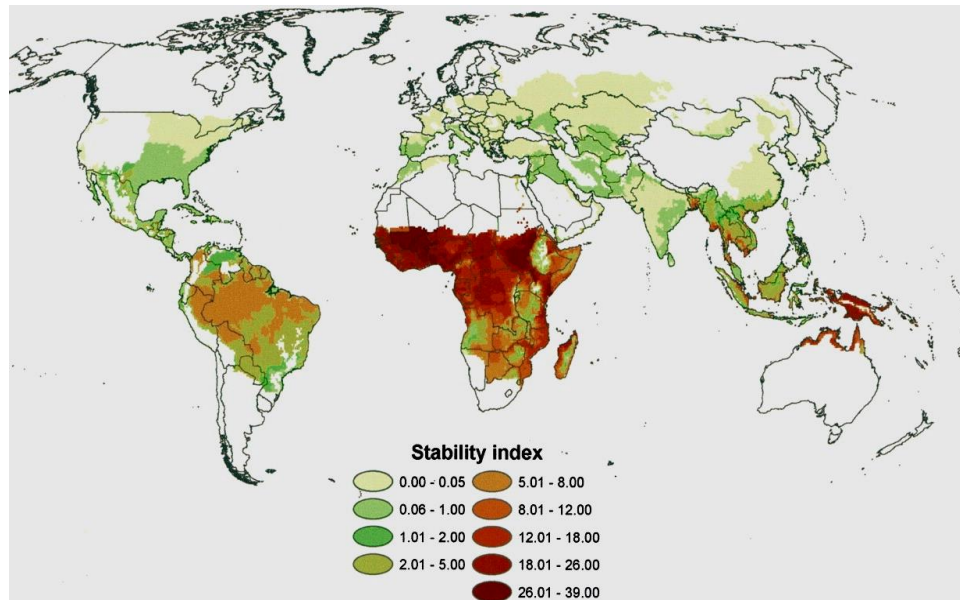
Fig1.6: The map represents malaria stability index, depictions of the intensity of malaria risk throughout the world (Robinson project) (Kiszewski *et al.* 2004).

Entomological inoculation rates (EIR) of malaria transmission in Africa are on the high side, ranging from 100 to 1000 infective bites per person per year (Diabaté *et al.* 2003). Malaria transmission carried out in Brazzaville (Congo) show that the EIR varies from more than 100 infective bites per person per year to less than 1 infective bite per person every three years. This represents almost the entire scale of malaria transmission seen in Africa (Trape and Zoulani 1987, Trape and Rogier 1996). In regions where mosquitoes are long-lived, transmission rate is also more intense (WHO 2011) and with intense transmission, up to 40% of the children may die of acute malaria (Malaria Statistics 1996).

1.1.6 Control Measures

The rationale behind vector control is population reduction of the primary vectors or complete eradication of such population. Diseases such as malaria and others like it are controlled chemically, physically, and biologically. In *An. gambiae s.s*, the physical modification (elimination of breeding sites) has not helped much as a control measure. Source Reduction (SR) conducted in Dar es Salam targeting the *An. gambiae* larvae achieved only 47 % reduction of malaria transmission and there is fear that in urban areas where breeding site is man-made larval control may even be smaller. Still in the same study, carried out in Tanzania and the Solomon Islands, two chemical control methods were used Indoors Residual Spraying (IRS) and Insecticide Treated Nets (ITNs) respectively. These did not achieve a 100 % reduction of malaria transmission (Ayesha *et al*. 2010) (Fig 1.7).
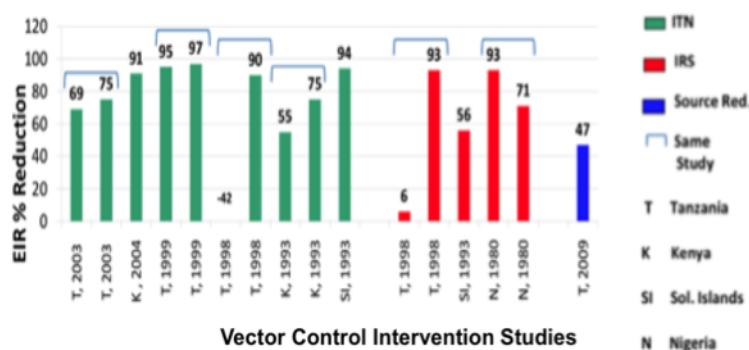
Fig 1.7: Vector control intervention (Ayesha *et al*.2010).

A comparative study on the use of IRS for malaria prevention, revealed that Entomological Inoculation Rate (EIR) was less than 1% when the IRS was used and its Protective Efficacy (PE) was 54% in Tanzania with younger children, 26% in Nigeria during wet the season, 88% in Pakistan and 31% in India (Pluess *et al.* 2010). ITNs are cost effective, and if used together with Long Lasting Insecticide-treated Nets (LLINs) and may achieve a

good transmission reduction (Enayati *et al.* 2009). To bring our dream of malaria eradication to reality, the Integrated Vector Management (IVM) programme seems to be the way out since the current measures in use are failing in various degrees, but with the SIT championing the course we may go far.

1.1.7 Insecticide resistance

Spraying of living house with insecticide like pyrethroids and carbamates, and screening sleeping beds with insecticide treated net with the hope of reducing malaria transmission is some of the preventive measures adopted. However, there is an increase in the resistance of some of these chemicals by the mosquitoes. The good effective and low costing insecticides, which are safe for humans, are very few (Trape 1987). Due to the increase in resistance problems, the use of chemical mixtures or rotations was made (Farenhorst *et al.* 2009). Nevertheless, with significant cross-resistance, these synthetic insecticides have become commercially less viable, which caused a move to biorational insecticides giving birth to the evolution of bioinsecticides/biopesticides. These biorational insecticides do not have residue problems, and in most cases are locally sourced and safer, though not as effective as chemical insecticides (Azucena *et al.* 2010). In Africa, the *An. gambiae, An. arabiensis* and *An. funestus* showing resistance to insecticides have become a major problem in the vector control programme (Farenhorst *et al.* 2009). Resistance to pyrethroid in *An. gambiae* is caused by high frequency of knock down resistant (kdr) gene and or enhanced metabolic mechanisms in *An. gambiae s.s* (Koudou *et al.* 2011; Greenwood and Mutabingwa 2002). The ITNs, which significantly reduced malaria-related morbidity and mortality, are increasingly applied in sub-Saharan Africa. Long-lasting insecticide nets (LLINs) were developed as a more sustainable solution to the problems faced with ITNs. They were coated with pyrethroid deltamethrin to a target dose of 55mg/m2 ($\pm$ 25%),

having a synergist Piperoxly Butoxide (PBO) incorporated with deltamethrin in the polyethylene roof. The target dose of deltamethrin in the sides of PermaNet 3.0 is 85 mg/m2 (± 25%) (Koudou *et al.* 2011). Despite all these desperate efforts made to control malaria transmission, a lot of work remains to be done.

1.1.8 Drug resistance

Drug resistances by parasites are one of the obstacles to overcome in the malaria control programme (WHO 2008). This drug resistance has caused some set back in the whole control programme and their negative affect is seriously being felt. In some part of the Southeast Asia like Thai-Cambodia, Drugs such as chloroquine, sulphadoxine-pyrimethamine and mefloquine, has been used for years in malaria control, but now the parasites have developed resistances to them, increasing the rate of infection in those areas (Greenwood and Mutabingwa 2002). Chloroquine has been the most successful drug ever used in the treatment of falciparum malaria due to low cost, low toxicity and high efficacy against susceptible parasites (Achudume *et al.* 2009). However, its resistance together with sulphadoxine-pyrimethamine is increasingly spreading widely even in Africa. Recently, the anti malaria drug Artemisinine is being resisted by the *Plasmodium* parasites. It is believed that if the Artemisinine resistance continues to spread like that of the old faithful chloroquine, the consequences will be great on the public health. Often, resistance is thought to increase with the use of single-drug therapies (monotherapies) than it will be with the combine therapies. With monotherapy, it is easier for the parasite to adapt and eventually overcome the obstacles presented by a single drug than a combination of drugs delivered together. Monotherapies are therefore the primary force behind the spread of Artemisinine resistance of *Plasmodium* parasite (WHO 2010).

1.2 Mating

1.2.1 Mating behaviour

The continual existence of any group of organism reproduction is a critical issue. In most organisms where sexual reproduction is required, a suitable mate must be found. In *An. gambiae,* several studies have been done on the different areas of reproductive behaviour such as mating, genetics, environmental effects, fecundity and fertility in relation to infection (Takken *et al.* 1998; Hogg and Hurd 1995; Hogg *et al.* 1996; Tripet *et al.* 2001; Diabaté *et al.* 2003; Ng ́habi *et al*. 2007; Gary and Foster 2006). Mating in flight is a unique feature of the dipterans (Clement 1999), and observation shows that *An. gambiae* mating takes place in flight during swarming (Diabaté *et al.* 2009; Howell and Knols 2009; Yuval 2006 and Charlwood *et al.* 2002b).

1.2.2 Swarming

Dipterans aggregate and move about actively in large numbers (Sullivan 1981) whenever they want to reproduce. This aggregate motion is a characteristic type of flight known as swarming, usually performed by organisms of similar size. At dusk male mosquitoes are seen forming swarms, which last for about 20-30 minutes (Manoukis *et al*. 2006) It starts off with lone males arriving at the swarm site (Clement 1999) and once swarming sets off, females enter the swarm and couples leaves the swarm pairs to complete mating. Different species swarm at different locations as well as display different swarming behaviours. While warm, windless weather favours and synchronizes swarming in male *Aedes* spp. (Nikolaeva 1976), different species of Anopheline form their swarm at different heights using markers as observed by Howell and Knols (2009). These swarm heights may also be related to position with swarm markers, While *An. gambiae* S-form males swarm near females' feeding sites under a clear view of the sky, *An. funestus* on the other hand makes

use of the horizon to swarm. The *An. merus* uses a ground marker, and *An. freeborni* uses both horizon and ground markers to swarm (Howell and Knols 2009). Mated males returned to the swarm after mating, and bigger males show greater mating success than did smaller ones (Yuval *et al.* 1992). In about 2 minutes before sunset in the evenings, *An. gambiae* males are seen swarming in zigzag flight 2-3m or 2-5m over horizontal contrast (Fig.1.8) such as footpaths, grassy areas or bushes. Swarms remain stationary, flying within a 1.5 m radius of an imaginary center throughout their duration (Charlwood *et al.* 2002; Diabaté e*t al*. 2006; Charlwood and Jones 1980). The virgin females approach the swarm later to mate with their conspecific males.

For *An. gambiae* female, mating usually occur once as recorded by (Tripet *et al.* 2003) and about 150 eggs are laid in batches within 3 days after a blood meal (Yaro *et al.* 2006; Gillies and Wilkes 1965). Thailayil *et al.* (2011) have shown in their laboratory experiment that the females of *An. gambiae* are not able to differentiate between spermed males and spermless males and mate as usual. They observed that *An. gambiae* females when mated with the spermless males still show the same-mated responses, such as laying large numbers of eggs and are refractory to a second insemination. This agrees with studies where injections of accessory gland proteins (ACPs) are able to induce post-mating behaviour in female *An. gambiae* (Shutt *et al.* 2010).

Fig 1.8: Pictures of representative swarm marker in *Anopheles gambiae s.s.* The arrow indicates the exact placement of the swarm in each site (Diabaté *et al.* 2009).

1.2.3 Assortative mating

Marchand (1984) have witnessed mixed swarms of sympatric species in the *An. gambiae*. Monospecific swarming in open spaces around houses at sunset has also been observed in *An. funestus* in southern Mozambique (Charlwood *et al.* 2003). In *An. gambiae s.s,* the two molecular forms swarms together, but are reproductively isolated. Studies have shown a high degree of assortative mating between these two closely related populations. The frequency of mixed swarm is low and may have been responsible for the low frequency of cross mating and hybrid within the forms (Tripet *et al.* 2001). There is complete or partial segregation in mixed swarm of *An. gambiae* (Diabaté *et al.* 2006, 2009). Mate recognition in swarms results to diverse swarming habit in the two forms, though they exist in sympatry (Diabaté et al. 2003). While the two molecular forms may co-exist in the same environment and swarm together for mating, they do not however cross mate with each other (Tripet *et al.* 2005).

In the field in Mali, Tripet *et al.* (2001) using the polymer chain reaction (PCR) analyzed sperm collected from the spermathecae of the wild females. They recorded strong assortative mating between the two forms. They also pointed out that if for any reason there is a cross mating between the two cryptic forms, the female would mate a second time. The second mating would be with its conspecific male (Tripet *et al.* 2003). The extent to which this assortative mating is occurring in the field and in between chromosomal forms remains an issue for discussion (Yawson *et al.* 2004; Lee *et al.* 2009).

In the laboratory, M and S forms easily mate to produce viable and fertile offspring (Diabaté *et al*. 2009).

1.2.3 Mating success

Anopheles male mating success depends on different parameters only determined in a few species, e.g. where size is a determining factor for mating success in the temperate species, in the tropical species it is still in-progress (Howell and Knols 2009). For instance, the larger males of *An. freeborni* are said to mate more than the smaller ones (Yuval *et al*. 1992). Charlwood *et al*. (2002, 2003) observed the reverse in *An. funestus*, and *An. gambiae* where size did not affect their mating success. The mating success of male *An. stephensi* decreases with age, as the number of spermatocysts decreases and the proportion of the testes occupied by the sperm reservoir increases with age. At the same time, the quantity of spermatozoa present in the sperm reservoir and post-gonad system together with the rate at which the male accessory gland secretions are replenished decreases with successive mating (Mahmood and Reisen 1994). In *An. gambiae,* during swarming when a female approaches a swarm, a group of males would dart in her direction (Charlwood *et al*. 2002; Marchand 1984). This is thought to be because of the female wing tone (Howell and Knols 2009). A similar effect (male wing tone) is also experienced by the female at the same time. Mate searching starts and suitable mates begin to pair up within the next

6.1minutes. Successful couples exit the swarm in an unstable slow movement slightly above ground level to mate. In the laboratory, apart from the colonization effect like fixation of alleles, normal mating behaviour may have been altered because of limited space in the cages. Therefore, modified mating behaviour is exhibited which may not occur in the wild type (Knop *et al.* 1987).

1.2.4 Insemination

Insemination is the actual sperm transfer that takes place when the orifice of the female spermathecal duct is pushed by the male gonapore and sperm together with accessory gland secretions are deposited into the female by insertion of the male genitalia into the female reproductive genitalia (Spielman *et al.* 1974). In *Anopheles*, the male approaches from the female and grasps her with his tarsal claws. He then swings his abdomen to hold her genitalia. An interlocking position is achieved, as soon as this is done and they fly off together to complete the insemination only locked by abdomen (Howell and Knols 2009). After each sexual activity, the male seminal vesicles are refilled with sperm and accessory gland secretions (Mahmood and Reisen 1982). In Ae*des taeniorhynchus,* most insemination takes place soon after sunset (Haeger *et al*. 1972). Most females will need to be 30-40 hours old at sunset before they will be inseminated. Nasci *et al.* (1989) documented that if *Ae. aegypti* females are mated by *Ae. albopictus* males, they will have small dead sperm cells in their spermathecae, but when inseminated by their own males, sperm cells are alive and present in larger quantities. Larger males of *An. gambiae* transfer more sperm to the females than smaller males, and in a small highly populated laboratory cage insemination rate in females are very high as opposed to the larger field population (Ponlawat and Harrington 2009). Upon insemination in the *An. gambiae* females are refractory to a second mating (Tripet *et al*. 2003).

1.2.5 Accessory gland secretions

They occur as proteins, which are simple peptides, pro-hormone-like peptides, and large glyco-proteins in D. *melanogaster* (Wolfner 1997). However, in *Culicine*, it is a mixture of fluids and granules (Clement 1999). Some of which are varieties of bioactive molecules that can change the female reproductive response. They are transferred during mating alongside with the sperm cells (Gillott 2003; Wolfner 1997). A large number of male accessory gland (MAG) proteins have been identified in numerous other insects as *Aedes* a*egypti*, honeybees, butterflies, and crickets (Sirot *et al*. 2008; Baer *et al*. 2003). These secretions help in sperm storage in the spermatheca, stimulate an increase in egg number and development, and modulate oviposition (Wolfner 1997). In *An. gambiae* they produce an unreceptive attitude to second mating or unwillingness to re- mate for some period (Tripet *et al.* 2003; Thailayil *et al*. 2011). Studies by Klowden (2001) have demonstrated that male accessory gland substances modulate female receptivity in most mosquito species, but they are not functional in the development of sexual refractoriness in *An. gambiae s.s* as the implantation of male accessory glands and the injection of gland homogenates did not affect the insemination rate of unmated females. However, Shutt *et al*. (2010) and Thailayil *et al.* (2011) have shown that indeed injection of male accessory gland protein influences the insemination rate in female *An. gambiae s.s.*

1.2.6 Mating plug

The male *Anopheles gambiae* mosquitoes are 'generous' and 'selfish', after mating, they leave the female with a parting gift called the mating plug (Clement 1999). This is a gelatinous coagulated mass of proteins interacting with enzymes and seminal fluids (Catteruccia 2009). This gelatinous substance is found in the oviduct of freshly mated female Anopheline as observed by Giglioli (1964) and it helps to secure sperm within the

female and ensure successful fertilization. Limited information is available on most of the specific roles played by these proteins. It is believed that it plays an important role in the reproductive success of female *An. gambiae* specie for the reason that they only mate once in their lifetime (Tripet *et al.* 2003). *Plugin* and *Transglutaminase* enzyme are the most abundant component of the mating plug. They are the major substrate for the male-specific proteins responsible for the coagulation of the liquid male accessory gland (MAGs) secretions (Roger *et al.* 2009). In *An. gambiae s.s* strains, a third protein have been named at the nucleotide level AgAcp34A-3. This protein is believed to have an important role in female fertility and post-mating responses (Mancini *et al.* 2011). Mating plug does not only deter second insemination as believed, but helps the female to store sperm correctly inside them (Roger *et al.* 2009). Removal or interfering with mating plugs in female *An. gambiae* may interrupt or cause total failure of reproduction (Catteruccia 2009).

1.3 Laboratory rearing

1.3.1 Environmental conditions

In nature, temperature, humidity, wind, and rainfall are some of the environmental factors that affect different biological process. For mosquitoes, processes like blood feeding, growth are greatly tied to temperature (Scott *et al.* 2000). An increase in rainfall results in an increase in relative humidity, which affects flight and host seeking behaviour in *Cu. nigripalpus* (Day and Curtis 1989). In *An. gambiae,* distribution and abundance are affected by rainfall, but may not have an effect on swarming behaviour (Ng'habi *et al.* 2005). Mosquito rearing in the laboratory involves the simulation of their natural environment. Mosquitoes are provided with the optimum ecological condition required for good and healthy living to ensure their availability for research. In as much as efforts are

made in order to mimic nature, rearing of some sibling species of *An. gambiae* complex under insectary conditions is still difficult as is the case for *An. quadriannulatus* (Mpofu *et al.*1993). In the laboratory, Shioa *et al.* (2008) reared adults of *An. gambiae s.s* under 28°C, 70-80% relative humidity and 16:8 photoperiod (L.D). However, Aboagye-Antwi and Tripet (2010) documented an average temperature of 26°C and 65-70% Relative Humidity (RH) for rearing of *An. gambiae s.s* in the Keele University insectaries. It has been observed that atmospheric moisture content (relative humidity) has an impact on the female reproduction in terms of egg laying in *An. gambiae s.s.* In the laboratory, the presence of wet oviposition pots initiates egg laying (Minakawa *et al.* 2002).

1.3.2 Nutrition

1.3.2 1 Larvae

In the laboratory, feeding regimes are more or less routinely carried out with definite time and proportion. Laboratory larval food for most *An. gambiae* is made up of fish food of various kinds (pellets and flakes) in different forms. The quality of the diet fed to the larva is important, as well as the quantity. The amount of food available at the larval stage significantly affects physiological processes in the *An. gambiae s.s* (Aboagye-Antwi and Tripet 2010). The quality of food fed to the larvae of *An. gambiae s.s,* affects the number of ovarian follicles (Reisen *et al.* 1982; Arrivillaga and Barrera 2004). Larval nutrition has a strong effect on reproductive traits like maturity and egg production. *An. gambiae* females, larvae reared at low food quality produced fewer eggs as adults than those reared on the high and improved food quality (Dimitriew and Rowe 2011).

1.3.2.2 Adults

Adult mosquito food in most insectaries ranges from different concentrations of sucrose, glucose, and fructose solutions. Sometimes, wet fruits like raisins are provided and in some cases, anti-oxidants are utilized in the diets to increase longevity and fecundity of the female. For example, methylparaben, found in the human diet was used to increase adult longevity of Anopheline (Howell and Knols 2009). Under laboratory conditions, *An. gambiae* female is provided with blood meal weekly or, as the case maybe to help in the ovarian development. The ovarian development takes place soon after feeding on a single blood meal. The activation of ovarian development and Oocytes in the *An. pharoensis* Theobald female was because of a blood meal, which provided nutrients to initiate the gonadotrophic cycle (El-Akad and Humphrey 1990b). These females often need several blood meals for a single gonadotrophic cycle. However, Lounibos *et al*. (1998), stipulates that the frequency of blood meals within a single ovarian cycle seems to be more important in Afro-tropical malaria vector *An. gambiae s.s* compared to Neotropical *Nyssorhynchus* species. It was shown that the first blood meal taken by the *An. gambiae s.s* female is utilized to increase metabolic reserve, and subsequent ones are then used for egg production (Charlwood *et al*. 2003). Adult *An. gambiae s.s* females fed on high quality food began producing eggs sooner than those fed on low food quality. They also produced a greater total number of eggs (Dimitriew and Rowe 2011).

1.3.3 Inbreeding

The genetic make up any organism plays a major role in both external and internal environmental processes towards the success of that group of organisms. Mating between closely related individual results in a situation known as inbreeding (Waser 1993). In population biology, it is necessary to gain a better understanding of the effects of

inbreeding for a meaningful study. In breeding problems may come as the accumulation of recessive deleterious traits (Vergeer *et al*. 2012) because of the similarity between the mate genome, however this only happens if these recessive genes are present in the two parents. The more closely related the mating pairs are the more homozygous deleterious genes they pass down to their offspring. If the accumulation of genes is dominated by heterozygous allele or homozygous allele the result is the same, reduced fitness and this is reflected in the entire population (Frankham *et al*. 2002). Munstermann (1994) observed reduced heterozygosity in colonized *Aedes sp*, when there was a close mating between siblings (brother-sister). Armbruster *et al*. (2000) disproved the speculation by other authors that inbreeding is at the extreme in the laboratory than in the field. Their observation in *Aedes geniculatus*, showed no significant interaction between inbreeding depression and environmental conditions. Studies on vector capacity in *Aedes albopictus* revealed that inbreeding only reduces larval survival and longevity in adult female but had no effect on the male longevity (O'Donnelly and Armbruster 2010). Inbreeding affects the genetic makeup of a colony. This is usually seen as a probability, as the level of homogeneousity of colonies continues to increase, they become strains whose genetic components are entirely different from the field populations, and the level of competitiveness is presumed to drop (Howell and Knols 2009). Two inbred strains of *Aedes triseriatus* went through 12 generations of intense inbreeding and after analysis for isozyme polymorphism, there was still a very high level of heterozygosity despite the heavy inbreed mating. The cause for this is because of certain factors like recessive lethal and natural recombination suppressors, which their processes are still a mystery to a certain extent (Benedict *et al*. 2009). Therefore, heterozygosity achieved at a higher level or retained in the same intensity as in the parent strains is because of selection. In *An. gambiae,* accumulations of

deleterious alleles because of colonization have a huge effect on the male reproductive organs (Baeshen *et al*. 2014).

1.4 Vector control projects

1.4.1 Sterile Insect Technique   (SIT)

Mating behaviour in male mosquitoes has become of great concern for the purpose of vector control in the Sterile Insect Technique (SIT) programme. In the SIT, the sterilization involves exposing male mosquitoes to ionizing radiation, mostly χ- or γ- that makes them sterile. Different strains of sterile male mosquitoes have been released for mating with wild females via the SIT control projects. The aim is population suppression or eradication. These releases have recorded are both successes and failures to a certain extent (Benedict and Robinson 2003). For the *Anopheles,* the first success was with the small-scale release of *An. albimanus* population using chemo-sterilized males aiming for population reduction. On a larger scale the project failed and this was believed to be because of migration of individuals from other areas to the barrier zones (Benedict and Robinson 2003). Three thousand, one hundred males of *An. culicifacies* were released in 1976 to mate with both wild and lab-reared females, competition for mating with the wild male population was high. In 1980, the number of males released was scaled up (7,500), the sterile males were found to be less competitive even though swarming, and mating was observed (Reisen *et al.* 1981a). In Burkina-faso between 1968 and 1969, the initial release of sterile male *An. gambiae* was made seeking population reduction, mating competitiveness was weak, though dispersal was high (Davidson *et al.* 1970). The ability of lab-reared males of *An. gambiae* to successfully mate and transfer sterile sperm to the wild female population has

been a limiting factor (Tabachnick 2003). *An. gambiae* male mating success remains a very critical issue for the SIT vector control programme (Takken *et al*. 2006).

1.4.2 Genetically Modified Mosquitoes (GMM)

With the increase in insecticide resistance by the sub-Saharan malaria vectors *An. gambiae s.s, An. arabiensis and An. funestus*, there has been a fresh awareness into research for the development of other methods of vector control based on the genetic manipulation of the vector (transgenesis) (Alphey 2002). The general idea is population replacement by consistent production, and release of genetically modified competitive male mosquitoes into the environment. The process of creating transgenic mosquitoes with practical application is now very possible due to existing expertise and technology. Two basic principles are in place, the first principle relies on the creation of transgenic *Anopheles* expressing some anti-malarial genes, which attack the parasite as it develops within the mosquito. The mosquito carrying this refractory would then be released into the environment, and mates with wild population females, hence spreading the refractory gene and disease transmission is disrupted (Alphey 2002; Alphey2014). The second is the Release of Insects with a Dominant Lethal Mutation (RIDL), which is effectively similar to SIT. It involves the release of male mosquitoes, which instead of being sterilized by radiation they carry a transgene, which trigger off mortality in their offspring (Alphey 2002; Burt 2014). The SIT and GMM projects sound great, but finding a suitable candidate has been very exhausting owing to the scanty information on the reproductive investments of *An. gambiae* males. The quantity and quality of sperm transferred in both field and laboratory is important as they may have reproductive fitness consequences relating to male mating competitiveness. Insufficient Acps and sperm transfer might also affect male reproductive success and increase the likelihood of the female re-mating. In order to

accomplish high success rate in the control programmes, the reproductive investments of suitable male candidates should be better or comparable the wild males. Generating and rearing males with such quality in the laboratory is one major goal of this research.

1.5 Aims and Objectives

1.5.1 Aims

The aim of this research project is to examine the reproductive investments of laboratory reared males of *An. gambiae*. The assessment of male reproductive investments such as sperm numbers transfer to females, the activity of the sperm transferred and the size and quantity of sex peptides transferred might assist in solving some of the problems associated with low mating competitiveness in lab reared *An. gambiae* males. This might in turn help to design a better vector control programmes.

1.5.2 Objectives

1. Development of a technique based on Real-Time quantitative polymer chain reaction (qPCR) to determine the amount of sperm transfer to females from field populations of *An. gambiae s.s.*

2. Quantification of sperm transfer in laboratory-reared male *An. gambiae s.s* male and female exposed to pre-mating hydric stress.

3. Assessment of sperm maintenance and sperm activity in laboratory reared female *An. gambiae s.s* exposed to post mating hydric stress.

4. To determine the effects of inbreeding and heterosis on sperm transfer and sex peptide in relation to male reproductive success.

5. To determine the inbreeding effects on survival of heterotic supermales and inbred males *An. gambiae s.s.*

1.6 Conclusion

Malaria disease is a pronounced global health burden, predominantly ravaging the poor and less privileged populations living in tropical and sub-tropical regions of Africa and Southeast Asia. The major vector is the *Anopheles gambiae* complex, and *Anopheles gambiae s.s* is the most significant human malaria vector. Prevention, treatment, and control are almost as old as the mosquito itself. Lack of effective control measures has not only resulted in a health problem, but also socioeconomic growths have been retarded in these endemic regions. Geographical and reproductive barriers have led to differentiation of several chromosomal and molecular forms. The two molecular forms (M and S) An. gambiae exist in sympatry, but there is reproductive isolation between them in many areas. Environmental factors as well as genetic factor affect mating success in *An. gambiae.*

Male mating success in *An. gambiae* will be relied upon heavily for the implementations of vector control projects like SIT and GMM programmes targeting population reduction for wide area coverage. Productivity in relation to mosquito quality is easy to measure in both field and laboratory conditions by counting the number of males produced per female, but a much more difficult task is to know how fit these males are. Now, only proxies of certain traits such as adult size, weight, and biochemical compositions are studied (Alphey 2010). Therefore, it is essential that detailed studies be carried out on male reproductive investment such as sperm numbers, sperm activity, and sex peptide transfer to determine male reproductive fitness cost. The development of sperm quantification technique will be an essential ecological tool in understanding the male mating investments of *An gambiae* in the laboratory reared strains as well as in the field population. The understanding of such a natural process will be critical to discover more effective measures to combat the problem of reduced mating competitiveness in laboratory reared males.

Chapter 2

General materials and methods

2.1 Mosquito strain

In this study, mosquito strains used were the Kil, an old inbred strain from the Marangu area in Tanzania that has been reared at the LSHTM research center since 1975. They were brought to the Patrick Manson Keele insectary in 1990 and many generations of it had been maintained. The Mopti strain (MRA-763) came as viable eggs maintained from the same strain at the Malaria Research in Mali and Reference Reagent Resource Centre (MR4). Dr. F. Tripèt and Prof. Greg Lanzaro from University of California, Davis collected the strains from the N'Gabacoro area of Mali in West Africa in 2003. The Tripèt research group maintains generations of these strains amongst others in the Patrick Manson insectary in School of Life Sciences at Keele University for research purpose. The Mopti males and Kil females were crossed to generate a hybrid and the F1 male progeny from the crossing was referred to as supermale. They were never maintained as a colony but were created from the parent strains when required. Kela 2006 was a field collected female samples from the huts in the Kela area of Mali in West Africa and had been preserved in 70% ethanol for about 6 years.

2.2 Mosquito rearing

2.2.1 Adults

Adult mosquitoes were maintained under constant temperature of 26±1 ºC and relative humidity of 75-80 %, as well as a constant photoperiod of 12h light and 12h darkness in the insectary (Patrick Manson Insectary of the School of Life Sciences at Keele

University). Our standard rearing cages were made up of 5liter volume white plastic buckets (20.5cm x 20cm). The tops were covered with white nettings to which the edges have been sewn with elastic bands. On one side of the bucket was an opening with sleeve this provides access in and out of the cage. The flies were maintained in stock density of 800-1000 per cage. Cotton wool impregnated with distilled water was placed on top of each cage with adult flies at all times. Inside the cage, 20-30ml of 5% glucose solution was provided in a small glass tube with folded brown filter paper that absorbs the solution. This provides a landing pad for the mosquitoes to perch and feed on (Fig 2.1). The glucose solutions were replaced every 3 days.
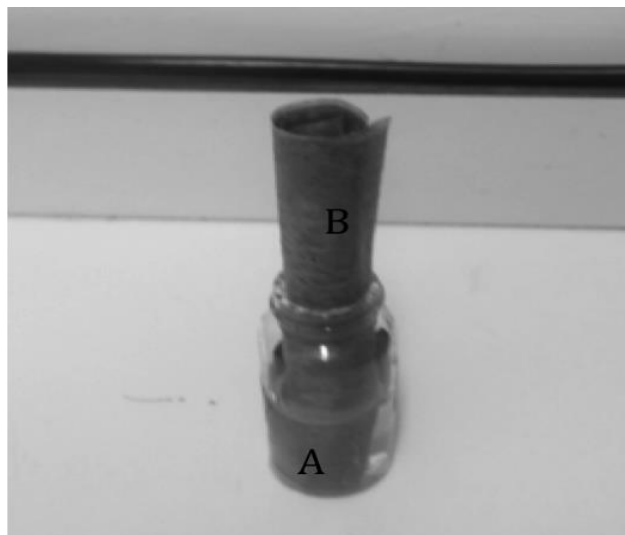
Fig 2.1: Adult fly feeding setup: A= 5% glucose solution in glass bottle; B= Brown filter paper soaked with glucose solution

2.2.2 Larval feeding regime

Much emphasis is laid on food in all living organisms, such that too little or too much gives a negative effect. Therefore, it is necessary that the right nutrition be given to achieve optimal effect. In mosquito, the quantity and quality of food given to the larvae reflect on the adult body size and their reproductive potentials (El-Akad and Humphrey 1990). Three separate larval feeding regimes were used during the course of this study.

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2.2.2.1 First feeding regime

This was an optimized working regime designed by a senior colleague to create female flies that are of good phenotypic quality. Hatched larvae were transferred to white coated metal rearing trays (33 x 23 x 5cm), 200 larvae per tray using 3ml pipette. To each of these trays, 1000ml of distilled water and one to two drops of liquifry (Interpet Ltd., Dorking, UK) were added on day zero. Subsequently, they were fed by adding 10mg of ground flakes of fish food (Tetra Werk, Melle, Germany) once daily for the next two days. For day 3, 20mg was added twice. Finally, 40mg are given twice daily until pupation. This regime was adopted for rearing and maintenance of mosquito colonies used in the stress experiment (Table 2.1).

Table 2.1: First larval feeding regimes adopted from Aboagye-Antwi and Tripèt (2010)

Day 0: liquifry one drop once a day

Day 1: 10 mg once a day

Day 2: 10 mg once a day

Day 3: 20 mg twice daily, morning and evening 40mg in total

Day 4: 40 mg twice daily, morning and evening 80mg in total

Day 5: 40 mg twice daily, morning and evening 80mg in total

Day 6: 40 mg twice daily, morning and evening 80mg in total

The second one was a slight modification of the first by another colleague still trying to improve on body size. The portions were altered slightly and measured out to fit better into their feeding needs. Each larval tray was fed one drop of liquifry once on day 1. 10mg of ground flakes of baby fish food once in the next two days. On days 4 and 5 they were fed 30mg of ground flakes of baby fish food once a day. From days 6 - 9, 30mg was added twice daily. To reduce the variations in the quantity of food given, the food was measured, weighed, and stored in small centrifuge tubes prior to the onset of the experiment (Table 2.2).

Table 2.2: Second larval feeding regimes adopted from Rowidah Baeshen 2013 (Unpublished thesis).

Day 1: liquifry one drop once a day
Day 2: 10 mg once a day
Day 3: 10 mg once a day
Day 4: 30 mg once a day
Day 5: 30 mg once a day
Day 6: 30 mg twice daily (morning and evening) in total, 60 mg
Day 7: 30 mg twice daily (morning and evening) in total, 60 mg
Day 8: 30 mg twice daily (morning and evening) in total, 60 mg
Day 9: 30 mg twice daily (morning and evening) in total, 60 mg

2.2.2.3 Third feeding regime

The last larval regime was another modification that aimed at improving the size and increase fitness at the same time. Hatched larvae are transferred to larval rearing tray as in the last two regimes. An initial 500lm of de-ionized water was put in the trays with one drop of liquifry this counts as day one. The second day, a flat spoon of grounded TetreMin flakes is given. Day 3, they were fed with one flat spoon of TetraMinBaby, and on day four, they were fed with 1/2 spoon of TetraMinBaby. On day 5, another 500ml of de-ionized water and one big spoon of the TetraMinBaby were added to the tray at about 9am. Approximately 6h later on the same day they were fed again with two different fish pellets, 6 beige Tetra Pond sticks (Tetra Werk, Melle, Germany) and 4 red sinking carnivore protein pellets (Hikari Tropical). The pellets would last for days 6-8. The pellets were evenly distributed at the four corners of the tray. They are slow dissolving would take care of their high nutritional requirements at this late larval stage, when they tend to feed voraciously. Pupation that sets off slowly from day 8 and on this day, they are fed with another big spoon of TetraMinBaby. As pupation increases, the portions of the food fed were gradually reduced (Fig 2.2).

Fig 2.2: Larval rearing trays covered with perforated Perspex lid. On top are the new larval trays, followed by the older ones with fish pellets. The bottom shelf contains larval trays with pupating larvae.

2.2.3 Pupae

Pupation starts gradually from the 8th-9th day after being added to the trays and pupae were picked daily from the larval trays by the use of a suction pump connected to a clean 500ml conical flask and sieved into small polystyrene emerging pots. Clean distilled water was added and the pot, transferred into the adult cage for emergence.
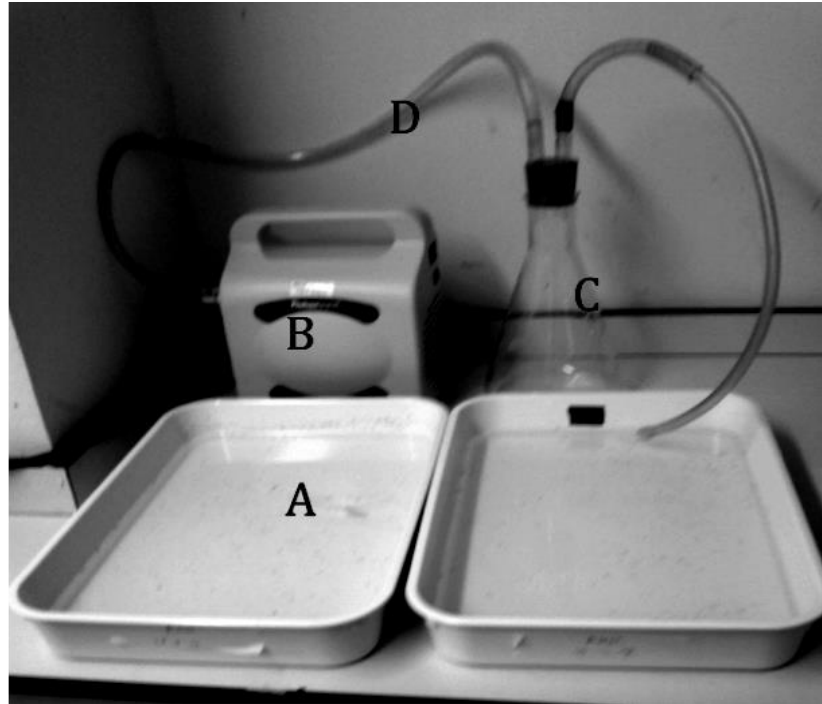
Fig 2.3: Setup for picking pupae on the insectary workbench;A= larval trays, mixed with pupa.;B= Vacuum pump; C and D= Conical flask with rubber tubing connected to the pump.

2.2.4 Sexing

Usually, virgin males or females are required for experiments, and it is a lot easier perform the sex separation as pupae (Sharma *et al.* 1972). Several studies have used different sex separation techniques such as oxygen requirements, body size, and grid system (Sharma *et al.* 1974; Papathanos *et al*. 2009). In this thesis, all virgin adult flies used were separated manually under the dissecting microscope (Lieca Microsystems GmbH, Solms, Germany) as pupae based on the morphological sex difference found on their paddle on the last abdominal segment (Fig 2.4). The tip of the male paddles is pointed and those of the females are flat (Barbosa 1968; Benedict 2007). Sexing was done only when virgin adult flies are required for experiments.
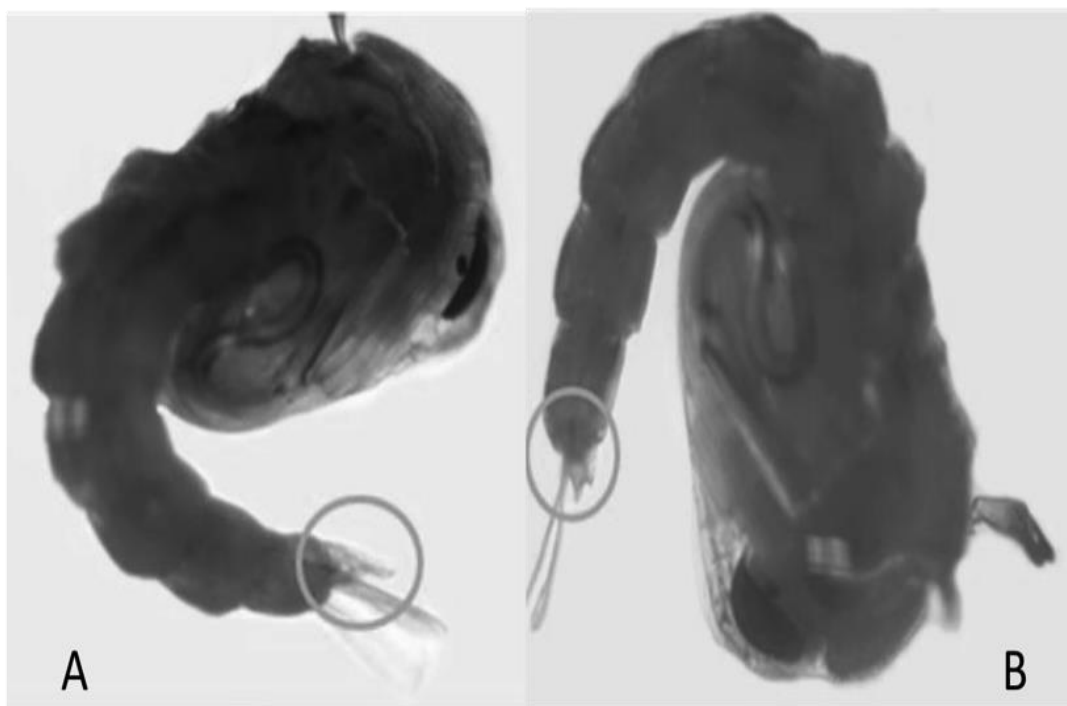
Fig 2.4: A= male pupae with pointed paddle. B= female pupae with flat paddle.

2.2.5 Blood feeding

Every week mature females from the colonies are fed with defibrinated horse blood (TCS Biosciences Ltd Ref.HB034) supplied through artificial membrane feeder (Hemotek® membrane feeding system, Discovery workshops, UK). Before feeding time, the bottom of the cage was cleaned and lined with brown paper to prevent haemolysis stains on the bottom of the cage. The sugar solution in the bottle was taken out for about 4-6h. A 0.5ml feeding reservoir which has been set at 38°C was used. Feeding last for about 30min and a fresh bottle of blood was used each week (Fig 2.5).
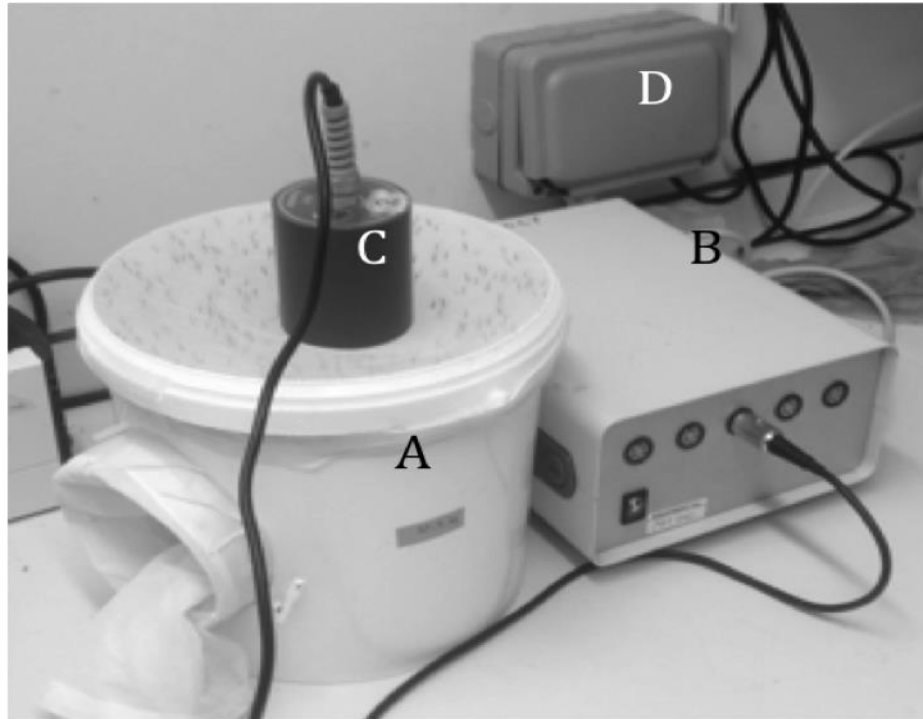
Fig 2.5: Blood feeding setup; A: Cage with blood feeding adult flies, B=Hemotek central heating source C=preheated blood feeding reservoir, D= Main socket

2.2.6 Production of virgin adults from Mopti and Kil parental strains

Female and male individuals used in the mating experiments (Chapter 5 Sections A, B and C) were produced using a standard protocol to minimize phenotypic variation among individuals and variance amongst experimental groups. To do so, 200 virgin Mopti male and 200 virgin Kil males were combined each with hundred virgin females of their strain in two separate cages. After mating, females were blood fed for 30 minutes using the Hemotek membrane blood feeder. Precisely 48 hours, post blood meal, gravid females were given an oviposition cup of egg laying. Two days afterwards, the egg pots were recovered and allowed to hatch. First larval instars were transferred to rearing trays as described above (Section 2.2.2). However, the second feeding regime was used for Adults in Sections A & B, while the third feeding regime was used for adults in section C). Upon pupation, they were collected and sexed in order to create virgin males and females used in all the mating combinations.

2.2.7 Production of hybrid supermales

The supermale was created by crossing, the male of a colonized Mopti strain (~10 years) with a much older Kil female strain (~35years) using the same procedure used to generate virgin males and females from the Mopti and Kil strains. Two hundred virgin Mopti males between 3-5 days old were combined with 200 virgin females of Kil and allowed to mate for two nights. Following the mating, females were blood fed for 30min using membrane blood feeder. Exactly 48 hours, post blood meal, gravid females were provided with a small polystyrene pot lined with clean white filter paper (0.03mm thick and 0.11cm wide) and half filled with de-ionized water for egg laying. Three days later, the egg pot was retrieved, first larval instars were transferred to rearing trays, 200 larvae per tray. The larvae were maintained like the rest of the other larval colonies using either second or third larval feeding regime (Table 2.2 or Table 2.3). Upon pupation, they were sexed, under a binocular dissecting microscope (Lieca Microsystems GmbH, Solms, Germany) as described in section 2.5.2 and transferred to two different cages in a medium sized polystyrene pot half filled with de-ionized water. The cages were provided with 5% glucose solution in a glass bottle with folded brown filter paper and on top of the cages were wet cotton wool pads with de-ionized water. The F1 male progenies from this crossing were referred to as 'supermales'.

2.2.8 Confounding factors

To avoid variations, during mosquito rearing the larval trays was swapped daily in all the experiments, in addition to the standardized feeding regime. The adult flies were supplied with the same type and amount of glucose solution throughout the experimental periods. The positioning of the experimental cages was changed daily in addition to the fixed insectary conditions to avoid environmental effects. During sample collection, the cages

are agitated and with mouth, aspirator a sweep is made to collect the mosquitoes. The aspirator also makes it easier to distinguish the right sex and count the sample.

2.3 Laboratory techniques

2.3.1 Spermathecae and sperm bundle dissection

Spermathecae dissections (preserved or fresh) of female mosquito samples were carried out under a binocular dissecting microscope (Lieca Microsystems GmbH, Solms, Germany). An individual is picked with a pair of tweezers and placed on a clean slide with a drop of PBS solution. Using the dissecting needles, the last abdominal segment of the mosquito was gently pulling out from the rest of the body while holding firmly the thorax with the left dissecting needle. The spermatheca is a dark brown organ located at the very last segment. It comes out with the other tissues and detritus. The unwanted materials are gently cleared away with great care to avoid damaging the spermatheca. The clean spermatheca was then gently lifted up with the tip of the pin and transferred to another slide for viewing (sperm activity).

For sperm bundle dissection (insemination status), as soon as the spermatheca is dissected out, it is transferred to another slide with a clean drop of PBS solution. The spermatheca is pushed gently to the edge of the solution drop, and carefully pressing down with one pin and using the other pin to give the spermatheca a tap from one corner to crack the capsule open. The sperm bundle is seen like a 'fluffy wet cotton wool' in fresh dissections (Fig 2.6), but in stored samples, it has the appearance of a solid gel.
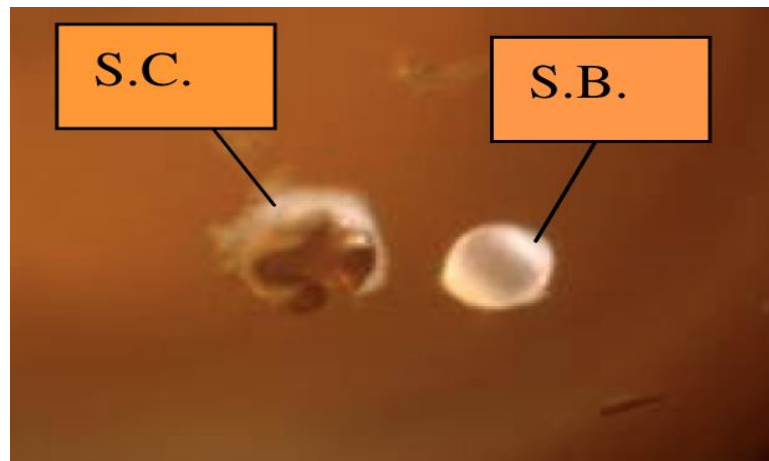
Fig 2.6: *An. gambiae* **spermatheca capsule (SC) and sperm bundle (SB)**
(Tripèt *et al*. 2001).

2.3.2 Measurement of sperm activity

Spermatheca from dissection was transferred to a phase contrast microscope set at x4 objective. Prior to this, the microscope was equipped with a Motic Images Plus camera (Motic China Group Co. Ltd) that was connected to a Laptop computer (MacBook Pro). Once a successful transfer has been achieved, the microscope was properly adjusted (set at x10 objectives) for better and clearer focus. The tumbling movement of the sperm bundle inside the spermatheca was then viewed on the laptop screen. The sperm bundle makes a rotation movement that was recorded as QuickTime movie (using the QuickTime player software on the laptop). The sperm bundle movement inside the spermatheca was observed for one minute and scored as low, medium, high, very high and no activity when there was no movement at all. The time for dissection and scoring of an individual fly took about 3 minutes sometimes less. This was to ensure that no sets of samples would last more than 15 minutes in the icebox to avoid the effect of ice on the sperm vitality. In addition, the researchers performing the dissections and sperm activity scoring were blind with regard to which treatment group was being processed at a given stage. To ensure that sperm activity could be recorded within 2 minutes of the start of the dissection, a team of two researchers

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has always performed the dissection and sperm activity scoring. The scoring was done later ranked from 1-5 (no activity to very high activity). The median sperm activity recorded over 1min of observation was used for subsequent analyses.

2.3.3 Genomic DNA (gDNA) extraction

Two techniques for gDNA extraction were used in this project; DNAzol method and ChargeSwitch (Invitrogen) method. The DNAzol method was used for gDNA extraction from whole body of single individuals for molecular form identification while the ChargeSwitch method was used on the spermathecae or sperm bundles.

2.3.3.1 DNA-zol: Whole body

In as much as the protocol of the manufactures were observed, there was a slight modification in terms of quantity used as the kit was designed for a much bigger organism. Using a plastic pestle, the whole body were homogenized in 100µl of DNAzol reagent in 1.5ml centrifuge tube. They were centrifuged at 10000g for 10min and supernatant transferred to a new tube. By the addition of 100% ethanol, the DNA was precipitated by another centrifuging at 1000g for 10min. The isolated DNA was then washed with 750µl of 70% ethanol and centrifuged at 10000 g for 10min twice. The supernatant was discarded after each wash by blotting the tubes in between tissue pads. At the end of the second wash, the tubes containing the DNA pellets were put in the incubator (37 °C) overnight to dry out. Next morning, 200µl of distilled water was added to the tube and vortex briefly to suspend the DNA. They were transferred into the permanent storage tube stored at -20°C freezer.

2.3.3.2 ChargeSwitch: spermathecae and sperm bundles

The manufacture's guide for ChargeSwitch gDNA micro tissue kit (Invitrogen) was followed, with slight modifications made on the working volume to fit into our requirement. Before starting, heat block was set at 55 °C, 500µl of lysis buffer plus 5µl of proteinase K were pipette into 1.5µl centrifuge tube. The dissected spermatheca or sperm bundle from female mosquitoes was then transferred into the lysis mix. The mixes were incubated on the heat block and vortex 10s every 15min for 1-2h, or incubated overnight without vortexing. When they were removed from incubation, 2.5µl of RNase A was added into the tubes and pipettes up and down gently to homogenize the mix. They were incubated at room temperature for 5min. 200µl of purification buffer was added, pipetting up and down gently to purify the DNA. Adding 20µl of magnetic beads that has been suspended by vortexing and pipettes up and down gently five times incubated at room temperature for 1min bonded the DNA. The tubes were then placed on a MagnaRack for another 1min and the supernatants carefully removed and discarded. Removing the tubes from the MagnaRack, 500ml of wash buffer was added, pipettes up and down twice to suspend the beads and place them back on the MagnaRack for a minute. The supernatants were removed with a pipette carefully. The washing of the beads was done twice. After the second wash, the tubes were removed from the MagnaRack, 75µl of elution buffer were added pipetting up and down gently several times to suspend the beads. They were incubated at room temperature for 5min and place on the MagnaRack for 1min. Elute containing purified DNA was removed and transferred into DNA permanent storage tubes and stored at -20 °C.

2.3.4 PCR

Polymerase chain reaction (PCR) is a common and often indispensable molecular biology technique in medical and biological research labs for diverse applications developed by Kary Mullis in the 1980's (Bartlett and Sterling 2003). The method is based on thermal cycling. A single or relatively few copies of a piece of DNA are amplified across several orders of magnitude to generate thousands of millions of copies of a specific DNA sequence of interest. In this thesis, all PCR experiments were done following a PCR protocol described by Scott *et al.* (1993). All the PCR reaction was performed in a 0.5ml thin walled 96 well PCR plate (Star lab). Each 25µl PCR tube contains 5µl of x5 buffers (Promega) 0.5µl (10nM) of deoxynucleotide triphosphates (dNTPs), 0.5µl of each of the two (forward and reverse) oligonucleotide primer pairs (Taqy and S23), 0.125µl of Taq Polymerase enzyme (Promega), 1.5µl of $MgCl_2$ (Promega) and 14.88µl of distilled water to give a volume of 23µl. Finally, 2µl of the gDNA template was added to get a final volume of 25µl. The amplifications were carried out using a 96 well BioRad DNA engine thermal cycler (PTC-200). The PCR was for 35 cycles with the following cycling periods: 95°C for 3min, initial denaturing, 95°C for 15s denaturing, 52°C-65°C for 30s annealing temperature (range) 72° C for 1.5mins for extension and 72 °C for 10mins final extension.

2.3.5 Electrophoresis

At the end of the thermal cycles, amplified gDNA fragments were electrophoresed on 1.8% agarose gel. To prepare the gel, 3.6g agarose + 20mls of 1x (TBE) buffer solution and 180mls of distilled water were heated in a microwave oven (shook at intervals) for 3 minutes to dissolve the agarose. It was kept at room temperature to cool to about 60°C. 2μl of ethidium bromide was added and the mix was given a vigorous shake. The gel was cast and allowed to set for 1h. 5μl of the PCR mix was loaded into the gel wells and run at 80 volts for 2h in 1X TBE buffer using a mini gel system (MP-250N). The gel was photographed using a Bio Imaging System (SynGene).

2.3.6 Real –Time quantitative PCR (qPCR)

TaqMan probe is an oligonucleotide with fluorescent dye on the 5'base and a quenching dye, on the 3' base. They are designed to hybridize to the internal region of a PCR product. The principle is based on the hybridization of probes, which relies on fluorescence resonance energy transfer (FRET) for quantitation. The TaqMan probe when illuminated, fires up the fluorescent dye and transfers energy to the adjacent quenching dye molecule without fluorescing (Fig 2.7) When the polymerase replicates a template in a PCR reaction to which a TaqMan probe has been added, the 5' exonuclease activity of the polymerase encounters and hybridizes with the probe. This divides the fluorescent and quenching dyes, and FRET (fluorescence resonance energy transfer) no longer occurs, resulting in fluorescence. The increase in fluorescence in each cycle is proportional to the rate of probe cleavage to target. For sperm number quantification throughout this thesis the TaqMan probe and principle was applied due to its specificity, i.e. binding to only target single DNA strands.

Boeckman *et al.* 2000
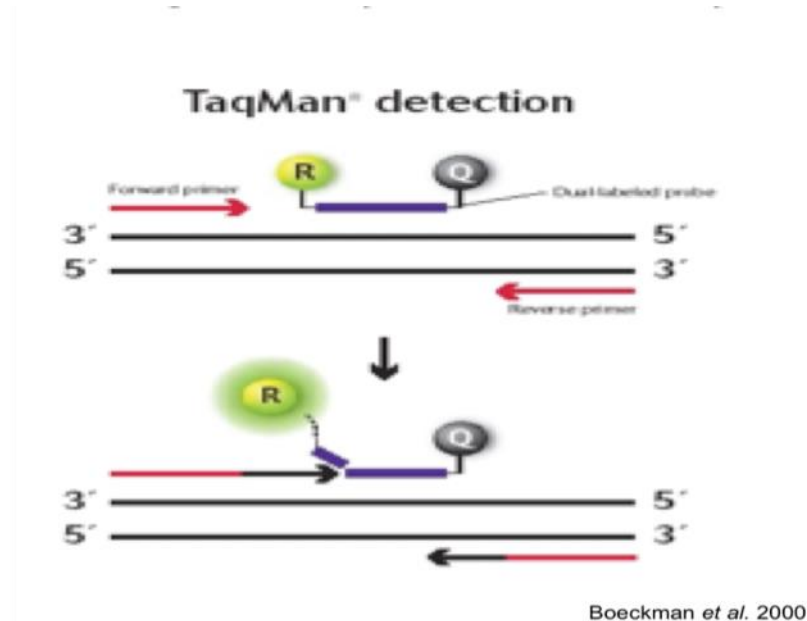
Fig 2.7: TaqMan chemistry mechanism: This probe is an oligonucleotide with a reporter dye attached to the 5' end and a quencher dye attached to the 3' end. If the probe is not hydrolyzed the quencher and the fluorophore remain in proximity to each other,. During the PCR, the probe anneals specifically between the forward and reverse primer to the PCR product. The polymerase carries out the extension of the primer and replicates the template to which the TaqMan probe is bound. The 5' exonuclease activity of the polymerase cleaves the probe releasing the reporter dye away from the close vicinity of the quencher. The fluorescence intensity of the reporter dye is then measured as a result.

2.3.7 Standard curve calculation using gDNA

Based on the genome size of *An. gambiae* (278,253,050 bps) a standard curve serial dilutions was made and used in estimation of sperm copy numbers transferred by male mosquitoes during mating. The calculation was done based on the AppliedBiosystem manual for creating a standard curve using a gDNA (AppliedBiosystem procedure for creating standard curves from gDNA). In this experiment, the target was sperm copy numbers between 10,000copies to 0 copies.

The mass of the gDNA per genome was calculated as:

m=[n][1.096e-21g/bp] where; n=genome size (bp)

$$m = \text{mass}$$

$$e^{-21} = x10^{-21}$$

Substituting for n, m= [278253050] *[1.096e-21g/bp] =3.04965e^{-13g}

[3.04965e-13] *[1e12pg/g]=0.304965= ~0.3

~0.3pg/genome divide by 1 copy y-DNA

p/genome=[0.3/genome] * [genome/1 copy= 0.3pg/1copy

i.e. 0.3pg of *An gambiae* gDNA contains 1 copy of y-DNA gene.

Now to calculate the mass of gDNA containing the copy number of interest,

(10,000 copies – 0 copies)

10,000copies x 0.3pg =3000pg(mass of gDNA)

Table 2.3:The copy number of interest multiplied mass of the haploid gene to get the mass of gDNA needed. The mass of gDNA needed (pg) multiplies by the volume of gDNA template pipette into each reaction gave the concentration of gDNA required.

Copy number	Mass haploid gene (pg)	Mass of gDNA (pg)	Reaction vol. (µl)	Final conc. gDNA (pg/µl)
10,000		3,000		1,500
250	Multiply	75	Divided	37.5
	By		By	
	0.3pg		2µl	
6.25		1.875		0.94
0.16		0.048		0.024
0.004		0.0012		0.0006

Therefore; from a gDNA extraction of 28.2ng/μl which equals 28,200pg/μl, Using the formula C1V1=C2V2.

28,200V1=1,500pg x 200μl

V1=1,500pgx 200μl/28200pg =10.6μl

Therefore, 200μl final vol. -10.6μl of gDNA =189.4μl

189.4μl of water (diluents) would be added to 10.6μl of gDNA to get 200μl final vol.

Table 2.4:A 40-fold standard curve dilution 4 step prepared from 28.2ng/μl gDNA extraction.

Dilution	Initial con. (pg)	Vol. gDNA (μl)	Vol. diluents	Final vol. (μl)	Final con. (pg/μl)	Resulting copy
Stock	28,200	10.6	189.4	200	1,500	10,000
1	1500	5	195	200	37.5	250
2	37.5	5	195	200	0.94	6.25
3	0.94	5	195	200	0.024	0.16
4	0.024	5	195	200	0.0006	0.0039

2.3.8 Measurement of wing lengths

The winglength of the mosquito has been reported in many studies to have a strong correlation with body size (Manoukis *et al.* 2006 and Jirakanjanakit *et al.* 2007). In studies where direct body size measurements cannot be taken due to changes in preservation methods the winglength are used to determine the body size. Also to correct for differences in body size that may exist and be a confounding factor in the experiments. The winglength of the entire adult mosquitoes used were measured. The wing was adhered to a clean slide

with a drop of PBS solution. Under a binocular microscope, calibrated with a 1mm eyepiece graticule the wing was measured from the from the alular notch to the distal wing margin ignoring the fringe. The measurement done was taken to the nearest 0.01mm.

2.3.9 Statistical analysis

All data were analyzed with JMP10 software (SAS Institute Incorporated. NC, USA 27513). Normality in the distribution of the data was checked using the Shapiro-Wilk Goodness of fit Test. All normally distributed data were analyzed parametrically using ANOVA and mean difference within experimental groups were tested and compared with Turkey HSD. Mean differences were presented with bar plots at 95% confidence interval throughout the thesis. Experimental outcome with $P < 0.05$, the null hypothesis for such experiment were rejected. Kruskal-Wallis and Wilcoxon test (non parametric) was used for all data that deviates from normal distribution and displayed as box plots. Where medians are used they are presented with lower whiskers constructed as 25th percentile -1.5* interquantile range and upper whiskers as 75th percentile + 1.5* interquantile range. Where data were ranked, Dunn's test was used to compare means between experimental groups. Proportions and frequencies were determined where appropriate. The main effects and interactions from the replicates within experiments were always statistically tested, but reported only when significant.

Chapter 3

**A novel approach to sperm quantification in *An. gambiae s.s* based on
TaqMan real-time qPCR**

3.1 Introduction

Genetic manipulation of colonized insects and pests for vector control measure had been acknowledged for years (Benedict and Robinson 2003). The Sterile Insect Technique (SIT) was one of the first biological vector control projects planned for a wide area purpose. The SIT project involves the release of millions of sterile male insects over a wide area to mate with wild females (Dyck *et al.* 2005). The released male is exposed to radiations that would bring some modifications in their sperm cells. The affected sperm cells become sterile and mating of release sterile males with wild females leads to infertile egg production, causing a decrease in female reproductive potential. Eventually, with the sufficient release of sterile males over time, the vector population will be suppressed (Knipling 1959). The sterile male release of *Cochliomyia hominivorax (Coquerel)* (screwworm) to eradicate the pest in North and Central America was one of the earliest success stories of such programme (Klassen and Curtis 2005). The first air operational release of sterile males was carried out in the 1990's in Unguja Island, Zanzibar for the suppression of *Glossina austeni* to free the cattle from nagana disease (Dyck *et al.* 2005). The SIT was used in the 1980s and 1990s to eliminate the melon fly *Bactrocera cucurbitae* (Coquillett) in Okinawa, Japan and all of Japan's southwestern islands (Klassen and Curtis 2005). In Chile, the SIT was used to purge the country of the Mediterranean fruit fly that was achieved by 1995 (Kuba *et al.* 1996).

The use of SIT has not been very successful with mosquito releases (Benedict and Robinson 2003). In the 1960's, sterile male release (SIT) of mosquitoes for population

reduction was attempted, and some basic issues, which needed to be dealt with, were encountered (Benedict and Robinson 2003). The mass production of males only with adequate reproductive quality would be huge task to overcome (Lines and Curtis 1985). Population suppression has been achieved only in a few cases with very large release ratios of sterile to wild mosquitoes. The largest release of sterile males for the control of mosquitoes was conducted in El Salvador and India, but was terminated prematurely due to politics and civil war in both countries respectively (Dyck *et al.* 2005). In addition to the opposition by local governments and local populations, a major failure encountered in the SIT project was that sterilized males often suffered from low mating competitiveness when compared to their wild counterparts (Curtis, 1978). The sterile male release of *An. culicifacies* Giles in Pakistan failed due to subnormal mating competitiveness shown by the sterile males (Asman *et al.* 1981). On the Pacific coast of El Salvador, chemo-sterilized males of *An. albimanus* were demonstrated to be as competitive as non-sterilized males in cage experiments, but upon release to compete with the wild males, they achieved only 25% mating competitiveness (Klassen and Curtis 2005). In Sudan, *An. arabiensis* males produced from the 68th generation of a laboratory strain were able to compete with non-sterile males produced from field-caught larvae and pupae for wild virgin females. The study was performed in large semi-field enclosure, only 71% mating competitiveness was witnessed in the radio-sterilized males against that of non-sterile counterparts (Hassan *et al.* 2011). In Nigeria, an enclosed field trial involving sterile males release of *An. gambiae* complex was carried out, the sterile males failed to mate with the wild females.

In the 1990's, the initiative of using Genetically-Modified Mosquitoes (GMM) to reduce malaria disease was founded on the concept that genetic construct will affect the vector competence of mosquitoes causing a reduction in the rate of parasite transmission (Takken and Scott 2003). Various approaches of the GMM projects with diverse molecular

biology are in progress and as well as field trials (Alphey 2013).

A GMM approach similar to the SIT is the Release of Insects with Dominant Lethal mutation (RIDL) (Alphey 2002). In RIDL, males carry a lethal gene construct, and mating with females results in the production unfit offspring that does not survive to maturity. The release of RIDL males in adequate numbers to mate with wild female population for a long time will eradicate the target population. It is intended that with RIDL, reduced vigor and low mating competitiveness suffered by SIT males due to gamma irradiation will be overcome (Alphey 2014). The fitness cost with regards to mating competitiveness in RIDL strains has been shown; Lee *et al.* (2009) conducted fully contained large enclosure experiments to evaluate the mating competitiveness of two RIDL strains of *Aedes aegypti* expressing an irrepressible dominant lethal gene. The result revealed a successful mating competitiveness between the males from a local wild-type laboratory strain of *Aedes aegypti* and the transgenic strains. The same RIDL males when released in open-field trials by Harris *et al.* (2011) in Grand Cayman Island, they showed a 44% reduction in mating competitiveness compared to wild males. Another recent study reported a low mating competitiveness in males that were competitive in large-cage experiments set in the laboratory. The transgenic males (OX3604C) of *Aedes aegypti* carrying a dominant female/lethal system were competitive in laboratory large-cage experiments, but in semi-field enclosure, the same males only achieve up to 59.1% of the mating competitiveness with wild-type males (de Valdez *et al.* 2011; Facchinelli, *et al.* 2011). In GMM release programmes, it might be likely that sperm investment and male fertility could affect the overall impact of release programme. For the success of the SIT project, it is critical that wild females mated by the sterilized males do not re-mate. A reproductively fit male should transfer sufficient sperm to avoid re-mating (Robinson *et al.* 2002). Thus, it has become essential to ascertain the amount of sperm in the reproductive organs of male and

female (Reichardt and Wheeler 1995). In the *Anopheles,* the amount of sperm transferred has been related to the male body size. For example, in irradiated and un-irradiated males of *An. arabiensis*, a weak positive correlation was found between sperm amount invested and male body size (Helinski and Knols 2009). In the lab colonies, larger males of *An. gambiae* would prefer to mate with larger females when given the choice and can inseminate up to 10 females per night. Thus, the amount of sperm produced by the male was not a determining factor for mating (Okanda *et al.* 2002). Determining the amount of sperms invested by the males during copulation might assist in understanding their reproductive success and will contribute in appreciating the mosquito mating biology as recent evidence suggests that variation in ejaculate investment might force some females to go back to swarms to mate again.

Many researchers have studied sperm numbers across insect species using different methods (Reichardt and Wheeler 1995). Ponlawat and Harrington (2009) manually counted sperm numbers transferred by of *Aedes aegypti* from dry slide samples under a phase contrast microscope. Helinski and Knols (2008) also performed a manual sperm number counting using the dark-field view microscope. Superseding manual sperm number counting is the counting of live sperm in an appropriate physiological buffer solution under the microscope using a haemocytometer (Keller and Passera 1992). A modified form of the haemocytometer is the fluorometer, which allows for efficiency and the procession of larger sample sizes (Reichardt and Wheeler 1995). The live/dead sperm viability kit (L-7011, Molecular Probe) has been used widely by many researchers to study sperm numbers. The sperm storage organ is crushed, covered with a slip, and immersed in the dye on a microscope and the slide is viewed instantly under a fluorescent microscope (Twig and Yuval 2005). The head of live sperm stains green and head of dead sperm stains red (Bernasconi *et al.* 2002; Nakahara and Tsubaki 2007).   In addition to the above-mentioned

method of sperm quantification, Tram and Wolfner (1999) utilized competitive quantitative PCR to measure and compared the amount of sperm transfer in *Drosophila melanogaster.* They estimated the sperm amount transfer by comparing the intensity of sperm signals with a competitor sperm amount, which they have categorized previously with PCR. The PCR products were analyzed on 5% ployacrylamide gels and the sperm DNA molecules matched with the various categorized DNA molecules to determine the sperm quantity of each sample.

Studies on cost of sperm investments exist in other insect species (Reinhold *et al*. 2002; Wedell *et al.* 2002), but none in mosquitoes. The sperm quantification methods used in other insects could only give an approximation of the sperm quantities (Holman 2009), but in Anopheline mosquitoes because of the intricacies involved in the spermatheca dissection these methods has not been used (Helinski and Knols 2009). Sperm number transfers in *An. gambiae* remain largely unexplored due to the complexity related to the bundle nature of sperm cells and also the uniformity in the dilution of samples for counting (Helinski and Knols 2009). Consequently, we have developed a new method for sperm number quantification based on TaqMan qPCR quantitative assay. The qPCR assay is less laborious and has room for analysis of a minimal starting quantity. It also offers the possibility of getting a more accurate result on sperm numbers using Y specific primers (Heid *et al*. 1996 Krzywinski *et al.* 2005). In our technique, the TaqMan probe was used instead of SyberGreen dye, due to its high specificity, binding only to targets. It is intended that this newly developed qPCR method based on the TaqMan probe will facilitate studies on male quality and ecology of reproductive investments. It will also highlight the importance of male investment size and reproductive trade-offs in *An. gambiae,* which currently is deficient. Taken for granted that the quantity of sperm transfer by the male *An. gambiae* during mating might have a fitness cost relating to male competitiveness,

estimating sperm investment will be beneficial in defining male quality and mating success. The understanding of mating competitiveness in males for the success of SIT and GMM base control programmes is of foremost importance (Takken *et al.* 2006). Therefore, the knowledge of sperm number investment is very fundamental to improving male competitiveness for progress in the SIT and GMM programme working towards male mosquito releases. At the time of this study, there was no published information on sperm number investment in field *An. gambiae s.s,* and using the newly optimized assay, we determined the sperm number investment in field captured females of M and S molecular forms of *An. gambiae s.s.*

3.2 Materials and Methods

3.2.1 Molecular form identification

Adult females were collected with mouth aspirator from huts in the Kela area (11º88'N, 8º45'W) in Mali, West Africa. They were taken to the laboratory at the Malaria Research and Training Centre Bamako Mali and stored in 75% ethanol. Thereafter, they were transported to Keele University UK. Genomic DNA extraction was performed on female carcasses using DNAzol method of extraction (Invitrogen, Carlsbad, CA, USA). Species diagnostic PCR was done as described by Fanello *et al.* (2002) was undertaken to differentiate the *An. gambiae s.s* from their sibling species *An. arabiensis*. The same PCR was utilized to differentiate the M-molecular forms from the S- molecular forms. In Mali, the two molecular forms (M and S) are known to exist in sympatry (Favia *et al.* 1997) hence; a molecular identification was very necessary. The M-form individuals belong to the Mopti chromosomal forms, and the S-forms are either Savanna or Bamako chromosomal form (Favia *et al.* 1997).

3.2.3 gDNA extraction (ChargeSwitch gDNA) for sperm number quantification

The female spermatheca was dissected by pulling out the last abdominal segment with a dissecting needle as described in Chapter 2. The dissected spermatheca was then transferred to a clean microscope slide for sperm bundle dissection as described in details in Chapter 2. The dissected sperm bundle was then transferred into a tube with 500µl lysis buffer containing 5µl of proteinase K in a 1.5ml centrifuge tube and incubated overnight at 55ºC. The next morning, 2.5µl of RNase A was added to each tube and gDNA extraction performed using the ChargeSwitch gDNA micro tissue kit (Invitrogen) following the manufacturer's with slight modifications (see Chapter 2). The purified gDNA was transferred into permanent screw cap DNA storage tubes and stored at -20 ºC for later use.

3.2.4 Optimization of TaqY primers and Probe-Y for qPCR assay

TaqMan Y-specific primers 'TaqY' and Probe Y were designed by Dr F. Tripet to amplify a short sequence of the Y-chromosome. They were short amplicon primer, short amplicons work better and give a consistent result when used on amplicon size of 50-150bp (Krzywinski et al. 2005). The primer pairs were optimized to amplify Y-chromosome sequence of 77 base pairs in length (Table 3.1). In PCR reactions, it is important to find an optimal temperature for the annealing stage. The TaqY primer temperatures were then optimized by performing a gradient PCR with an annealing temperature range of 52-65ºC for 30s (Cal. Tm for TaqY primers were 57.75ºC) on a standard PCR. Each tube contains 12µl of TaqMan Universal Master Mix ll no UNG, 1.5µl TaqYf and TaqYr, 7.5µl of water and 2.µl of gDNA template was added to make the final volume 25µl each in 0.5ml in 96 well PCR plates. The amplifications were performed in 96-well thermal cycler (BioRad DNA engine, PTC-200). The PCR cycling period was for 35 cycles. At the end of the thermal

cycle, the PCR products were electrophoresed in 1.8% agarose gel and good amplifications bands were observed at higher temperature 61.6-65.0ºC.

Table 3.1: Detail sequence of TaqY primers and ProbeY used for the optimization of sperm quantification technique .

Primer/Probe	Primers/Probe sequence	Length (bp)	Target size (bp)
TaqY forward	5'-TTACCACGCTGGCAAATGC-3'	19	77
TaqY reverse	5'-CGTGCAACAGCTCGTGATC-3'	19	
Probe Y	5'-AGATGGATGCGGCGT-3'	15	

The probe-Y was optimized using the TaqY primers to determine the appropriate volume and concentration that will yield an optimal result. The Probe-Y was 15base 5`-AGATGGATGCGGCGT-3` in length and the G/C content was 60% to ensure appropriate specificity. The Probe-Y Tm was higher than the annealing temperature used during thermal cycling and no Gs at the 5'end to avoid arrangement that quenches reporter fluorescence after cleavage. The Probe-Y was marked with FAM as the reporter fluorescence dye and TAM as the quencher dye. The starting volume was 1.25µl of 10nM with 0.25µl of 50/50nM of primers. The qPCR reactions (Table3.2) were made on a 0.5ml MicroAmp optical 96-well PCR reaction plate and were sealed with MicroAmp optical adhesive film, centrifuged at 2000rpm for 4min at 4ºC. The thermal cycling was performed on an AppliedBiosystem StepOnePlus RT-qPCR instrument and the thermal cycling conditions were; 2min at 50ºC, 10min at 95ºC, and 40cycles of 0.15min at 95ºC and 1min at 60ºC. Different volumes and concentrations of Probe-Y and TaqY primers

were tested following the guideline in AppliedBiosystem protocol for allelic discrimination assays. The concentration of the probe was usually in the range of 10nM to compete for binding to their target with the amplification primers that are found in greater concentration (Bustin 2000). After several attempts without amplification, the standard dilution 100pm/µl without further dilution gave the desired amplification.

Table 3.2 Details of the qPCR for mix per reaction tube for the optimization of probes/primers used for quantification of sperm extracted from the spermatheca of female *An. gambiae s.s*

Reagent	Reaction volume (µl)
TaqMan Universal Master Mix II	10
TaqY forward primers	0.25
TaqY reverse primers	0.25
Probe Y	1.25
Water	6.25
gDNA template (sample)	2
Total	20

3.2.5 Optimization of standard curve

Using the ChargeSwitch extraction protocol, a large amount of gDNA was also extracted from 3 adult males for use in standard curves for sperm copy number quantification. The whole body of 3 Mopti males was crushed and homogenized in 500µl of lysis buffer with 5µl of proteinase K. All other steps were as described above and in Chapter 2. After the extraction, the concentration of the gDNA extracted was determined on a Nanodrop 1000 spectrophotometer (Labtech International Thermo Scientific). The purity was measured based on the ratio of absorbance at A260/280nm (wavelength DNA/ wavelength Protein)

and samples with A260/280 values below 1.8 discarded. The DNA extracts were then pooled and the mean concentration of the mixture was measured.

Based on the measured concentration of the male DNA mixture and the known theoretical haploid genome size of *An. gambiae* (278,253,050bp) (Vector base genome) a standard curve dilution was prepared. Calculations were done as described in the 'AppliedBiosystem procedure for creating standard curves from gDNA' (Chapter 2). Standard curves were made using 4 large steps of dilution to account for the possible large variation in the amount of sperm detected (i.e. ranging from absence of sperm to many sperm). In *Drosophila*, for example, up to 4,690 sperm were counted by direct counting in a fixed female reproductive tract (Gilbert 1981). Tram and Wolfner (1999) using a method of competitive quantitative PCR pre-dating modern qPCR techniques, estimated 154-15,400 sperm in the female reproductive tract following a single insemination. In this experiment, expected sperm copy numbers were assumed to range from 0-10,000. Therefore, as a starting point, a 40-fold standard curve dilution was prepared from the gDNA extraction from the whole body of the male mosquito. The initial concentration of DNA was 28.2ng/µl (28,200pg/µl) (Table 3.3).

Applying the formula $C1*V1=C2*V2$ gave $28,200 * V1= 1,500pg * 200µl$; therefore $V1= 1,500pg * 200µl/28200pg = 10.6µl$. Consequently, 189.4µl of water (diluents) was added to the 10.6µl of gDNA to get a concentration of 10,000 haploid genome copies per microliter in a final volume of 200µl (Table 3.3).

Table 3.3: 4-steps standard curve dilution prepared from 28.2ng/µl of gDNA extraction from male *An.*
gambiae s.s

Dilution steps	Start conc. (pg)	Vol. gDNA (µl)	Vol. diluents (µl)	Final vol. (µl)	Final conc. (pg/µl)	Resulting Copy #
Stock	28,200	10.6	189.4	200	1,500	10,000
1	1500	5	195	200	37.5	250
2	37.5	5	195	200	0.94	6.25
3	0.94	5	195	200	0.024	0.16
4	0.024	5	195	200	0.0006	0.0039

3.2.6 Estimation of sperm number

The qPCR mixes for sperm number estimation in Kela 2006 females were based on the
TaqMan Universal Master Mix II (AppliedBiosystem) and prepared in accordance with
the AppliedBiosystem guidelines that we further optimized (See Chapter 2, Table 3.5).

**Table 3.4: Optimized working qPCR mix per reaction tube for sperm number quantification using
gDNA extracted from the female spermatheca of** *An. gambiae*

Reagent	Reaction volume (µl)
TaqMan Universal Master Mix II	10
TaqY forward primers	1.5
TaqY reverse primers	1.5
Probe Y	1.25
Water	3.75
gDNA template (standard dilutions)	2
Total	20

Two independent replicates of the 40-fold and 4-step standard curve were loaded on each plate of samples. All the reaction was conducted on a 0.5ml MicroAmp optical 96-well PCR reaction plate. The PCR plate was sealed with MicroAmp optical adhesive film and centrifuged (Hettich Zentrifugen Universal 32R) at 2000rpm for 4min at 4ºC. The PCR reaction plate was set on an AppliedBiosystem StepOnePlus qPCR for quantification using a Y-specific TaqMan probe combined with the described dilution curve quantification (see Chapter 2), Using 40 PCR thermal cycling conditions; 2min at 50ºC, 10min at 95ºC, and 0.15min at 95ºC and 1min at 60ºC. At the end of the 40 cycles, the baseline and threshold were manually adjusted following the recommendation described by AppliedBiosystem. The cycle threshold values (Ct) of the standard curve and samples were saved for later statistical analyses using the JMP10 software (JMP 10, SAS Inc. USA). Ct values for the dilution curve standards were plotted against their respective 40-fold dilution. A line of best fit was made for each of the two dilution curves using a log-quadratic equation (Fig 3.1). The best quadratic equation as assessed by the highest r^2 was then used to calculate the sperm number equivalent to 2µl of each individual samples matching the measured Ct values.   The sperm numbers were log transformed to normalize the data. The transformed data were then multiplied by 75µl which was volume of gDNA extracted from one spermatheca.
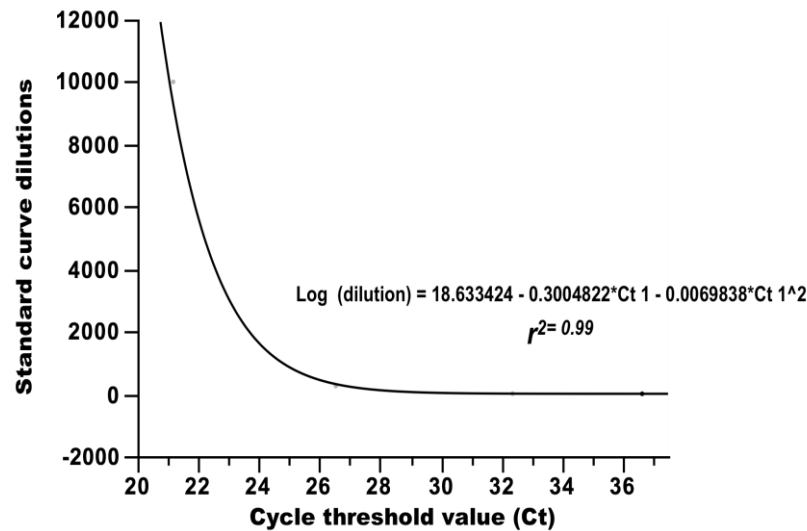
Fig 3.1: Example of a transformed fit log-quadratic curve of standard curve dilution of gDNA extraction of whole body of male An. gambiae plotted against their Ct values of to generate a quadratic equation for sperm copy number calculation.

3.2.7 Repeatability test of the qPCR method for sperm number quantification

It is important that an individual obtains similar results from an experiment involving repeating measurements of same parameter with the same method given that the conditions are identical (Bailey *et al.* 2007; Bartlett and Frost 2008). Dissimilarity in the results might be because of reasons like intrinsic distinctiveness of the instruments used and not due to poor accuracy in the method or observer (Petrie and Sabin 2005). Thirty gDNA samples extracted from the female spermathecae were utilized to test the repeatability of the qPCR method for sperm number quantification. The qPCR reaction mix was prepared as described previously with two independent replicates of the standard curve dilutions. All were loaded on the same plate and thermo cycled on AppliedBiosystem StepOnePlus Real Time Thermal Cycler. The best-fit log-quadratic equation generated with the standard curve (see above) was then used to translate Ct values into their sperm number equivalent. The entire process was replicated a second time under the same conditions and the Ct values and sperm copy number from both plates

plotted against one another. Pearson's correlation coefficient (r) was used to measure the repeatability of the method (Vaz *et al.* 2013).

3.3 Results

3.3.1 Reliablity of sperm quantification technique

There was a significant relationship between the two Ct values (Linear regression: n= 30, T= 11.2, $P < 0.001$). The Ct 1 value plotted against the Ct 2 values showed a strong positive correlation between the two values (Pearson's correlation coefficient: n= 30, r= 0.90, $P < 0.001$) (Fig 3.2). The same analysis was conducted on the estimated sperm number after transformation of the Ct values into their sperm number equivalent based on the standard curve dilution equation. A significant linear relationship was found (Linear regression: n= 30, T= 15.7, $P < 0.001$) and the correlation coefficient showed a strong agreement between both plates (Pearson's correlation coefficient: n= 30, r= 0.91, $P < 0.001$). The sperm copy number estimated in the first plate was strongly correlated to the sperm copy number estimated in the second plate (Fig 3.3). The strong agreement between the repeated measures confirms that our qPCR sperm number estimation were significantly repeatable under the similar condition.
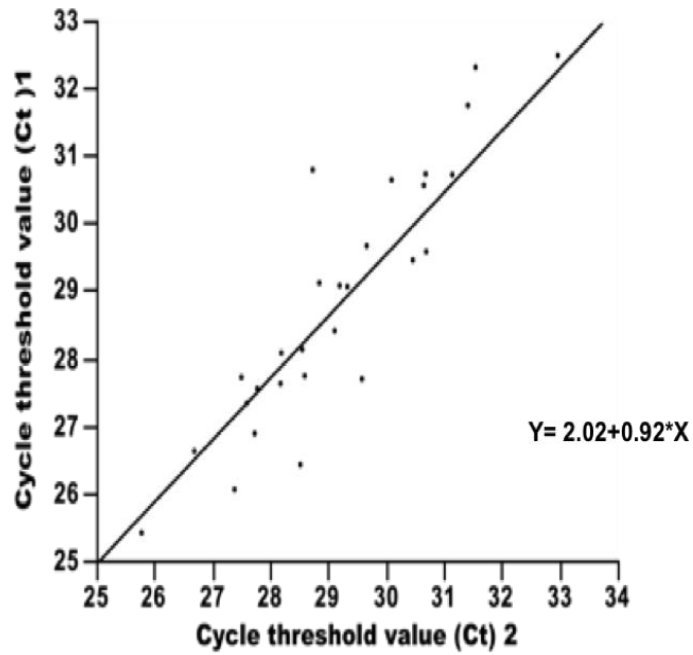
Fig 3.2 : Plot of the Cycle threshold value (Ct 1) against the repeated measure Cycle threshold (Ct 2) of gDNA extracted from female spermathecae of *An. gambiae* for the estimation of sperm number transferred to female during mating (Linear regression: n= 30, P < 0.001).
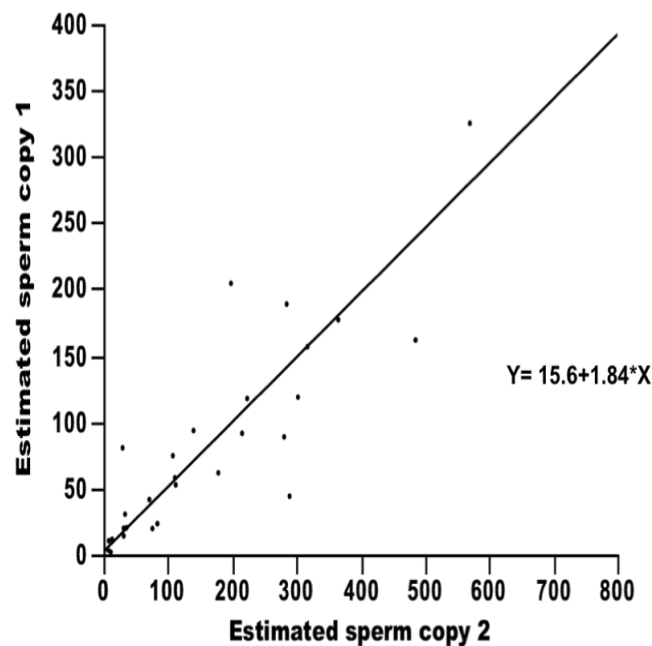
Fig 3.3: Plot of the estimated sperm number from plate 1 against the repeated measure estimated from sperm number plate 2 based on 2 μl from the gDNA extraction from sperm on. Ct values were transformed based on standard curve dilution of quadratic equation, to get their respective sperm number equivalent (Linear regression: n= 30, P < 0.001).

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3.3.2 Estimation of sperm numbers from field-captured M and S molecular forms of

An. gambiae

The overall, mean estimated sperm numbers in Kela 2006 females was 2849 (95% CIs= 1975-3724). The mean sperm numbers estimated in the M-molecular females was 2427 (807-4046) and mean sperm numbers estimated in the S-molecular form females was 3120 (2002-4238) (Fig 3.4). There was a wide variation in the frequency distribution of sperm numbers between the M and S molecular forms (Fig 3. 5), but no significant difference was found between the estimated sperm numbers in the two forms (ANOVA: n= 125, $F_{1,125}$= 0.05, P= 0.820). The qPCR quantification of sperm content in the spermathecae revealed 99% insemination rate.
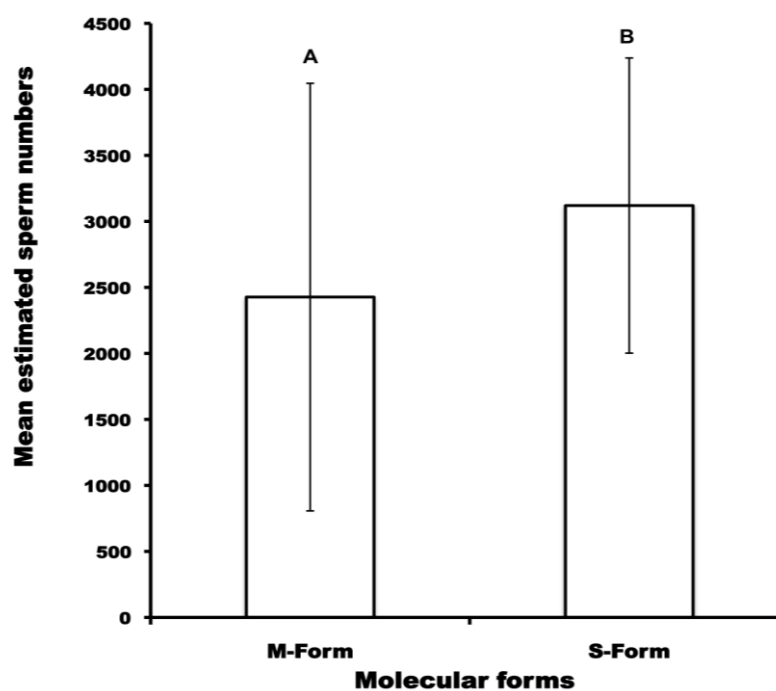
Fig 3.4: Mean estimated sperm number in the female spermathecae of M and S molecular forms of field-captured *An. gambiae s.s.* Different letters show significant difference.

M-molecular form
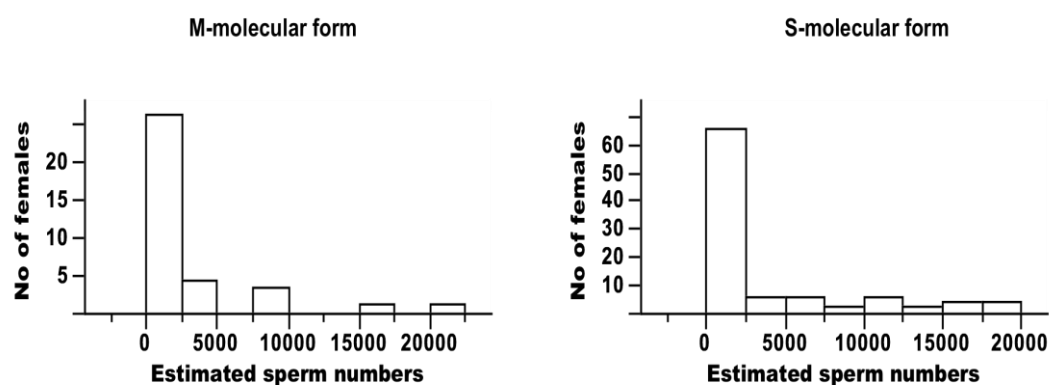
S-molecular form

Fig 3.5: Frequency distribution of estimated sperm numbers in the spermathecae of female M and S molecular forms of field captured *An. gambiae s.s*

3.3.3 Female body size

The mean female body size for M forms was 2.86mm (2.80-2.93) and for S form was 2.92 (2.88-2.96) (Fig 3.6). Overall, no significant correlation was found between the female body size and the log-estimated sperm numbers in the whole dataset (Linear regression: n= 125, T= 0.86, *P*= 0.390), or within each form (*P* > 0.73 in both cases) (Fig 3.7).

M-molecular form
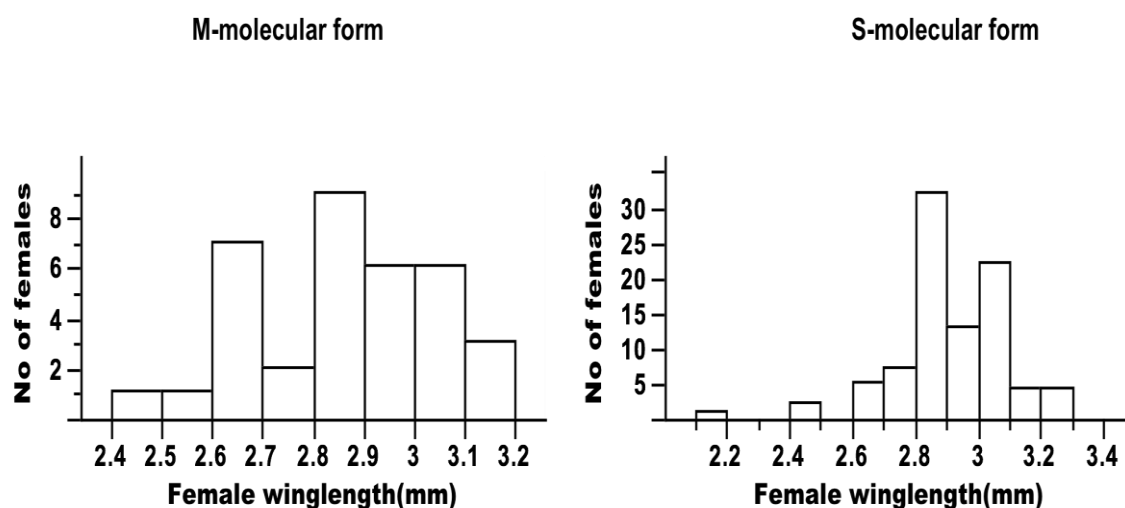
S-molecular form

Fig 3.6: Frequency distribution of female body size (winglength measurement) of M and S molecular forms of field captured *An. gambiae s.s*
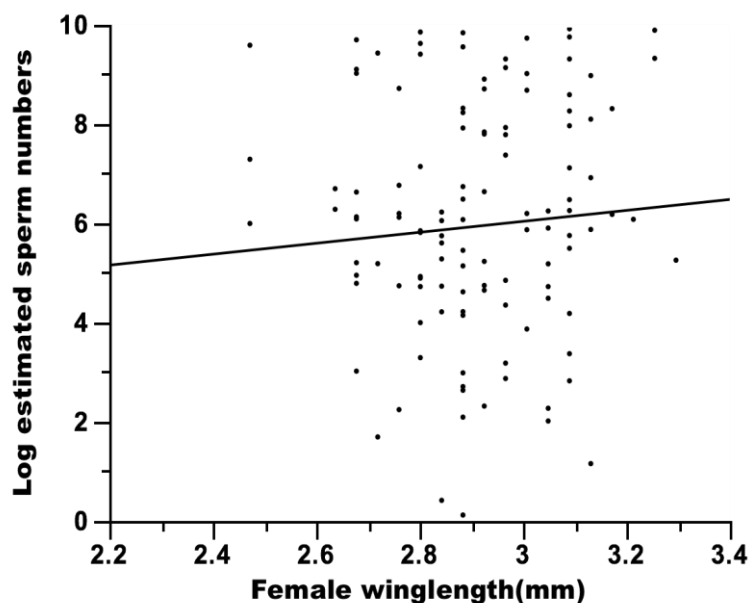
Fig 3.7: Correlation between Log-estimated sperm numbers and female body size of the field captured *An. gambiae s.s*

3.3.4 Identification of Molecular forms

Overall, 132 females were characterized; the PCR molecular identification, analysis revealed 26.5% of the females to be M-molecular form, 68.2% S-molecular form, and 5.3% were unidentified (Fig. 3.8).
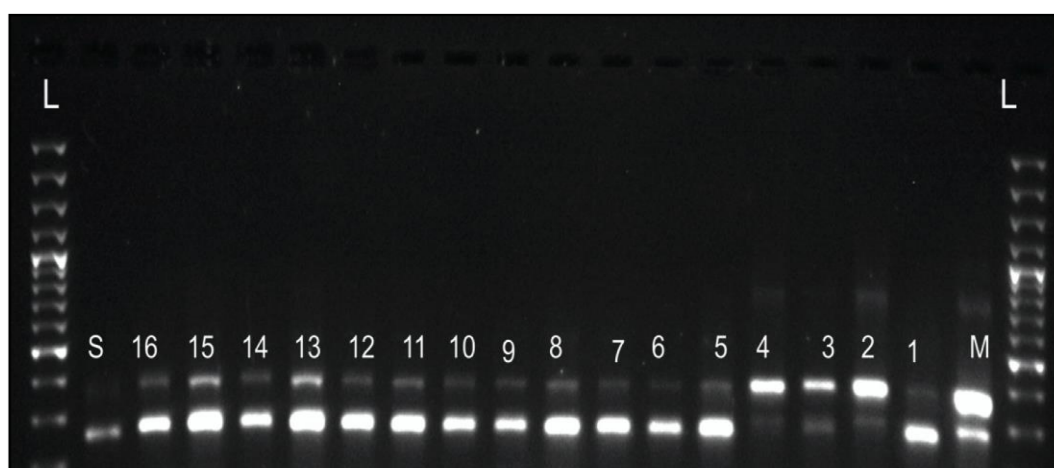
Fig 3.8: A sub sample of PCR molecular identification of field-captured female Kela 2006. The PCR product from female body of Kela 2006 *An. gambiae* was electrophoresed in 1.8% agarose gel L= 100bp DNA weight marker. M= control for M-molecular form. S= control for S molecular form. Wells 2,3 and 4= M-forms, and wells 1 and 5-16= S-form.

3.4 Discussion

In this study, we developed a new technique to quantify sperm numbers using a qPCR based on TaqMan assay. We used the technique to detect mating frequency in field captured females of *An. gambiae s.s* and observed that there was a very high frequency of mated females of M and S caught inside the huts in the Kela area of Mali. We applied the technique to quantify sperm numbers in the spermathecae of the field caught M and S molecular forms of Kela 2006 and we found a huge variation in the sperm content in the female spermathecae. This variation might be because of many undetermined factors such as male age, first mating, or even sperm size.

Sperm transfer or retained in the male and female organs across many organisms has been counted using various methods (Holman 2009). Most of these methods have downsides either in the preparatory process before the sperm counting or during the actual counting, which eventually results in inconsistency in the results (Holman 2009). Dissecting the sperm storage organ and using the appropriate type of physiological buffer is one challenge posed by these methods (Reichardt and Wheeler 1995). More so, fixing and counting the sperm is time consuming and laborious and sometimes difficulties in preparing a homogeneous spread and large sampling error can lead to rough estimates (Tram and Wolfner 1999). New qPCR based on TaqMan Probe circumvents some of the limitations of other sperm counting methods. It is not laborious, and is not dependent on particular physiological buffers. Most importantly, it allows for the quantification of all sperm content of the spermathecae thanks to the use of Y specific primers and using a Probe Y with high specificity. The spermathecae with very little or no sperms in them were equally detected as the qPCR requires very minimal starting template (Bustin 2000). Furthermore, our method has an advantage over the quantitative PCR method used by Tram and Wolfner (1999) for determining sperm numbers, there was no need for gels. This

qPCR assay can be applied to further assays where quantitative quantification is necessary. A strong positive agreement was found between the repeated measures of the same samples under the similar condition, which confirms that our qPCR sperm numbers quantification was reliable. It has been recommended that values above 0.90 be regarded as high correlation, while 0.80 is seen as moderate, but values below that are not good enough or poor (Vincent *et al.* 1999). Here, repeatability was equivalent to 0.95 suggesting good reliability of the technique. In most subsequent studies (see Chapters 5), we nevertheless measured sperm amounts through two independent runs.

The use of the PCR method to observe mating status in *An. gambiae* has been shown previously. Tripet *et al.* (2001) used PCR to detect cross mating in field-collected females of M and S forms from the same area in Mali. Ng'habi *et al.* (2007) used quantitative PCR with Y specific primers to differentiate between mated and virgin females of *An. gambiae* and the sibling species *An. arabiensis*. Utilizing our qPCR assay with both Yprimers and Probe Y, we observed a very high-mated frequency in the females caught inside houses (99%). Mating induces behavioural changes such as host-seeking behaviour in the females of *An. gambiae s.s* (Gillott 2003; Baldini *et al.* 2013). The presence of a large number of mated females inside and round living quarters suggests that they were there to take a blood meal, which they need for egg development. This is consistent with other findings that host seeking behaviour is one of the post-mating changes displayed by mated females of the *An. gambiae* (Baldini *et al.* 2013).

We quantify sperm numbers from the female spermathecae, despite the fact that the female samples had been kept in ethanol for about 6years. We found that the average amount of sperm in the spermathecae did not differ within the molecular forms. Due to the unavailability of practical tools for sperm quantification, no published data were available for comparison from past field or lab studies in *An. gambiae s.s* at the time of this study. In

An. arabiensis there are no data available from spermathecae, but Helinski and Knols (2008) measured sperm number in the testis by manual counting under a dark view microscope. They found $8214\pm467SE$ in 12-day old males and $5022\pm375SE$ in 3day old males. In *Aedes aegypti*, the mean number of sperm was counted from the spermathecae of field females using a phase contrast microscope, and the sperm number observed ranged from 1154 to 1892 in the spermathecae of females mated by 1-10 old males (Ponlawat and Harrington 2009). This was less than the overall mean sperm number (2849) that was quantified from the spermathecae of Kela females.

Huge variations in sperm number was observed in the spermathecae of the females. It is not clear why there is this wide variation. Natural variations in the number of sperm found storages organs of different mosquito species have been documented (Ponlawat and Harrington 2007; Helinski and Knols 2009). These variations have been be linked to both ecological and biological factors like male age and size. Effect of age on male reproductive fitness is a biological occurrence documented across a wide variety of organisms (Ponlawat and Harrington 2007). In *Ae. aegypti* older males produce and store more sperm and consequently transfer more sperm numbers to their female mates (Ponlawat and Harrington 2007, 2009). In *An. arabiensis* older males also produced a larger sperm number in their testes than younger males (Helinski and Knols 2009). It might be that some of the Kela females were mated with older males result in large sperm content in their spermathecae. On the contrary, in the Mediterranean fruit flies, the number of sperm stored by the female is dependent on the age of the male mate, females mated with older males store fewer sperm amount than those mated with younger males (Taylor *et al.* 2001). Naturally, there are variations in the age of the males as well as females in the field, and we have no idea of the age of the females or their male mates. So if the male age affects sperm uptake and storage in the females, it could be that females here may have been

mated with older males with large sperm transfer, but retained just few, which result in the variation in the sperm amount quantified.

Multiple mating caused depletion in the level of ACPs in fruit flies (Bloch- Qazi and Wolfner 2003). Females mated with males with depleted ACPs stored few sperm even though they received the usual amount of sperm (Tram and Wolfner 1999). In the *An. gambiae* sperm uptake and preservation, by females require sufficient transfer of MAGs that will help them to store the sperm very well (Roger *et al.* 2009; Catteruccia 2009). Perhaps, some of the females were mated with males that transferred insufficient amount of MAGs to assist them store more sperm. More so, in the laboratory larger males of *An. gambiae* can inseminate up to 10 females per night (Okanda *et al.* 2002). The females with few sperm content could have been mated with males that have had several mating in one night, resulting in low sperm transfer. In field multiple mating and cross mating have been reported to occur in a 1.2% of *An. gambiae* M and S the population (Tripet *et al.* (2001). This could also influence the sperm numbers. If the initial mating was from male of a different molecular form, the female will most likely re-mate with males from its own form (Tripet *et al.* 2003). The second mating would invariably increase the quantity of sperm content in the spermathecae of the female. Egg laying could be another possible explanation for the wide sperm number variation. There is a possibility that some of the females caught have already used some of the sperm for fertilization of their eggs and so the sperm quantified were just left over from the fertilization process.

Male sperm investment maybe another factor that influenced sperm variations in the study group. Ejaculate size depends on the mating chances the males have during the mating period (Wedell *et al.* 2002). The variation in the sperm number here might be as a result of males with less mating chances just wanting to give it all at the only available opportunity they have. Secondly, it has been demonstrated in other species that male

reproductive investment can be influenced by the size of the female, i.e. male allotting more sperm to bigger females than smaller ones. If males allocate sperm according to the female quality, they would increase investment in the first mating in females with superior quality (Reinhold *et al.* 2002; Scarf *et al.* 2013). These suggest that the females with very high amounts of sperm numbers might have been first mating or virgin females (Reinhold *et al.* 2002; Ponlawat and Harrington 2007). Despite the huge overall variation observed in the sperm numbers quantified from the spermathecae of these field caught female, there was no difference in sperm number variations in the two molecular forms.

In conclusion, the results suggest that the qPCR technique is reliable under comparable conditions and can be used to detect sperm numbers from close to zero numbers to thousands of numbers. It can facilitate studies on reproductive ejaculate size without the fear of the long process involved in organ dissections and sample preparations. Long time preservation would not be a barrier in the sperm number estimation. Thus, the technique can be implemented in both fresh and stored samples. Huge sperm variations observed in the study group could be explained by a whole lot of undetermined factors such as male age, female quality and number of mating.

Chapter 4

Effects of hydric stress on sperm investment in laboratory reared males of *An. gambiae s.s*

4.1 Introduction

Adult mosquitoes are challenged with diverse problems as they emerge. Desiccation is one such major problem, mosquitoes hardly ever have regular access to water because water availability is seasonal in a wide variety of environments. Water deficits in their tissues can result in stress that may interfere in their physiological processes (Aboagye-Antwi and Tripet 2010). The accessibility of water during the adult stage of male and female mosquitoes is very fundamental in enabling them to cope with dehydration trauma. For instance, adult females of *An. gambiae* reared in constant contact with water prior to a severe desiccation challenge resists that challenge better than those reared with partial access to water (Aboagye-Antwi and Tripet 2010). The abundance and geographic distributions of the M and S molecular forms of *An. gambiae s.s* is affected by seasonal changes (Favia *et al.* 1997; Toure *et al.* 1996). In Mali, S molecular forms correspond to Savanna and Bamako chromosomal forms while the M molecular form corresponds to the Mopti chromosomal form (Lee *et al.* 2009). Mopti M form is more flexible to environmental changes than the S forms (Toure *et al.* 1994). For instance, in Mali, where the two forms occur in sympatry the Mopti M form widens its habitation from the humid savannah in southern Mali to the Sahel and South-Saharan areas. It also has the ability to reproduce in the rainy season as well as in dry season wherever larval breeding sites are accessible. This flexibility in environmental adaptation found in the Mopti forms has been ascribed to its 2R inversion polymorphism (Toure *et al.* 1994). The abundance of a particular type of chromosomal inversion arrangement depends on prevailing season. The a

and bc chromosomal inversion occurs in high frequency amongst the Mopti forms of *An. gambiae* with relatively high abundance in the dry season. Whereas Savanna and Bamako S forms without these inversions (a and bc) are better adapted to wetter regions in Mali (Touré *et al.* 1998). There has been evidence suggesting that the M molecular form of *An gambiae* aestivates (summer diapause) to survive the long dry seasons in the Sahel, while the S forms and the *An. arabiensis* migrates and returns after the first rains to reestablish their population (Lehmann *et al.* 2010; Huestis *et al.* 2011, 2012).

In the laboratory, desiccation affects the *Anopheles* species differently. Two sibling species *An. arabiensis and An. gambiae s.s,* were exposed to the same level of desiccation challenge, the result showed that *An. arabiensis* have significantly higher desiccation resistance than the S form of *An. gambiae s.s* (Gray and Bradley 2005; Lee *et al.* 2009). The differences in desiccation resistance in individuals are dependent on factors affecting individual's phenotypic quality (morphological, and behavioural) (Aboagye-Antwi and Tripet 2010). More so, biochemical and physiological properties (bulk water and catabolism of lipids and glycogen) are very essential to desiccation resistance and these are dependent on body size of the mosquito (Aboagye-Antwi and Tripet, 2010). Desiccation affects males and females of *An. gambiae,* but the females survive better than the males regardless of the molecular form (Lee *et al.* 2009). Females of M molecular form can survive up 50h without food or water under dry condition (Lee *et al.* 2009).

Abundant information focusing on desiccation resistance in other insects such, as *Drosophila melanogaster* is available, but very little has been documented regarding similar cases in *An. gambiae* (Gibbs 2002: Gibbs and Vanier 2004; Archer *et al.* 2007). The few available studies on *An. gambiae,* has not been able to explain fully the physiological effects of hydric stress (Aboagye-Antwi and Tripet, 2010). Therefore, it is important to gain a better understanding of the degree to which desiccation affects the

reproductive process in the laboratory-reared mosquitoes as environmental stress like hydric deficits might result in fitness disadvantage.

Decrease in survival and subsequent reproduction because of present reproduction trade-off is charged to cost of reproduction (Dao *et al.* 2010). Mating and or sperm allocation has been shown to be costly for males. It increases their mortality and decreases their chances of future reproductive success (Cordts and Partridge 1996; Dao *et al.* 2010; Caballero-Mendieta and Cordero 2013). In many insects, the amount of reproductive ejaculate is a function of male fitness (Caballero-Mendieta and Cordero 2013). As male mate frequently, their sperm become depleted and insemination rate drops (Scharf *et al.* 2013). In insect with sperm rivalry, like beetles and *Drosophila*, males are aware of the risk encountered by their sperm and invest a lot more to give their sperm some fertilization chance (Wedell *et al.* 2002). Under stress or harsh environmental conditions, reproduction cost could be influenced negatively or positively. For instance in the beetle *Callosobruchus maculatus*, harsh conditions like heat shock stress results, in 19% reduction in male ejaculate size (van Lieshout *et al.* 2013). In butterfly, smaller males of *L. aripa* produce large ejaculated size despite the longevity cost of them because transferring large ejaculate size has a positive effect on female fecundity (Caballero-Mendieta and Cordero 2013). Stress response aids the organisms to activate energy to combat or break away from a danger in so doing increasing its probability of survival. Overcoming an immediate danger using all the available resource results in directing resources away from areas not directly concern with present risk (Selye 1975). Perhaps, under hydric stress condition that is likely to have a negative effect on survival and hence affecting future reproduction. We assumed that *An. gambiae* males if stressed might invest more in their current reproduction than future reproduction, because more investment in current reproduction might be the strategy that will maximize their lifetime reproductive success.

In *Callosobruchus maculatus* females under water stress mate more frequently to obtain water (Edvardsson 2007). Meaning that females chose to mate more with males that will transfer larger ejaculates for them to acquire the water they need for survival. Females of *An. gambiae* under similar stress condition might become more open to sperm uptake than usual for survival.

In dry season, adult M forms of *An. gambiae s.s* witness-reduced reproduction to survive aestivation (Huestis *et al.* 2011; Yaro *et al.* 2012; Lehmann *et al.* 2014). The number of females experiencing gonotrophic dissociation increases from 5% to 45% over the dry season oviposition reduces from 70% in wet season to 20%, and egg batch drops from 173 to 101 despite the availability of water (Yaro *et al.* 2012). Nevertheless, insemination rate and blood feeding are the same in both seasons (Yaro *et al.* 2012). This situation suggests that mated *An. gambiae* females under stress might trade reproduction for prolonged existence, i.e. keeping the sperm for nourishment and maintenance of the body and not for reproduction.

Sperm plays a very fundamental role in male reproductive success and increase in sperm number is regarded as a significant part of reproductive success many in insects, (Baer and Boomsma 2004). Several studies have counted sperm numbers stored and transferred to female insects using methods like haemocytometer (Keller and Passera 1992), fluorometer (Reichardt and Wheeler 1995), and dead and live sperm viability kits (Bernasconi *et al.* 2002; Hunter and Birkhead 2002; Twig and Yuval 2006; Nakahara and Tsubaki, 2007; Radhakrishnan and Fedorka 2011). In *An. gambiae s.s*, information on the sperm number is scarce, and published data are not available on hydric stress and its implications on reproductive investments. Here, we investigated the effects of pre-mating hydric stress on male sperm investment. The males of *An. gambiae* were subjected to hydric stress for several hours before being combined with their non-stressed females for

an overnight mating. In a reciprocal experiment, females were exposed to the same pre-mating stress condition before overnight mating with non-stressed males to study the female strategy in terms of sperm uptake under stress. The sperm transferred to the female spermathecae were quantified using the newly developed qPCR assay because the existing sperm counting techniques do not generate precise estimates of the sperm number (Holman 2009). In a different study, we examined the impact of partial access to water on sperm activity and storage in females stressed for several hours daily for 7 days after mating. It is anticipated that results from this study would reveal important trade-offs between hydric stress and sperm investment and maintenance in males and females of *An. gambiae*. Such trade-offs could explain some of the extensive variation in spermathecal sperm content observed in natural populations (Chapter 3). In addition, these studies might highlight important novel aspects of male reproductive biology with important implications for vector control programmes in which male competitiveness and sperm transfer play an important role.

4.2 Materials and Methods

All adult males and females used in this experiment were 3-4-day old. They were reared based on the first larval feeding regime described in Chapter 2. All individuals were sexed as pupae and kept in separate cages as virgin male and female adults under standard insectary conditions (see Chapter 2).

4.2.1 Sperm transfer in hydric-stressed males

A first experiment was conducted to test the potential effects of hydric stress experienced by males prior to mating on their reproductive investment in terms of the sperm transferred to females and insemination rates. Virgin Mopti males were transferred to 3 mating cages

(50 per cage) and either kept under normal conditions or stressed for 4hrs and 8hrs. The stress involved replacing the 5% glucose solution in the cages with 2 sugar cubes and the wet cotton wool pads on top of the cages with dry ones for 4h (2pm-6pm) or 8h (10am-6pm) respectively prior to mating. At the end of the stress period, each experimental male group was combined with 100 virgin non-stressed Mopti females for overnight mating. The rationale for the excess of females (1:2 ratio) was to create variations in the male overnight insemination rates under stress. The next morning, all the females were collected and stored in 75% ethanol for dissection later. Two replicates of the experiment were conducted with independently reared mosquito cohorts. Overall, 900 adult mosquitoes were used (300 males and 600 females) (Table 4.1).

Table 4.1 Overnight-mating combinations of virgin Mopti males of *An. gambiae* exposed to pre mating hydric stress for 0h, 4h and 8h.

Cages	Stress duration	Mating combination
A1 and A2	4h	50 stressed males x 100 non-stressed females
B1 and B2	8h	50 stressed males x 100 non-stressed females
C1 and C2	0h	50 non-stressed males x 100 non-stressed females

4.2.2 Sperm uptake in hydric-stressed females

A reciprocal of the male hydric stress experiment was conducted to assess the effect that hydric stress no sperm uptake in female prior to mating. The setup was identical to the stress male. Virgin Mopti females were transferred to 3 mating cages (100 per cage) and were either kept under normal conditions or stressed by swapping the 5% glucose solution inside the cage with 2 sugar cubes and replacing the wet cotton wool pads that on top of the cages with dry ones for 4h (2pm-6pm) or 8h (10am-6pm) respectively. Each group of

females were later combined with 50 non-stressed virgin Mopti males for overnight mating. Two cages of the experiment were conducted with independently reared mosquito cohorts. Overall, 600 females, and 300 males were used to set up 6 mating cages, three per treatment group (Table 4.2).

Table 4.2: Overnight-mating combinations of Mopti females of *An. gambiae* for pre mating hydric stress for 0h, 4h and 8h.

Cages	Stress duration	Mating combination
A1 and A2	4h	100 stressed females x 50 non-stressed males
B1 and B2	8h	100 stressed females x 50 non-stressed males
C1 and C2	0h	100 non-stressed females x 50 non-stressed males

4.2.3 Sperm storage and vigor in hydric stressed females (post mating hydric stress)

To investigate the effect of hydric stress on sperm storage and sperm activity, Mopti females mated overnight were given limited access to water for the next 7 days. To do so, 50 virgin Mopti females were combined with 100 virgin Mopti males for overnight mating in a cage. The rationale for the excess male (ratio 2:1) was to ensure that all the females were mated before the next morning. The cages were provided with 5% glucose solution for food and wet cotton pad for water. Following a successful overnight mating, the next morning the males were removed from the cages. The stress was carried out by replacing 5% sugar solution with 2 sugar cubes and swapping the moist cotton pads with dry ones on top of each cage for 4h (2pm-6pm) and 8h (10am-6pm) respectively (Table 4.3). The exchange was made at exactly the same time daily for 7 days. Two replicates of the whole experiment were conducted with independently reared mosquito cohorts

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Table 4.3: Overnight mating and stressing cages for Mopti females of *An. gambiae*. After the overnight mating, the males were removed and females stressed daily for 0h, 4h and 8h for 7days.

Cages	Mating Combinations	Daily post-mating stress in females (hr)
A1and A2	100 non-stressed males x 50 non-stressed females	4hrs
B1and B2	100 non-stressed males x 50 non-stressed females	8hrs
C1and C2	100 non-stressed males x 50 non-stressed females	0hrs

4.2.4 Spermathecae dissection and determination of sperm activity

On the morning of day 7, subsamples of females were collected for sperm activity. They were transferred into a small polystyrene pot covered with a mosquito net and fastened with elastic rubber. For the dissection, a random subset of 6 females was obtained from each treatment cage and placed in another small-netted polystyrene cup of ice to knock them down partially. To prevent possible bias, subsets were taken consecutively from each treatment group and the order of treatment groups reversed after each cycle of 18 dissections. More so, researchers carrying out the dissections and sperm activity scoring were blind with regard to which treatment group was treated at a particular stage. To make sure that sperm activity might be recorded within 2 minutes from the start of the dissection, two researchers always performed the dissection, and sperm activity scoring. The dissection was performed at room temperature under a binocular microscope (Leica Microsystems GmbH. Solms, Germany) with dim light to prevent heating up the sperm prior to measuring its activity. The sperm activity was video-recorded for one minute using a Motic images plus camera (Motic China Group Co. Ltd). Then saved as a QuickTime movie on a MacBook Laptop Computer (Apple Inc.) (See full details in chapter 2). The

median sperm activity at the end of 1min observation was used in statistical analyses. The female bodies were preserved in another tube for winglength measurement.

4.2.5 Spermathecae dissection of stored samples

Stressed males and females (stress male reverse) samples were preserved in ethanol. The dissection was done following the protocol for stored samples as described in chapter 2, but further dissection of sperm bundle was performed to determine mating status. As soon as the spermatheca was dissected out, it was transferred to another slide with a clean drop of PBS solution. The spermatheca was pushed gently to the edge of the solution. Using the dissecting pin, the spermathecal capsule was squeezed gently from both sides to break it open. Sperm when store in ethanol coagulates (Tripet *et al.* 2001). Sperm bundle of mated females was picked up with the dissecting pin and transferred to a 1.5ml centrifuge tubes (containing 500µl of lysis buffer plus 5µl of protease K). The mix was incubated overnight on a heat block (55ºC) and subsequently gDNA extraction the next morning. The body of the females was preserved in another tube for winglength measurement.

4.2.6 gDNA extraction for sperm quantification

The genomic DNA (gDNA) extraction from the spermatheca for both stored and fresh samples was made using the ChargeSwitch gDNA micro tissue kit (Invitrogen). The manufacturer's protocol was applied with slight modification on the volume (see Chapter 2). The extracted gDNA was transferred to permanent DNA storage tubes with screw caps and stored at -20 ºC for sperm number quantification. The gDNA used for the standard curve dilution were also extracted the same way from the whole body of Mopti males. The concentration and DNA purity was determined using the Nanodrop 1000 spectrophotometer. Samples with A260/A280 below 1.8 were regarded as contaminated. A

mixture was made from uncontaminated samples and was used for the standard curve dilution (3 separate tubes mixed together).

4.2.7 qPCR sperm quantification

Sperm quantification using qPCR method was conducted on the samples (stored and fresh). A subset of samples (20 females from each cage) per treatment group in the 3 different experiments was taken. Each qPCR reaction tube (see Table.4.4) for sperm number quantification was based on the TaqMan Universal Master Mix II (AppliedBiosystem) and prepared in conformity with the AppliedBiosystem guidelines (Chapter 3 for details).

Table 4.4: Details of the qPCR mix per one reaction tube for sperm quantification of gDNA extract from female spermathecae of *An. gambiae*.

Reagent	Reaction volume (µl)
TaqMan Universal Master mix II	10
TaqY forward primers	1.5
TaqY reverse primers	1.5
Probe -Y	1.25
Water	3.75
gDNA template (sample)	2
Total	20

Two independent standard curve dilutions were prepared as described in Chapter 3 and loaded together with the samples on a MicroAmp optical 96-well PCR reaction plate. The PCR plate was sealed with MicroAmp optical adhesive film and centrifuged at 2000rpm for 4min at 4ºC. The PCR reaction plate was thermo cycled following the AppliedBiosystem' TaqMan assay settings on AppliedBiosystem StepOnePlus Thermal

Cycler (Table 4.5). At the end of 40 cycles, the cycle threshold values (Ct) of both samples and the standard curve generated were exported to the JMP 10.0 software for statistical analyses.

Table 4.5: Temperature duration and number of cycles used for qPCR sperm quantification of gDNA extract from the female spermatheca of *An. gambiae*.

Stage	Temperature (ºC)	Time (minutes)	Nber cycles
1	50	2	1
2	95	10	40
3	95	0.15	40
4	60	1	40

4.2.8 Statistical analysis

All data were inspected for normality in the distribution and heterogeneity of variance. The data with normal distribution were analysed parametrically. The mean differences were compared and displayed as bar plots. Mean are presented with 95% confidence intervals throughout. The data that deviated from normal distribution was analyzed non-parametrically and displayed as box plots. Where medians are used they are presented with lower whiskers constructed as 25th percentile -1.5* interquantile range and upper whiskers at 75th percentile + 1.5* interquantile range. Cage effect within experimental groups were tested for statistical difference, but is only reported when significant. All analyses were carried out using JMP 10 software (SAS Institute, Inc. USA).

4.3 Results

4.3.1 The effect of pre-mating hydric stress on sperm transferred by males

Randomly selected subsamples of 40 females were dissected in each of the 3 treatment groups (0h, 4h and 8h) and analyzed for the effect of stress on sperm transfer. There was a significant main effect of treatment and replicate (ANOVA: treatment: n= 119, $F_{2,118}$= 10.71, $P < 0.001$; replicate: $F_{1,118}$= 28.87, $P < 0.001$). There was a significant effect of interaction between treatments and the quantity of sperm transferred in the two replicates ($F_{1,118}$= 8.46, P= 0.004). A post-hoc Tukey test performed on the least square means, showed that the 8h stressed males transferred significantly more sperm numbers than 0h males (Tukey: n= 79, $P < 0.001$) and 4h stressed males (Tukey: n= 79, P= 0.003). The mean sperm number transfers by 8h stressed male was 10690 (95% CIs: 12568-8201), 4h stressed male transferred 5171 (3219-7530), and the 0h stressed male transferred 2857 (737-4983) (Fig.4.1).
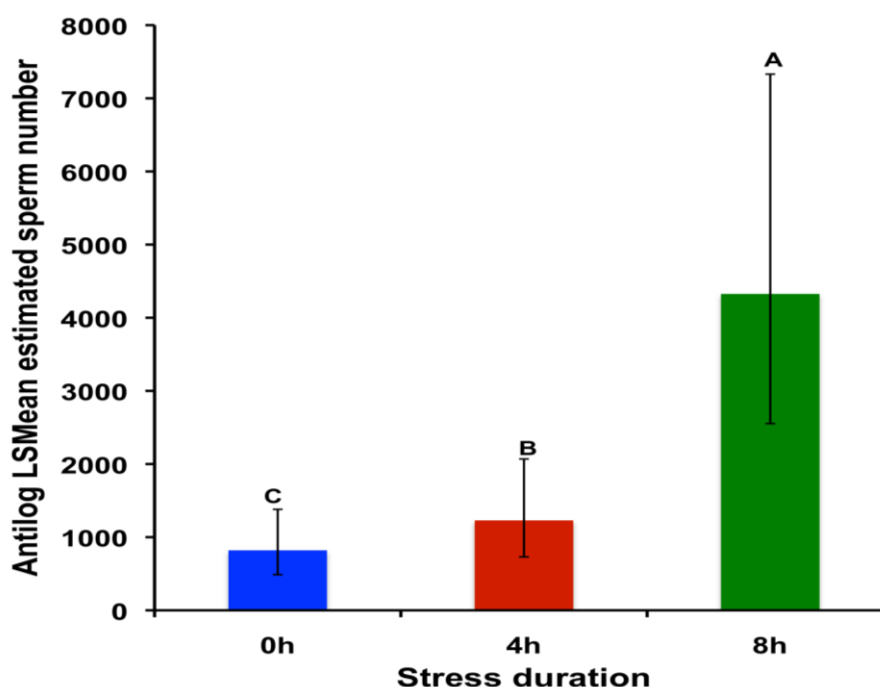
Fig 4.1: Antilog LSMean estimated sperm number transferred by Mopti males stressed for 0h, 4h, and 8h in an overnight mating with non-stressed Mopti females. Each error bar was constructed at 95% confidence interval of the mean. Different letters represent significant difference.

The frequency of mated and unmated females in the 3 treatment groups showed that 52.6% of the females dissected were mated and 47.3% were unmated. There was no significant difference between the frequency of mated and unmated females in all the treatment groups (Pearson Chi-square: n= 120, χ^2= 12.00; P= 0.290). Overall, no significant linear relationship was found between the female body size and log mean estimated number of sperm transferred (regression: n= 119, T= 1.73, P= 0.110) (Fig 4.2). The overall mean female body size was 3.02mm (95%CIs= 2.99-3.04).
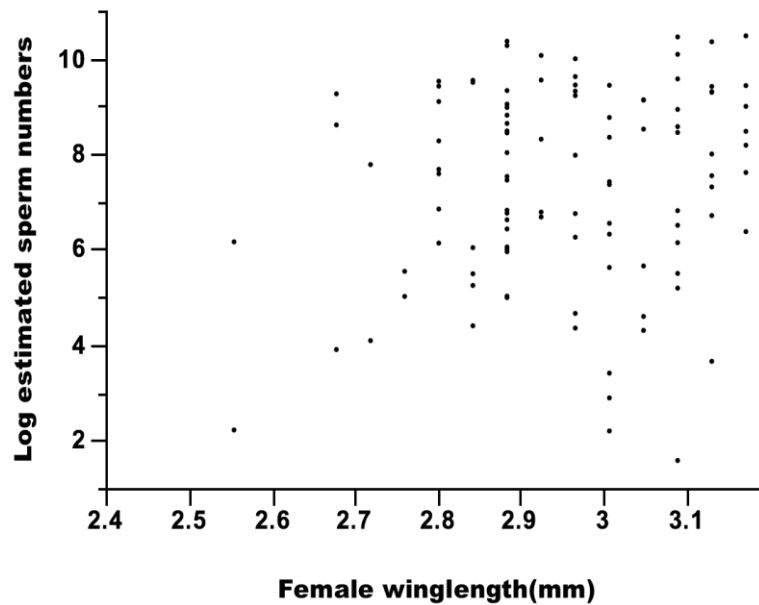
Fig 4.2: Relationship between female body size and mean estimated sperm number in females mated by stressed males.

4.3.2 Effect of pre-mating hydric stress on sperm number uptake by females

There was no effect of pre-mating hydric stress on sperm uptake by females in all treatment groups and replicates (ANOVA: treatment: n= 119, $F_{2,118}$= 1.67, P= 0.190; replicate: $F_{1,118}$= 0.92, P= 0.330). The mean sperm numbers in the spermathecae of the 0h stressed females were 3087 (95% CIs: 1655-4519), the 4h stressed females was 4845 (2603-7187) and 8h stressed female was 4658 (3168-6148).

Overall, the mean female body size was 3.01mm (2.98-3.03). The frequency of mated and unmated females between the treatment groups revealed no significant difference (Pearson Chi-square: n= 120, χ^2= 12.00, P= 0.290). The 0h stressed treatment group had 64% of unmated females and 34% of mated females, the 4h and 8h had 55.5% and 52.5% unmated females with 40.5% and 44% mated respectively.

4.3.3 Effect of 7d post-mating hydric stress on sperm activity in the spermatheca

Subsample of 40 females from each treatment group was dissected for sperm activity (Fig 4.3) and sperm quantification after 7 days stress. Insemination rate in the stress females was 100% in all groups. The 7 days stress revealed no significant effect on sperm activity (Kruskal-Wallis: n= 120, df= 2, χ^2= 4.82, P= 0.091). This was true for both replicates (Kruskal-Wallis: $P > 0.200$ in both cases). The median sperm activity for 0h treatment was 4 (25th- 75th interquantile range = 2-4), 4h was 4 (3-4), and 8h was 3(1-4) (Fig 4. 4).
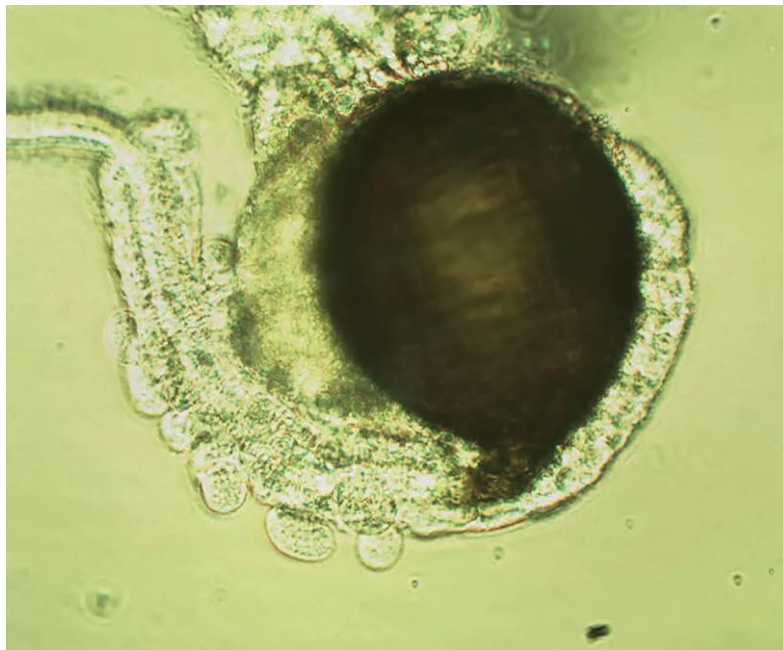
Fig 4.3: The movement of sperm bundle in the freshly dissected spermathecae of mated female *An. gambiae s.s*
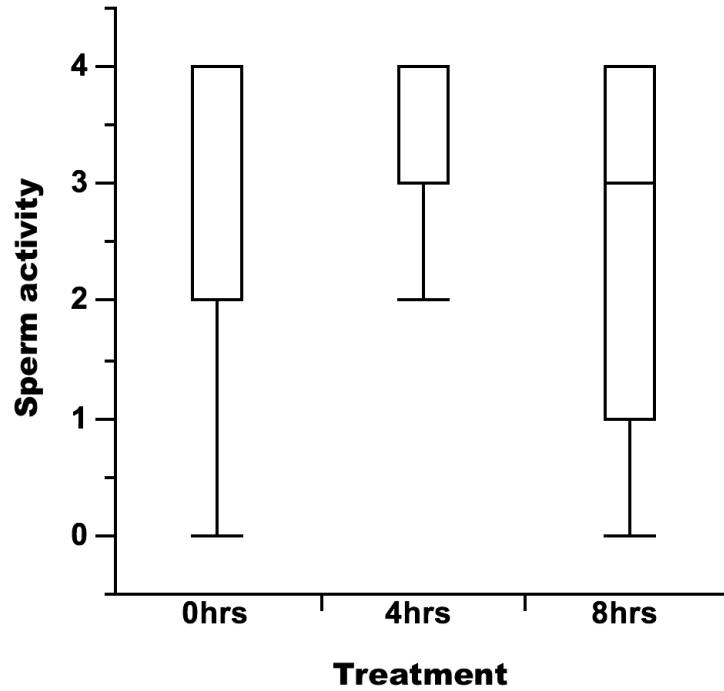
Fig 4.4: The median sperm vigor observed in overnight-mated Mopti females that were stressed for 7days (0h, 4h and 8h). In the boxes, lower and upper edges represent the 25th and 75thpercentiles and whiskers are equivalent to 25th- 75th quartile ± 1.5 * interquantile range.

4.3.4 Effects of 7d post-mating hydric stress on sperm maintenance in the spermatheca

There was no significant effect of stress on sperm quantity retained by females in the 3 treatment groups (ANOVA: n= 119, $F_{2,118}$= 0.93, P= 0.40). But, replicate and the interactions between stress and the sperm number retained by the females in the 3 groups was significant (ANOVA: replicate; $F_{1,118}$= 11.14, P < 0.001; interaction; $F_{1,118}$= 3.73, P= 0.003). The 0h stressed females retained 780 (95% CIs= 425-1108), sperm in their spermathecae, 4h stressed females retained 1007 (545-1469), and 8h stressed females retained 1404 (921-1888) (Fig 4.5).
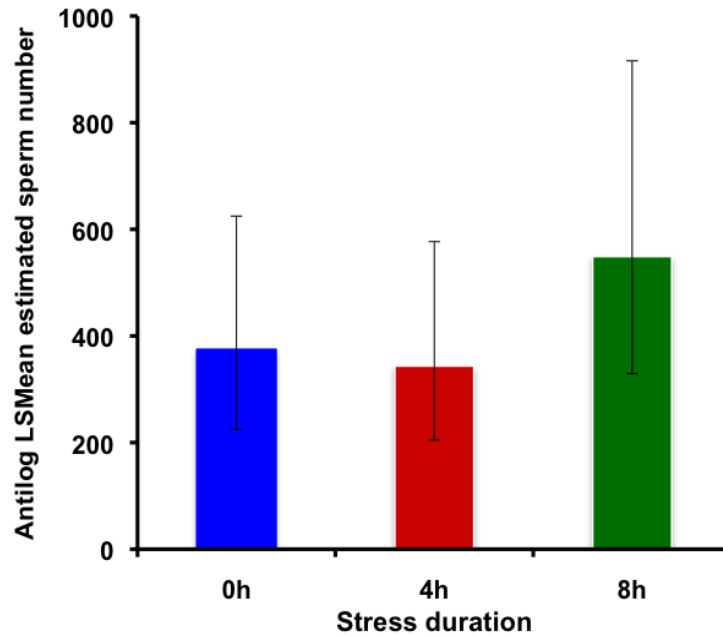
Fig 4.5: Antilog of log LSMean estimated sperm numbers retained by Mopti females stressed for 0h, 4h and 8h for 7 day after an overnight mating with non-stressed Mopti males. Each bar was constructed at 95% confidence interval.

Overall, correlations conducted between sperm number and female body size showed no a significant correlation ($P= 0.81$). Within treatments, the 4h treatment group showed a significant correlation between the log sperm number and female winglength (Spearman; $p= 0.32$, $P= 0.047$) (Fig 4.6).
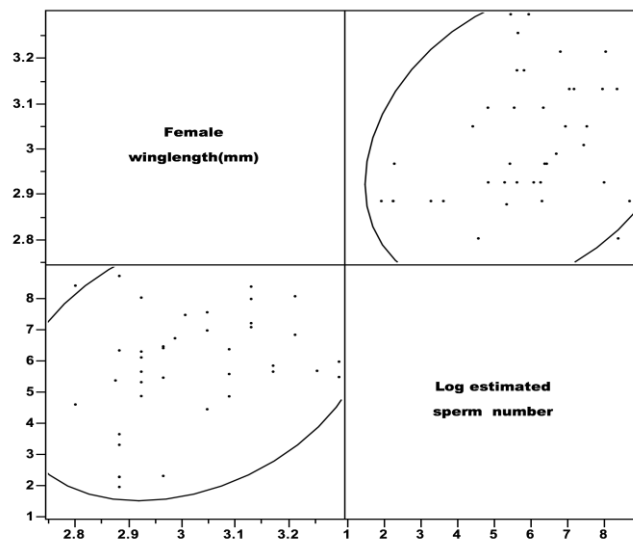
Fig 4.6: Correlation between female body size and log estimated sperm numbers in the spermathecae of 7 days stressed females in 4h treatment group.

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4.4 Discussion

In this research, two aspects of the effects of stress were investigated, first the consequence of water deprivation before mating on sperm investment by males and sperm uptake in the females of *An. gambiae s.s* and secondly, the effect of hydric stress on sperm activity and maintenance of mated females of *An. gambiae s.s.* We found that the hydric stress had a positive effect on the male's current investment. Increase in stress duration results in an increase in sperm amount transferred by the males, whereas the pre-mating hydric stress had no effect on sperm uptake in the female. In the second experiment focusing on sperm activity and sperm keeping, the 7 days post-mating hydric stress had no effect on sperm activity and sperm number maintenance in all the treatment groups.

Physiological stress tolerance such as water deprivation in *An. gambiae* have been noted in several studies (Aboagye-Antwi and Tripet 2010; Aboagye-Antwi *et al.* 2010; Fouet *et al.* 2012). However, no study has provided a comprehensive effect of stress on reproductive investment. High male mating investment is frequently related to reduced longevity (South *et al.* 2009). Stress response assists individuals to activate energy to combat or escape from a danger in so doing enhancing its likelihood of survival (Selye 1975). We then assumed that hydric stress before mating in the males of *An. gambiae* would activate an additive response in the males causing them to invest more on their current reproduction since the chances of survival and future reproduction will be limited. Our assumption was correct. There was a change in balance between current and future investment in reproduction towards investing more in the current reproduction. The stress conditions induce the males to allocate more sperm to ensure future reproductive success. This is consistent with the review by Snook (2005). The degrees to which males can increase sperm amount allocation are subject to their physiological conditions. Gathering all resources to overcome an apparent danger results in channelling resources away from

other areas not immediately involved in overcoming the risk (Selye 1975). The more stressed the males are, the more sperm they transfer (the 8h treatment groups transferred the largest sperm quantity). This is an adaptive response because the main concern is to overcome the threatening emergencies and so all reserve needs to aim at this. In addition, it has been suggested that there is an energy trade off connecting reproduction and continued existences in *An. gambiae* males. The males that mate first are the most energetic ones (Ng'habi *et al.* 2009). Large sperm number transfers by *An. gambiae* males under stress condition might be a high reproductive cost of siring more offspring. Our stressed males might want to give it all (transfer more sperm) while still have the energy to swarm and mate in so doing producing more offspring. Blay and Yuval (1997) made a similar observation in stress males of Mediterranean fruit fly. The stressed males transferred significantly more sperm to their mates than those without stress.

Female insects play a major role in reproductive investment per progeny than males because they control sperm movement inside their reproductive tract (Snook 2005). In dry season about 45% of females of *An. gambiae* experience gonotrophic dissociation thus decrease in reproduction (Yaro *et al.* 2012). We then hypothesized that if *An. gambiae* females are threatened with water stress, they might become more receptive to sperm intake to ensure survival (Edvardsson 2007). Unfortunately, this hypothesis were dismissed by the lack of significant evidence in the quantity of sperm received by stressed female in our experiment. No difference in sperm numbers accepted by the stressed females compared to non-stressed females was found.

In *An. funestus* the body size of the wild virgin females was comparable the to that of mated females Charlwood *et al.* (2003) in our experiment we had a similar observation. The body sizes of the inseminated females were comparable to the body size of the un-inseminated females. A laboratory experiment in *An. gambiae* recorded a

positive relation between male body size and female choice during mating the males would prefer to mate with larger females when given the choice (Okanda *et al.* 2002). No such correlation was found between males and females in our treatment groups. The males did not mate with females of any specific size. Furthermore, the sperm numbers uptake by females showed no significant correlation with the female size.

Sperm degradation has been suspected a couple of weeks after mating in the spermathecae of *Drosophila melanogaster* resulting in a decrease in sperm vitality (Bernasconi *et al.* 2002). In our experimental groups sperm activity was still comparable between the stressed and non-stressed females 7 days of post-mating water stress, suggesting no effect of stress on sperm activity. However, there was a slight difference in the mean sperm activity across our experimental groups but this was not significant.

Female sperm storage is very central to reproductive success given that sperm stored from a single mating can be used over a period of two weeks and are sometimes used for nourishing the reproductive tracts (Sivinski 1980; Bloch- Qazi and Wolfner 2003). The female flies can utilize sperm transferred straight away or stored it for later use (Tram and Wolfner 1999). Sperm storage needs both the sperm and ACPs (accessory gland protein secretions) for proper preservation since females that were mated by males with depleted ACPs stored few sperm even though they received the usual amount of sperm (Tram and Wolfner 1999; Bloch- Qazi and Wolfner 2003). No decrease in sperm numbers was noted in the stressed female and non-stressed female. The 7 days post-mating manipulations of access to water in mated females showed no decrease in the sperm number stored. Suggesting that the mated stressed female did not exchange sperm keeping for overall body maintenance and survival as we predicted (Bloch- Qazi and Wolfner; Huestis *et al.* 2011; Yaro *et al.* 2012). We suppose that females might have other mechanisms in place for body maintenance and sperm keeping. For instance, the presence

of Acps36DE has been associated with effective sperm keeping in fruit fly (Bloch- Qazi and Wolfner 2003). Stressed females in our experiment could receive sufficient Acps36DE during mating for competent sperm storage in so doing confirming the findings of Bloch- Qazi and Wolfner (2003). Failure to detect a significant difference in sperm number stored after stress in our experimental groups suggests that probably more time is required for noticeable effect to be seen. Perhaps a period of over two weeks must elapse before any noticeable change can be observed in stored sperm since some insects can comfortably store and use sperm received during the mating for a fortnight (Bloch- Qazi and Wolfner 2003). The lack of decrease in sperm number stored after 7 days of stress did not support the work of Twig and Yuval (2005) where sperm number in the spermathecae of fruit flies declined with time.

Finally, no association was observed between sperm number and sperm vitality in our entire treatment group. Reproductive success outcome such as oviposition and hatching rate was not measured because we did not have time to do so. Thus, further study is recommended towards measuring reproductive success and sperm vitality.

Chapter 5

Inbreeding, heterosis and reproductive investments

5.1 Introduction

Malaria control is one of the major problems facing the world health sector (WHO 2013). About 10% of the world population living in Africa live with the disease (Luke and Hoffman 2003; Fischer *et al.* 2008). In the past, the conventional methods of control, which involve the distribution of insecticide treated nets (ITN), large-scale indoor-residual spraying (IRS) and use of anti-malaria drugs have been effective in lowering the transmission rate of malaria in endemic countries (Lengeler 2004; WHO 2010). However, the recent increase in mosquito resistance to insecticide (Abilio *et al.* 2011) and the harmful side effects of the insecticides on the environment have introduced a great need for new control measures in endemic regions (Crampton *et al.* 1994; WHO 2010). More so, with recent indication of metabolic cross-resistance in *An. gambiae s.s.* (Mitchell *et al.* 2012), there is an urgent need to improve existing control measures and to develop new novel approaches to malaria control.

The sterile insect technique (SIT) has been successfully used in the field to control a range of pest species (Benedict and Robinson 2003). Unfortunately, the technique has largely failed in mosquitoes due to lack of competitiveness of radiation-sterilized males (Alphey 2002; Benedict and Robinson 2003). The success of the sterile insect technique (SIT) depends on the inundative release of sterile males to mate with wild female population and transfer sterile sperm that ultimately results in infertile eggs and no offspring produced (Benedict *et al.* 2009; Klassen and Curtis 2005). The lack of competitiveness of the sterile male mosquitoes has been linked to their mating behaviour (Howell and Knols 2006). The inability to locate and inseminate their mate in swarms

(Diabaté *et al.* 2009) as well as changes in male reproductive organs due to inbreeding and colonization (Baeshen *et al.* 2014) may have contributed to the poor mating success (Benedict and Robinson 2003) documented in sterile males. A current alternative method to the sterile male release technique is the incompatible insect technique (IIT), which belongs to so-called self-limiting strategies (Burt 2014). The method hinges on the use of Wolbachia bacteria infection of the released males. Cytoplasmic incompatibility induced by Wolbachia bacteria in the sperm transferred to females during mating subsequently kills all embryos (Werren *et al.* 2008). This method has successfully been used against the filariasis vector *Culex quinquefasiatus* (van den Hurk *et al.* 2012). In 2010, researchers focusing on *Aedes polynesiensis* control were able to induce sterility in males using a similar approach (Alphey 2014). Other advances to the IIT include the two versions of genetically modified mosquito (GMM) strains that make use of the tetracycline-repressible methodology also know as RIDL system (Burt 2014, Alphey 2014). In the first version, the mosquito carries a lethal positive feedback loop construct formed by transcriptional activator (tTAV). In their binding site, the tTAV produces high levels of protein that is toxic to the mosquito. The addition of tetracycline into the mosquitoes diet creates a feedback loop of tTAV that prevents negative effects on lab-reared individuals in so doing homozygous males are maintained (Burt 2014). These homozygous males reared with the tetracycline diet are released to mate wild type females and successful fertilization results in the death of all heterozygous progeny. Depending on where the transgene insertion took place in the genome, the effect can be felt either in the larval or early pupae stage (Phuc *et al.* 2007). Currently semi-field trials are in progress in Brazil and Malaysia for the control of *Aedes aegypti* (Alphey, 2014). In the second version of the method, only the female progeny of releasing male dies and the male progeny survives to carry on the construct to the next generation and causing death of up to the third female generation (Fu *et al.* 2010).

The IIT and RIDL tetracycline repressible systems require males with good mating competitiveness as well as high fertility and fecundity. In other words, high insemination rate and good mechanisms for ensuring the successful sperm transfer are fundamental to the success of these methods. The rationale is to ensure that insemination takes place and adequate amount of vigorous infected sperm cell is transferred and secured for the destruction of targeted embryos. The same rationale may apply to radiation-sterilized males in classic sterile mosquito releases in which it was suggested that partial sperm transfer by unfit males could result in females re-mating with non-sterile males thereby decreasing the impact of male releases (Alphey 2013).

An. gambiae males swarm at dusk for mating (Charlwood *et al.* 2002b; Diabaté *et al.* 2009). Swarming is a unique feature of the dipterans (Clement 1999; Charlwood *et al.* 2002b; Howell and Knols 2009). Females of *An. gambiae* enter swarms to mate with their conspecific males (Diabaté *et al.* 2009; Manoukis *et al.* 2006). In some insects, mating induces different type of physiological and behavioural changes in the females, for instance in *Drosophila*, mating result in change in the courtship behaviour of the female, refractoriness to further copulation and reduces her attractiveness to the male (Wolfner 2002; Rezaval *et al.* 2012). In *Aedes aegypti,* the transfer of 'matrone' to females (a male hormone) during mating triggers of a refractory attitude in the females and this is also true in the case of *Culex tarsalis* (Takken *et al.* 2006).   In the Anopheline, successful mating results in post-copulatory responses such as host seeking, egg laying and refractoriness to subsequent mating (Gillott 2003; Roger *et al.* 2008). Mating success is dependent on different factors in different mosquitoes. The ability of the males to mate several times is dependent on male size and its ability to find food (in the field plant nectar) (Takken *et al.* 2006). In the *An. gambiae*, factors such as body size, age and nutrition affect mating success. For instance, in lab-reared *An. stephensi* mating success decrease with age, as the

number of spermatocysts decrease and the proportion of the testes occupied by the sperm reservoir increases with age. The quantities of spermatozoa present in the sperm reservoir and the rate at which the male accessory gland secretions are replenished decreases with successive mating (Mahmood and Reisen 1994). In *An. freeborni,* larger males tend to mate more than the smaller males (Yuval *et al.* 1992), whereas in *An. funestus* and *An. gambiae* size does not determine mating success (Charlwood *et al.* 2002b, 2003). On the contrary, Ng'habi *et al.* (2005) noted that medium-sized *An. gambiae* males were more successful in getting mates than the smaller or larger males. In a recent study, mated males of both M and S molecular forms of *An. gambiae* collected from two different field locations were found to be bigger than unmated ones in (Maïga *et al.* 2013). In addition, Cabrera and Jaffe (2007) suggested that pheromone release and acoustics might contribute to mating success in individual males of *An. gambiae* while Diabaté *et al.* (2011) proposed that the swarm size foretell mating success than swarming site.

The number and quality of sperm in the reproductive organ of male insects are major contributing factors to reproductive success (Helinski and Knols 2009). Increase in sperm number is regarded as a significant part of reproductive success in many insects (Baer and Boomsma 2004). Numerous studies have been conducted in order to determine factors responsible for the mating success in diverse insect taxa. Sperm quantity has been recognized as one of the most important traits contributing to higher mating success, as chances are that big sperm quantity enhances fertilization (Kelly 2008, Rowe and Pruett-Jones 2011). Sperm longevity, motility, and speed may have also contributed to mating success (Hunter and Birkhead 2002). Sperm number has been investigated using different techniques such as manual counting under microscopes as demonstrated by Ponlawat and Harrington (2009) and Helinski and Knols (2009), fluorometer used by Reichardt and Wheeler (1995) and Live/dead sperm viability kit (L-7011) designed by Molecular Probe

(Twig and Yuval 2005; Bernasconi *et al.* 2002; Nakahara and Tsubaki 2007). In *Aedes aegypti*, larger males and older males are known to transfer more sperm to females than smaller and younger males and they have higher reproductive success (Ponlawat and Harrington 2009). In laboratory colonies of *An. gambiae* larger males tend to prefer larger females for mating (Okanda *et al.* 2002). The *An. gambiae* females have a high reproductive rate that enhances their success as a vector (Roger *et al.* 2009). The ability of the females to acquire sufficient sperm and utilize it after a single mating is an advantage to their mating success and the ability to restore damaged sperm cell implies that they have a good method for ensuring the viability of the sperm received during mating (Roger *et al.* 2008). Sperm allocation and or ejaculate transfer is a costly investment for male insects (Scharf *et al.* 2013). Across many insect species, the reproductive cost for sperm transfer is paid by reduced longevity (Wedell *et al.* 2002). For instance, the small male butterflies *L. aripa* is willing to pay the physiological price of reduced longevity for large ejaculate production and ensure female fecundity (Caballero-Mendieta and Cordero 2013). In *An. gambiae,* sperm numbers remain largely unexplored due to difficulties associated with spermatheca dissection (Helinski and Knols 2009). Very little is known about the actual sperm numbers transferred by males to the females during mating. The available information on sperm quantity is currently inadequate, mores so, there are no published data on the reproductive cost of small or large sperm investment by the males.

In the *Anopheles* female, only one spermatheca is found and all the sperm transferred to the female during mating is stored there (Clements 1999). In a comparative survey of the Anopheline, it has been proposed that sperm maintenance in the spermathecae depends on the size of the females and the volume of their spermathecae (Klowden and Chambers 2004). It has been suggested that sperm length and size could be linked to reproductive success since females of Anopheline tend to select the larger sperms more for fertilization

than medium or shorter sperms (Klowden and Chambers 2004). Sperm obtained from the spermathecae of female *An. arabiensis* were larger than the ones from the male testes (Helinski and Knols 2008). In laboratory colonies of *An. gambiae,* sperm length was found to continuously decrease with the age of the colony (Baeshen *et al.* 2014). It was shown that when these mosquito colonies were refreshed with individuals from the field, the sperm lengths were greatly enhanced. Therefore, the decrease in sperm length with colony age is associated with inbreeding, which could negatively affect reproductive fitness in most colonized strains (Li *et al.* 2008).

The *An. gambiae* females are largely monogamous i.e. mating only once in their lifetime (Tripet *et al.* 2003). During mating, the male transfers both sperm and Accessory Gland Proteins (ACPs). The functions of the ACPs (which are produced in the accessory glands) include allowing free sperm movement and physical barrier to re-mating, at least for a while (Gillies 1956; Tripet *et al.* 2005b). It helps for proper sperm storage (Gillott 2003; Sirot *et al.* 2008; Roger *et al.* 2009). The ACPs are rich with series of proteins that trigger off behavioural changes such as host seeking, post reproductive responses like egg laying and refractoriness to re-mating (Gillott 2003; Shutt *et al.* 2010). The ACPs are also referred to as Male Accessory Gland secretion (MAGs). The MAGs are deposited at the entrance of the female atrium in the form of gelatinous substance called the mating plug (Gillott 2003; Baldini *et al.* 2013). Though mating plugs have been identified in a vast number of animal species, efforts being made to identify their role but substantial facts are still lacking about some of their definitive roles (Birkhead and Moller 1998). Early researchers (in the 1960's) who studied the Anopheline believed that mating plugs serve no purpose, but were only seen as evidence of their Dipterans lineage (Giglioli and Mason 1966). In the 1970's, it was suggested that mating plugs in the *An. gambiae* serves as an obvious wedge to second insemination (Parker 1970). Further studies, suggested that

mating plugs may not just be for preventing re-insemination, but might also be to secure the sperm in the spermatheca and guarantee reproductive success by the male (Gerberc 1970). Roger *et al.* (2009) observed that female mosquitoes without the mating plug were unable to retain sperm in their spermathecae.

The mating plugs of the *An. gambiae* measures about 0.5mm long and resembles a transparent gel when freshly dissected (Gillies 1956). The shape of the mating depends largely on how much pressure it experiences in the atrium during dissection (Gillies 1956). Mating plugs were only observed in mated wild females just after a blood meal, but were never seen in virgin female mosquitoes. This helps to differentiate nulliparous females from the unfertilized ones (Gillies 1956). Under laboratory conditions, double plugs are occasionally produced, indicating attempted fertilization by more than one male (Gillies 1956). In the field, double plugs are absent (Giglioli and Mason 1966) because females never seem to return to the swarm after mating (Charlwood *et al.* 2003). Studies on the composition and formation of the mating plug in the *An. gambiae* by Roger *et al.* (2009) highlighted some of their importance. With RT-PCR and proteomics analysis, they were able to identify 27 sex-peptides that form part of the mating plug, 15 of these proteins are of male origin, 6 are of female origin and the remaining 6 are found present in both male and female reproductive tissues (Roger *et al.* 2009). The most abundant of the sex-peptides are *Plugin* and *Transglutaminase* and they make up the outer layer and matrix of the mating plug (Pondeville *et al*. 2008; Roger *et al.* 2009). Principally the cross-linking activities between the enzyme *TGase* and the *Plugin* forms the mating plug. In addition to the mating plug transferred, is a steroid hormone 20-hydroxy-ecdysone (20E) (Pondeville *et al.* 2008). They are produced and stored in the MAG and conveyed as part of the mating plug to the female during mating. The 20E hormones are necessary for activation of vitellogenesis a procedure essential for egg development (Pondeville *et al.* 2008). The

presence of 20E in the female reproductive tract stimulates the expression of heme peroxidase HPX15 (IMPer) genes that are important for female fecundity (Pondeville *et al.* 2008; Shaw *et al.* 2014). The expression of HPX15 genes in the female reproductive tract of *An. gambiae* protects the sperm from possible oxidation damage and the up regulation of the gene in the female reproductive tract several days after mating ensures long term fertility particularly if the mosquito blood feeds (Shaw *et al.* 2014). Also in the female reproductive tract, the Mating Induced Stimulator of Oogenesis (MISO) protein interacts with 20E hormones to induce increase in egg laying in mated blood fed females (Baldini *et al.* 2013). The expression of MISO in the female reproductive tract is regulated by Ecdysone Receptor (*EcR*).

Inbreeding takes place when related individuals with the same alleles mates to produce offspring (Waser 1993). This usually led to the accumulation of homozygous alleles, which ultimately results in reduced fitness (Michalczyk *et al.* 2010). Reproductive traits such as sperm qualities are particularly prone to inbreeding depression and have been studied in many organisms (Zajitschek *et al.* 2009). Inbreeding has been reported to have a negative effect on a wide diversity of organism in both captive and wild populations (Keller and Waller 2002; Lacy 1993). The decrease in fitness due to inbreeding effect is generally assumed to be because of blending effects of dominance and over dominance at recessive loci (Charlesworth and Charlesworth 1999). Inbreeding depression is well described in many insect species, particularly in *Drosophila* but the degree to which inbreeding affects different species varies among populations (Fox *et al.* 2007). A common argument has been that that inbred individuals are more sensitive to environmental stress than are out bred individuals, since stress enhances the manifestation of deleterious recessive alleles (Fox *et al.* 2011). In adults of *Drosophila melanogaster*, inbreeding led to reduced resistance to desiccation and inbred adults experience relatively great decrease in

productivity than outbred ones when exposed to heat stress (Dahlgaard and Hoffman 2000). Inbreeding depression is likely to manifest more under stressful environment than stress free environment (Armbruster and Reed 2005). For instance, inbreeding has been shown to increase with the stresses of the environment, to the extent of stress explaining as much as 66% of the difference in inbreeding depression (Fox and Reed 2011). Inbreeding primarily has been studied from a survival point of view across many taxa (Saccheri *et al.* 2005). For instance, inbreeding affects adult longevity in male *Aedes* but not the female (O'Donnelly and Armbruster 2010). In burying beetle *Nicrophorus vespilloides* direct effects of inbreeding has been linked to overall reduction in offspring survival (Mattey *et al.* 2013). The lifetime offspring production for adult *Callosobruchus chinensis* was reduced by 11%, but no significant effect of inbreeding was seen on survival (Harano 2011). Reproduction is closely associated with fitness and thus can significantly decrease due to inbreeding depression (Saccheri *et al.* 2005). The effect of inbreeding on reproductive traits like sperm quality has become of primary interest owing to the role of sperm quality in competition for fertilization success (Zajitschek *et al.* 2009). Inbreeding effects are easily noticeable in male reproductive investments, implying that inbreeding depressions are more severe in males than females (Ala-Honkola *et al.* 2014). For instance, there are indications that inbreeding negatively affects sperm quantity in *Poecilia reticulata* 'guppy' leading to reduction in male fitness (Zajitschek *et al.* 2009). In *Drosophila*, inbreeding affects male fertility, but has no impact on the sperm storage ability in females (Ala-Honkola *et al.* 2014).

Heterosis is defined as the occurrence of superior progeny from the genetic mixing of two inbred parents (Birchler *et al.* 2003). More often than not is attributable to the masking of recessive deleterious alleles in heterozygous genotypes (Pekkala *et al.* 2014). In homozygous genotypes, recessive deleterious alleles are unmasked and the advantages

of heterozygosity in over dominant loci are lost (Charlesworth and Willis 2009). Hybridization amongst populations that are genetically different is known to potentially improve the effects of inbreeding and boosting heterozygosity in the population (Whitlock *et al.* 2002). Furthermore, the hybridization of two inbred lines of *Drosophila melanogaster* results in increase in mean and maximum life span (Vaiserman *et al.* 2013). Many studies have looked at the effects of heterosis on mating speed in males or success in mating competitiveness (Hughes 1995), but very little has been done on male fertility in the absence of mating competition (Fry *et al.* 1998). *In An. gambiae,* hybridization of two strains resulted in enhanced fitness in progeny up to 20th generation. Two strains of *An. gambiae* Mbita and Ifakara strain were hybridized and female fecundity was increased due to heterosis (Menge *et al.* 2005).

Survival of high temperature stress is a heritable adjustable thermal acclimatization characteristic in many organisms. Individuals survive heat stress by basal or induced thermo resistance (Arias *et al.* 2012). In *An. gambiae* factors such as body size, genetic background and sex affects the level of desiccation resistance experienced (Fouet *et al.* 2012). Owing to decrease in overall fitness and genetic variations, inbred individuals are expected to show a decrease in survival and lifespan under most ecological conditions as compared with outbred individuals (Valtonen *et al.* 2011). For instance, in *Harmonia axyridis Pallas* (Ladybird beetle) the survival of two isofemale lines and hybrid lines were compared under laboratory conditions. The hybrid lines survived significantly higher than the two-isofemale lines (Seko *et al.* 2012). Survival in the dry season is critical to *An. gambiae* mosquitoes, particularly those living in the very xeric environment (Lehmann *et al.* 2010). The M form aestivates during the long dry season in the Sahel to survive desiccation whereas the S form migrates and return later soon after the rain starts (Lehmann *et al.* 2010; Huestis *et al.* 2011, 2012).

5.2: Section A: Impact of heterosis on sperm and sex-peptide transfer by hybrid supermales in large-group mating experiments

5.2.1 Introduction

In a recent study, Baeshen *et al*. (2014) crossed two laboratory strains of *An. gambiae* to generate a hybrid outbred 'supermale' with large sperm lengths to highlight the changes in male reproductive traits that have been affected due to inbreeding. Here, we hypothesize that inbreeding might affect insemination rates as well as the amount of sex peptides transferred with the sperm. We compare the mating success (insemination rate) of hybrid supermales and that of the inbred parental strains used to generate them in large-group overnight mating experiment. Sperm numbers transferred by hybrid male and inbred males of *An. gambiae* were quantified using quantitative PCR, and vitality of sperm was determined using the newly developed 'in vivo' method of investigating sperm vitality (vigor). We also examined the quantity of the *Plugin* and *Transglutaminase* proteins transferred by supermales and inbred males using ELISA method. Finally, the reproductive success of females mated to the different male treatment groups was measured.

5.2.2 Methods

5.2.2.1 Large group mating cages

All adults (Kil, Mopti and hybrid supermales), used were produced and maintained as described in Chapter 2. Four mating combinations were made with a 2:1 male to female ratio. In the first two cages, 200 virgin supermales were combined with 100 virgin females of the Mopti parental strain or the Kil parental strain. In the other two mating combinations, 200 virgin Mopti males were combined with 100 virgin Mopti females and 200 virgin Kil males were combined with 100 Kil females. The mating combinations were

set up in three replicates and all the adults used were between 3-5d old. The combination was done between 3-5pm to give the mosquitoes time to recuperate before swarming and mating at about 7pm. Following a single night of mating by 8am, sub-samples of females were removed from each mating combination cage for the measurement of different male reproductive investments. A first random sub-sample of females was taken for determination of sperm vitality. These females were transferred to another cage in the insectary for 24h to allow the window period required for sperm activation in *An. gambiae* (Roger *et al.* 2009). A second sub-sample was stored in ethanol for sperm number estimation using qPCR and assessment of mating frequencies at a later stage. A third random sub-sample of female was taken, knocked-down on the ice, and dissected immediately for sex-peptide quantification via ELISA, The remaining females were left in their original mating cages, blood fed and used for the estimation of female fecundity.

5.2.2.2 Determination of sperm activity

Measurement of sperm vitality followed the procedures described in chapter 2. After 24h, the sub samples of females collected for sperm activity were transferred into a small polystyrene pot covered with mosquito net and fastened with elastic rubber. For the dissection, a random subset of 10 females was collected from a given pot and placed in another small-netted polystyrene cup of ice to knock them down partially. In order to avoid possible bias, subsets were taken sequentially from each treatment group and the sequence of treatment groups inverted after each series of 40 dissections. The researchers performing the dissections and sperm activity scoring were blind with regard to which treatment group was being processed at a given stage. The median sperm activity recorded over 1min of observation was used for subsequent analyses.

Surprisingly, we observed that in some spermathecae, sperm activation had not taken place after the concept of 16-17h sperm activation (Roger *et al.* 2009). On average, sperm activation had not yet taken place in 44.3% of females and there was no significant difference in the proportion of females without activated sperm in their spermathecae after 24h within treatment groups (Chi-Square: n= 193, df= 3, χ^2= 4.686, *P*= 0.196). Therefore, analyses of sperm activity were done on spermathecae where sperm cells were active.

5.2.2.3 Measurement of spermatheca size

After recording the sperm activity, the spermathecae size was measured based on digital image generated from the QuickTime movie. The picture was imported into ImageJ 1.44 software and the surface area was measured on the screen using the build-in measuring tools. The measurement was done twice, the average taken and the surface measured in pixels was converted to mm.

5.2.2.4 Quantification of sperm via qPCR

The sub-sample of females for sperm quantification was dissected using the protocols described in Chapter 2. Due to storage in ethanol, the sperm bundle coagulates (Tripet *et al.* 2001). Sperm bundles were transferred to a 1.5ml centrifuge tube containing 500µl of lysis buffer plus 5µl of protease K for overnight incubation on a heat block (55ºC) and subsequent gDNA extraction. The gDNA extraction was made using the ChargeSwitch gDNA micro tissue kit (Invitrogen)(see Materials and Method Chapter for full details). The qPCR mix was based on the TaqMan probe assay and prepared according to the AppliedBiosystem guidelines with some optimizations (See Chapter 3 and Chapter 4, Table 4.4 for details of the qPCR mix per one reaction tube for sperm quantification). The qPCR reactions were run on an AppliedBiosystem StepOnePlus Real Time Thermal Cycler

following AppliedBiosystems' TaqMan assay settings and 40 qPCR cycles (Chapter 4 Table 4.5). At the end of the run, the amplification curves of the samples and standard curves were analyzed to determine their cycle threshold (CT). They were saved and exported to JMP 10 software. The linear equation generated was used to convert each sample CT value into sperm number equivalent (Chapter 3). The whole process was done twice, the means of the 2 technical replicates were taken, and log transformed to normalize the data. The transformed data was later multiplied by 75ul, which was the volume of the gDNA extraction from one spermatheca. The transformed mean value x75ul gave the final sperm number estimated for each sample.

5.2.2.5 Quantification of *Plugin* and *Transglutaminase* by ELISA

Quantification of accessory gland proteins was made jointly with fellow a PhD student Rowidah Baeshen. The sub samples of females for ELISA were collected and dissected immediately after the overnight mating (8am). Females were picked randomly directly from the mating cage from each mating combination. The tip of their abdomen (last two abdominal segments) was cut off making sure that the plugs were still there by visual inspection under the dissecting microscope. It was then transferred to 1.5ml centrifuge tubes containing 220µl PBS solution and proteinase inhibitor (PI) and frozen at -80C for later use. To prepare standard curves for *Plugin* and *Transglutaminase* quantification, ten 4-day-old Mopti males were used and their last abdominal segments containing the accessory glands were cut off and transferred to a 1.5ml centrifuge tube containing 880µl and 440µl PBS solution and proteinase inhibitor (PI) respectively. The cuticles and tissues of the mosquitoes were ground in a sonicating machine for 30s' to break them down (Sonicator: Vibra-cell 300W High Intensity Ultrasound Processor, Sonic& Material, Inc, Connecticut, USA, transferred to a sonicating water bath on ice for 10min at high

frequency (Bioruptor At UCD-200, diagenode), and then transferred to -80 freezer for 10min to cool down. The samples were centrifuged at 13000rpm for 15min at 4ºC and the supernatant collected for quantification by ELISA. All plate preparation steps followed standard ELISA procedures (Wigby *et al.* 2009). The antibodies used were Affinity-purified polyclonal against *Plugin* (AGAP009368) and (AGAP009099) (GenScript Corp., Piscataway, NJ 08854, USA.) For absorbance readings, 100µL of HRP substrate (TMB liquid substrate system, Sigma-Aldrich, St Louis, MO, USA) was added to each well and the plates incubated in the dark for 10min for *Plugin* and between 20-40min for *Transglutaminase*. Reactions were stopped by adding a 100µl stop solution (9% H_3PO_4) and read at 450nm using a Labsystems Multiskan plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Two independent standard curves were prepared based on six 2-fold dilutions and loaded on each plate. The average of the two dilutions were taken if both curves are good, otherwise, only the good dilution curve was used to generate an equation used to compute the quantity of proteins. The standard curve fittings were done using JMP 10 software (SAS Inc. USA). Based on the standard curve calculations, sample readings were translated into 'male accessory gland equivalents'.

5.2.2.6 Female fecundity and fertility

After blood feeding, the sub samples for the female fecundity were transferred into single oviposition tubes for egg laying. Food was provided in the form of cotton wool soaked in 5% glucose solution on top of each tube. The following day the tubes were checked for eggs that were laid. The number of eggs on each filter paper was counted under a dissecting microscope. Thereafter, egg papers were washed with de-ionised water into 300ml plastic tray for hatching. Each tray was allowed 4 days for hatching before the larvae were counted.

5.2.2.7 Data analysis

The entire data set was checked for normality in distribution as well as the heterogeneity of variance. Where data distribution was normal, the mean differences were compared using parametric analysis and displayed as bar plots. Means are presented with 95% confidence intervals throughout. If the data distribution deviated from normal, a non-parametric approach was employed and a box plot used for display. Where medians are used they are presented with lower whiskers constructed as 25th percentile -1.5* interquantile range and upper whiskers as 75th percentile + 1.5* interquantile range. The main effects and interactions from the replicates within experiments were always statistically tested, but reported only when significant.

5.2.3 Results

5.2.3.1 Hybrid supermale and sperm activity

The activity of sperm in the spermatheca was compared between the four mating combinations and a significant difference was found (Kruskal-Wallis: n= 107, df= 3, χ^2= 8.067, P= 0.045) (Fig 5.1). Pairwise comparisons between experimental groups revealed that Kil males mated with Kil females had significantly lower sperm activity than Supermales mated with Kil females (Dunn's test: n= 50, Z= 2.651, P= 0.048). Supermale mated with Mopti females and Mopti male mated with Mopti females had intermediate sperm activity and did not differ significantly from other groups (Dunn's test: $P > 0.136$).
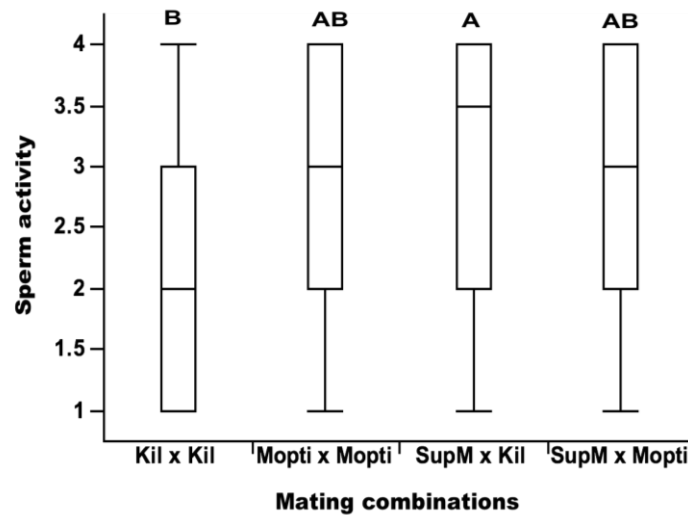
Fig 5.1: Median sperm vigor observed in the four experimental combinations; Kil x Kil, Mopti x Mopti, SupM x Kil and SupM x Mopti. Across the four boxes, horizontal lines in the boxes indicates the median active sperm value for each treatment group, lower and upper edges represent the 25th and 75th quartiles. The whiskers are equivalent to 25th- 75th quartile ± 1.5 * interquantile range.

The overall sperm activity of inbred males versus supermales was compared, the analysis revealed that the sperm numbers transferred by the hybrid supermale were significantly more active than the sperm cells transferred by the inbred male (Mann-Whitney: n= 107, df= 1, χ^2= 4.0, P= 0.045). The median (25th-75th percentiles) active sperm in inbred male was 2 (1-4) while that of the supermales was 3 (2-4) (Fig 5.2).
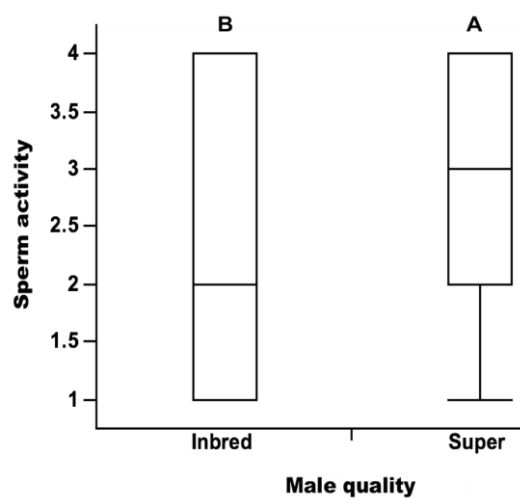
Fig 5.2: Active sperm values of inbred male and hybrid supermale used in the mating combinations. Horizontal lines in the boxes indicate the median active sperm value for each male quality. Lower and upper edges represent the 25th and 75th quartiles and whiskers are equivalent to 25th- 75th quartile ± 1.5 * interquantile range.

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5.2.3.2 Spermatheca size

Overall, 191 spermathecae were dissected and measured in the four mating combinations. Their mean difference was compared using one-way ANOVA. The spermathecae size of the Kil females were significantly larger than that of the Mopti females (ANOVA: n= 191, $F_{1,189}$= 5.0, P= 0.003). The mean spermathecae sizes (Fig 5.3) were 0.049mm (95%CI= 0.047-0.050) for Kil females and 0.046 mm (0.045-0.048) for Mopti females. Overall, no significant difference was found in spermathecae size within the 4 treatment groups (ANOVA: n= 191, $F_{1, 189}$= 2.49, P= 0.06).
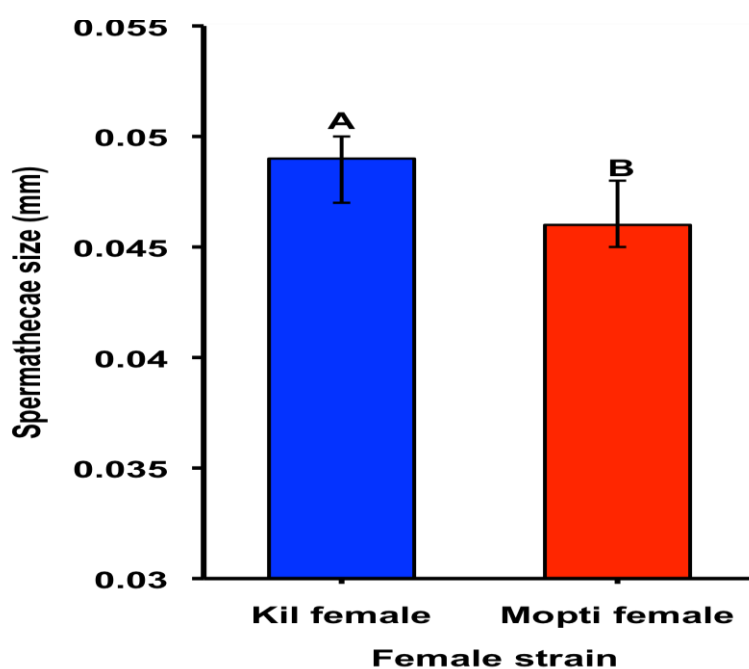
Fig 5.3: Mean spermathecae size of Kil and Mopti females used in the mating combination of the four experimental groups. Error bars were constructed at 95% confidence interval of the mean. Level of significance were presented with different letters

The Female body size (winglength measurement) did not differ between the 4 mating combinations (ANOVA: n= 286, $F_{3, 285}$= 0.15, P= 0.93) (Table 5.2) or, between female strains (Mann-Whitney test: n= 286, χ^2 = 0.001, P= 0.978). The median (25th-75th percentile) body size of the Kil female was 2.93 (2.88-2.97) and that of Mopti female, 2.93(2.88-3.00).

5.2.3.4 Estimated sperm numbers

The quantity of sperm amount transferred by males and stored in the female spermathecae in the four mating combination was analysed by 2-way ANOVA. There was a significant effect of replicate and treatment on estimated sperm numbers (ANOVA: replicate: n= 85, $F_{1,84}$= 56.5, $P < 0.001$; treatment: n=85, $F_{3,\ 84}$= 3.40, P= 0.022). Pairwise post hoc Tukey HSD tests conducted on the least square means (LSM) revealed that the old inbred Kil female mated with Kil male retained significantly more sperm than Mopti female mated by Supermale (Tukey: n= 46, P= 0.04) (Fig 5.4). Supermale mated with Kil female and Mopti mated with Mopti female transferred intermediate sperm amount and did not differ significantly from other groups (Tukey: n= 45, P= 0.40) (Fig 5.4). Across all mating combinations, there was a significant effect of female strain and replicate (2-way ANOVA: female strain: n= 85, $F_{1,84}$= 9.3, P= 0.003; replicate: n= 85 ,$F_{1,84}$= 57.8, $P < 0.001$) Kil females stored more sperm in their spermathecae than Mopti females (LSM: Kil= 5.76 (5.47-6.05) and (LSM: Mopti= 5.40 (5.11-5.64)
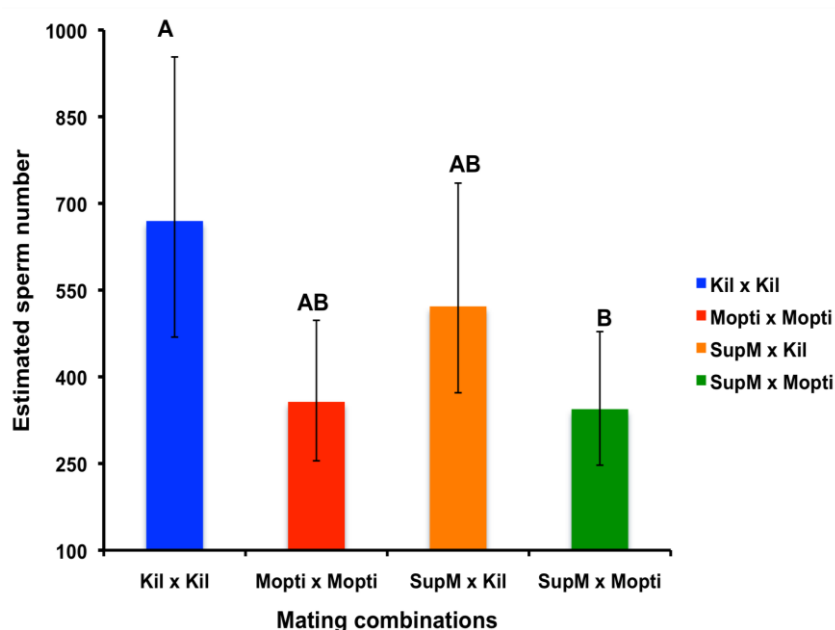
Fig 5.4: Antilog of Log least square mean (LSM) of the estimated sperm number transferred by Kil, Mopti and hybrid supermale in four mating combinations. Error bars were constructed at 95% confidence interval of antilog of LSM. Level of significance were presented with different letters.

5.2.3.4 Correlations between sperm numbers, sperm activity, spermathecae size, and body size.

The relationship between sperm number, sperm vigor, spermathecae size, and body size (winglength), was examined using Spearman correlations. The result of these correlations conducted on the overall experimental groups revealed no significant relationship between the four variables analyzed $(P > 0.05$ in all cases) (Fig 5.5). The same correlations, when conducted within each treatment, revealed a single isolated positive correlation between sperm numbers (Log) and spermathecae size in supermales mated with Kil females (Table 5.1). This relationship was not found in the other mating combinations.
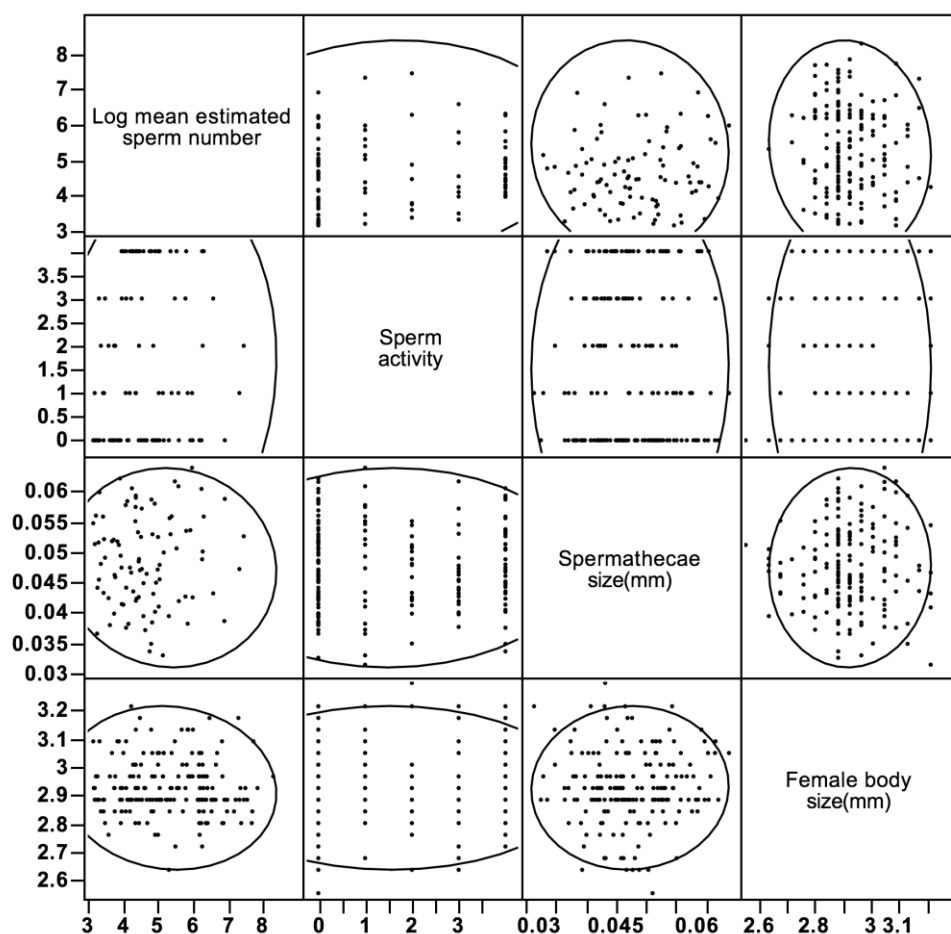
Fig 5.5: Spearman correlations of sperm number, sperm activity spermathecae size and body size on the four mating combination.

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Table 5.1: Spearman's correlation of sperm vigor, sperm amount, spermathecae size and winglength in the hybrid supermale x Kil female treatment group.

Variable	By variable	Spearman ρ	P-value
Sperm activity	Log mean estimated sperm number	0.11	0.60
Spermathecae size (mm)	Log mean estimated sperm number	0.54	0.006*
Spermathecae size (mm)	Sperm activity	-0.01	0.93
Female body size (mm)	Log mean estimated sperm number	-0.22	0.14
Female body size (mm)	Sperm activity	-0.19	0.19
Female body size (mm)	Spermathecae size (mm)	0.07	0.66

5.2.3.5 Estimation of *Plugin* and *Transglutaminase* by ELISA

5.2.3.5.1 Plugin

The mean quantity of *Plugin* transferred by Kil, Mopti and Supermale measured in females less than 24h after mating and expressed as MAG equivalents (see methods) was compared in the four mating combinations. No significant difference was found between groups in the quantity of *Plugin* transferred (Kruskal-Wallis: n= 122, X^2= 2.29, df= 3, P= 0.520)(Fig 5.6). The overall median (25th-75th percentile) amount of *Plugin* transferred was 0.47 (0.27-1.26) MAG equivalent. Mopti males mated with Mopti female transferred 0.40 (0.28-0.83) equivalents, Kil mated with Kil 0.43 (0.22-1.90), Supermales mated with Kil female 0.62 (0.28-2.15), and Supermales mated with Mopti female 0.50 (0.24-1.00).
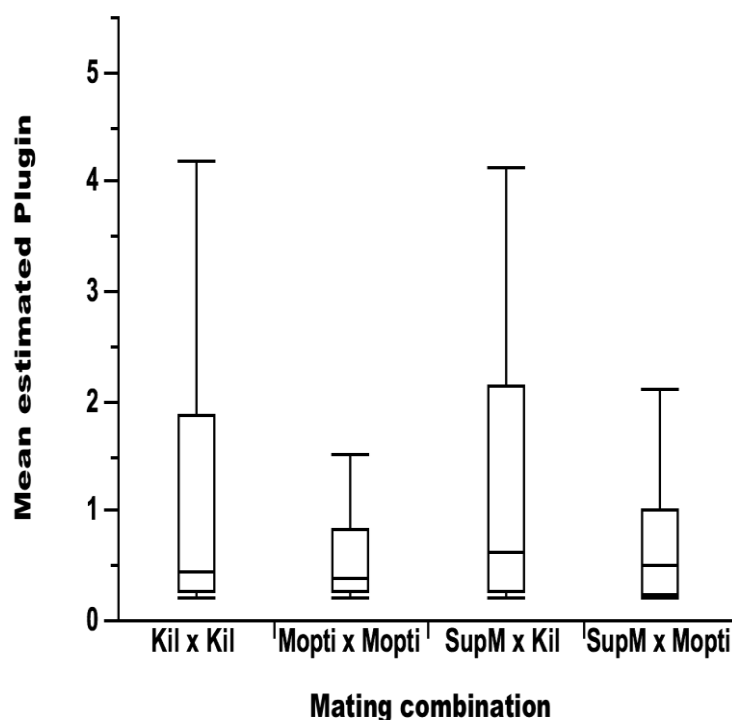
Fig 5.6: The mean estimated quantity of *Plugin* from the tip abdomen of female Kil and Mopti in four mating combinations less than 24h after mating. *Plugin* quantities are expressed as the equivalent of the mean quantity of protein found in one male accessory gland. Across the four boxes, horizontal line in the boxes indicates the median *Plugin* quantity for each treatment group. Lower and upper edges represent the 25th and 75th quartiles and whiskers are equivalent to 25th- 75th quartile ± 1.5 * interquantile range.

5.2.3.5.2 Transglutaminase

The mean quantity of *Transglutaminase* transferred by males in the four mating combination was compared, a significant difference was found (Kruskal-Wallis: n= 143, X^2= 9.81, *df*= 3, *P*= 0.020). Pair-wise comparison between experimental groups showed that Mopti females mated with Mopti males transferred significantly higher level of *Transglutaminase* than the kill female mated with its own male (Dunn's pair-wise comparison: n= 72, *Z*= 3.11, *P*= 0.011). The quantity of *Transglutaminase* transferred by supermales when mated with Mopti or Kil females was intermediate level and did not differ significantly from the previous mating combinations (n= 71, *Z*= 0.02, *P*= 1.00). The overall median (25th-75th percentile) *Transglutaminase* transfer was 0.41 (0.27-0.66) MAG equivalent. Mopti mated with female Mopti 0.49 (0.33-1.53), Kil mated with Kil 0.331 (0.27-0.50), Supermale mated with Kil female was 0.383 (0.27-0.65) and Supermale mated with Mopti female was 0.493 (0.27-0.66)(Fig 5.7).

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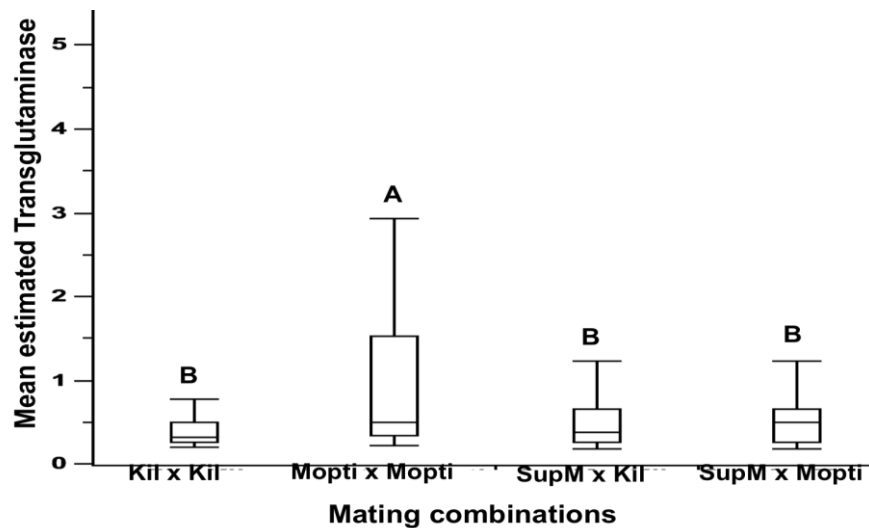
Fig 5.7: The mean estimated quantity of *Transglutaminase* from the tip abdomen of female Kil and Mopti in four mating combinations less than 24h after mating. *Transglutaminase* quantities were expressed as the equivalent of the mean quantity of protein found in one male accessory gland. Across the four boxes, horizontal line in the boxes indicates the median quantity of *Transglutaminase* for each treatment group. Lower and upper edges represent the 25th and 75th quartiles and whiskers are equivalent to 25th- 75th quartile ± 1.5 * interquantile range. Level of significance were presented with different letters.

5.2.3.6 Female fecundity

The number of eggs laid by the mated female and the number of hatched larvae was used to assess the reproductive success of the males in each mating combination. There was no significant difference in the mean number of eggs laid in the four mating combinations (ANOVA: n= 72, $F_{3, 71}$= 0.18, P= 0.910)(Fig 5.8). However, the number of females that lay eggs varied between experimental groups and the number of eggs laid per female was between 19 to 125 eggs. The overall mean number of eggs laid in the four mating combinations was 63.12 (57.35-68.89) eggs. No significant difference was found in the number of larvae that hatched between the four experimental groups (ANOVA: n= 72, $F_{3,71}$= 0.26, P= 0.860) (Fig 5.9). Overall mean number of hatched larvae in the four mating combination was 37.33 (32.02-42.64). The mean number of larvae hatched in the Kil mated with the Kil mating combination was 39.39 (28.04-50.75), Mopti mated with Mopti mating combination had 35.88 (22.38-49.41) hatched larvae. Supermale mated with Kil female mating combination had 39.57 (30.21-49.00) hatched larvae and Supermale mated with Mopti female 33.94 (23.52-44.37).

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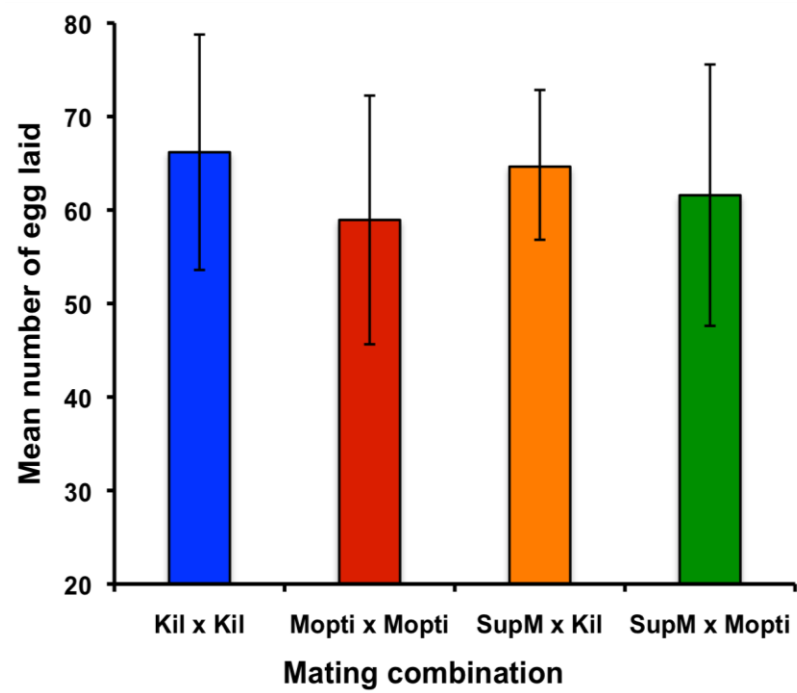
Fig 5.8: Mean number of eggs laid by Kil and Mopti females in the four mating combinations (Kil x Kil, Supermale x Kil, Supermale x Mopti and Mopti x Mopti). Error bars were constructed at 95% confidence interval of the mean.
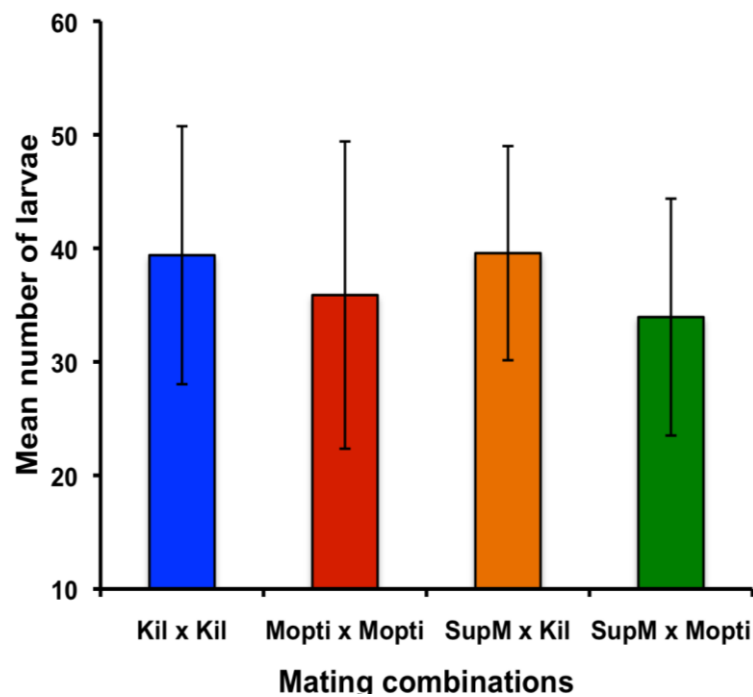
Fig. 5.9: Mean number of larvae hatched per group of eggs laid by Kil and Mopti females in the four mating combination (Kil x Kil, Supermale x Kil, Supermale x Mopti and Mopti x Mopti). Error bars constructed at 95% confidence interval.

Table 5.2: The mean and median values of all the variable parameters measured in the four mating combinations (Kil x Kil, Mopti x Mopti, Super male x Kil, and Super male x Mopti). Values in brackets are 95% confidence intervals or median interquantile range (25th- 75th percentile), and sample sizes are in italics.

Variables	Kil x Kil	Mopti x Mopti	Supermale x Kil	Supermale x Mopti
Mean Ct Values	33.96 (35.03-32.90)	34.66 (35.42-33.91)	33.64 (34.52-32.76)	35.08 (35.87-34.30)
	45	*47*	*46*	*48*
Mean sperm Number	620.07 (967.98-277.16)	400.11 (534.55-265.67)	461.16 (688.34-234.02)	308.76 (421.80-195.77)
	45	*47*	*46*	*48*
Spermathecae size (mm)	0.048 (0.049-0.046)	0.046 (0.047-0.044)	0.049 (0.051-0.045)	0.047 (0.048-0.043)
	49	*49*	*47*	*46*
Female body size (mm)	2.92 (2.95-2.89)	2.93 (2.96-2.91)	2.93 (2.96-2.90)	2.93 (2.96-2.90)
	73	*73*	*70*	*70*
Median sperm activity	3 (2-4)	4 (3-5)	4.5 (3-5)	4 (3-5)
	50	*49*	*47*	*46*

5.2.4 Discussion

This is the first study to examine sperm activity 'in vivo' in the malaria mosquito *An. gambiae* and to experimentally explore the effects of hybrid vigour on sperm activity and quantity in colonized strains of *An. gambiae*. Differences were observed in the sperm activity. The sperm amount transferred and stored by the females, and the quantity of sex-peptide transferred with sperm. Sperm activity was low in the old inbred Kil females mated with their own inbred males, but improved when they were mated by hybrid supermales. Overall, the hybrid supermales transferred sperm that were more active than that of the inbred males. These results suggest that inbreeding negatively affects sperm quality and that heterosis could be used to restore sperm quality in inbred lines.

Sperm quality such as sperm length and viability has been considered in many insects (Joly *et al.* 2004). In seed feeding beetles, inbreeding often affects sperm morphology or physiology (Fox *et al.* 2011). In *Drosophila* species, two types of sperm are produced, the short sperm cells and the long sperm cells (Pasini 2010; Joly *et al.* 1989) and sperm competitions have often been negatively associated with inbreeding in them (Mack *et al.* 2002). In *Drosophila melanogaster*, sperm viability is central to their reproduction (Radhakrishnan and Fedorka 2011) and sperm viability is higher in the promiscuous species than in their monogamous relatives, meaning that selection of sperm is more on the active sperm. Sperm competition and sperm viability correlated with success rate (Garcia-Gonzalez and Simmons, 2005) and in the absence of sperm competition, sperm selection by the females might be a means to choose the best male because males with active sperm sire more offspring (Birkhead *et al.* 1999). In *D. simulans* sperm viability was not affected by inbreeding (Okada *et al.* 2010). The *An. gambiae* mosquitoes produce sperm of variable length, which has been associated with reproductive fitness (Voordouw *et al.* 2008a; Klowden and Chambers 2004). Females of *An. gambiae* tend to select the larger sperm for

fertilization than smaller or medium sperm (Klowden and Chambers 2004). Baeshen *et al.* (2014) recently generated an out-bred supermale with long sperm length, which they suggested might have a fitness consequence on male reproduction.

Sperm activity varied across our four mating combinations. The old inbred Kil males mated with their own female recorded the lowest sperm activity, and when coupled with the heterotic males the vigor improved. Thus, increase in sperm activity suggests heterosis. The heterosis here may cause a positive fitness consequence of male investment. Unfortunately, it is not very clear what controls sperm activity in *An. gambiae.* Moore *et al.* (2004) establishes a negative correlation between sperm viability and testis size. In a different study, a very strong correlation was seen between sperm activity and female oviposition (Voordouw *et al.* 2008b). They suggested that sperm viability might lead to increase in fecundity resulting in male reproductive success. In *D. melanogaster,* male genotypes affected sperm viability stored in females 4 days post insemination, suggestive of a genetic basis for sperm viability (Civetta *et al.* 2008). Sperm viability is heritable in *T. oceanicus* implying that some factors that impact on its activity are genetically transferred traits which may be vulnerable to inbreeding or selection pressure (Simmons and Roberts 2005). As shown previously, the heterotic supermales have longer sperm length because of heterosis compared to the shorter sperm lengths of the inbred Kil males (Baeshen *et al.* 2014). The short sperm lengths observed in the inbred Kil might have a reproductive fitness cost which might eventually result in less number of progeny produced. The heterotic supermales have long sperm lengths, and we observed that these long sperms were more active than the inbred male strains. Therefore, heterosis can be used to revert the effect of inbreeding on sperm quality in colonized strains of *An. gambiae,* making them possible candidates for vector control projects. In addition, Mopti males (10yrs+) that are less inbred than the Kil males (35yrs+) transferred sperm that had intermediate activity.

This suggests that inbreeding starts much later in colonization and it requires more than 240 generations for the inbreeding effect to manifest, contrasting to the another report by Reisen (2003) that colonized mosquitoes turn into inbred within a few generations.

Overall, in the four mating combinations only 55.7% of the females had active sperm in their spermathecae 24h post mating. This observation was not in agreement with the speculated 16-17h necessary for sperm activation in *An. gambiae* (Roger *et al.* 2009). In other insects, it has been identified that sperm are active in certain sites of the reproductive tract and inactive in others (Werner and Simmons 2008). In *Drosophila*, sperm are only active in the seminal vesicles, ventral receptacle, and spermatheca but never active in testis (Werner and Simmons 2008). The activation of sperm in *Bombyx mori* was as results of some peptidase (Werner and Simmons 2008). However, in mosquitoes it is not yet known what activates sperm. This calls for further research on the subject.

In determining sperm activity, Werner and Simmons (2008) suggested that sperm activity might be affected by methodological factor such as the composition of the dissecting buffer; while Voordouw *et al.* (2008b) speculates that the time needed to dissect the spermatheca might cause a reduction in the sperm activity. In our experiment, we devise a novel type of 'in vivo' assay to determine sperm activity. The assay measures the sperm activity 'in situ' in the spermatheca (see the method section). With this assay, sperm mortality is very unlikely since the sperm cells were not exposed directly to the dissecting buffer and or damages due to sperm bundle dissection. More so, the likelihood of producing artifacts in terms of mortality within the treatment groups is reduced. In contrast to other sperm viability methods such as the live and dead sperm viability kit (Twig and Yuval 2005; Radhakrishnan and Fedorka 2011) where sperm could be damaged during

dissection and preparation for staining, resulting in inaccurate or underestimation of live sperm (Holman 2009).

In sperm quantification, inbred Kil males transferred more sperm numbers than the heterotic supermales and Mopti males. This indicates that not all reproductive traits in this inbred strain have been negatively affected by inbreeding. This result however is surprising given the large body of evidence suggesting that inbreeding affects sperm production and number. For instance, in seed-feeding beetles, inbred males transfer 17-33% fewer sperm than the outbred males (Fox *et al.* 2011). The females of *D. simulans* mated with their inbred males had a decline in the number of eggs laid indicating that inbred males may be transferring fewer sperm number (Okada *et al.* 2010). Here, the large sperm amount transferred by the inbred Kil males can be associated with selection pressure to adapt to the insectary rearing and mating conditions. In a review by Snook (2005) selection in sperm competition have been documented in many species and males tend to give in to ejaculates containing greater number of sperm to assist them balance selection for sperm traits against variance in the fertilization. Baeshen *et al.* (2014) documented selection of reproductive traits in lab-reared males of *An. gambiae*. They noted differential changes in the testes and male accessory gland size due to inbreeding. In the same study, they also documented changes in sperm quality such as sperm length. Though inbreeding affects some reproductive traits of this strain, sperm transfer and retention were definitely not affected. The old inbred Kil strain transferred more sperm than the less inbred Mopti males and heterotic supermale. There are two probable explanations for this result. Foremost, the inbred Kil males produce short sperm length due to inbreeding (Baeshen *et al.* 2014). We found these short sperm to be less active and we suspect that they might be infertile too, which may finally result in decreases in reproductive fitness. Therefore, in order to compensate for their short and less active sperm and increase their chances of the

production of more progeny, they transfer more sperm number. Secondly, it could be that the old inbred Kil females are more open to sperm uptake owing to long years of colonisation. They may possibly have adapted more to lab-rearing condition, whereas the Mopti females are less inbred and are not quite adjusted. Large spermathecae size was also observed in the inbred Kil females, (0.049mm) and the Mopti females (0.046mm). We have associated this large spermathecae size to be a laboratory selection trait, that enables Kil females to store the much sperm they uptake. A similar relationship has been documented by earlier research where the volume of spermathecae correlates with the sperm quantity that the females can retain (Klowden and Chambers 2004).

Sperm transfer and uptakes contribute actively to influence reproductive success. The amount of sperm in the male testes has been speculated to count towards reproductive success (Helinski an Knols 2009) and the presence of sperm in the spermathecae after mating has been proposed to trigger off female refractoriness to second insemination (Klowden 2006). With our newly developed assay based on qPCR, we quantify the actual sperm number transferred by the males and stored by the females. The qPCR technique offers a better likelihood of obtaining a more accurate result in sperm number transfers than the other existing methods like the manual counting sperm using phase contrast microscopes (Perez-Staples and Aluja 2006; Nakahara and Tsubaki 2007).

The mating plug is a very important part of male reproductive investment to ensure mating success. In that respect, there might be a correlation between the mating plug size and the amount of sperm quantity transferred. However, due to the transient nature of the mating plug, the quantity mating plug was measured using the two most important components (*Plugin* and *Transglutaminase)* that make up the matrix and the shell of the plug. The amount of seminal fluid transferred to females during mating can be affected by inbreeding in some species (Simmons and Beveridge 2011). The *Plugin* and

Transglutaminase proteins present in the female reproductive tracts were examined. In the first protein *Plugin*, there was no significant difference in the quantity of *Plugin* estimated across the treatment groups, the heterotic supermales, and inbred males transferred comparable amount of proteins to the females during mating. A significant difference was found in the quantity of the second protein. The less inbred Mopti males transferred more *Transglutaminase* that the heterotic supermales and the old inbred Kil males. This result indicates that probably the more *Transglutaminase* transfer by the Mopti males was an adaptive trait evolved by the strain to encourage proper sperm storage in their females. *Transglutaminase* has been reported to stimulate good sperm storage rather than act as a physical barrier to second mating (Roger *et al.* 2008). The fact that the much-inbred Kil strain, which transferred more sperm, had less of the *Transglutaminase* suggests no impact of inbreeding and secondly no effect of heterosis was witnessed. The Mopti stain being closer to the wild type might just be producing more *Transglutaminase* to adapt to lab rearing, ensure proper sperm storage and increase female fertility to avoid inbreeding effects.

No substantial difference was found in the number of eggs laid by females mated by the heterotic supermales compared to the inbred males and likewise in the number of larvae they produced. This implies no effect of heterosis and inbreeding. The setup was an overnight mating in large group cages, and not all the males might be very ready to mate. To ensure that all females were inseminated overnight we used a sex-mating ratio of 1:2 that is 100 females to 200 males and insemination rate was above 95% across all mating combinations. This is consistent with another study in *An. gambiae*, where similar sex-mating ratio was used (Gary *et al.* 2009). They used 1:2 female to male sex ratio (30 females to 60 males) and achieved 82% insemination rate over 5 mating days in the presence of sugar water. Sugar water according to Gary *et al.* (2009) increases

insemination potentials in 3day-old males. The idea that sugar water increases insemination rate in 3day-old males received the support of Charlwood and Jones (1979), they had 80% insemination in 3 mating nights using the same sex-mating ratio of 1:2 (50 females to 100 males in four replicates). Similarly, all our experimental groups were aged 3-5days and had access to 5% sugar solution. Despite the high insemination rate observed in our mating cages, only 14.5%-25% of the females lay eggs. Overall, 40 females lay eggs, and the mean number of eggs laid was 63.12 (CIs= 57.35-68.89). The average number of eggs per female was 19 to 125 eggs. There have been studies on the number of eggs laid in *An. gambiae*. Takken *et al.* (1998) related the number of eggs laid by the female to their body size and blood meal taken. They found that small females (2.63mm) take small blood meals (1.5μl) and lay 14 of eggs, while the large females (3.02mm) take large blood meals (2.0 μl) and lay 24.3 number of eggs. Hogg *et al.* (1996) observed 81.6 mean numbers of eggs in *An. gambiae* s.s females. In our experiment, after one mating night the females had a blood meal. No difference was found in the number of eggs laid by the females mated by the heterotic supermales compared to the inbred males and likewise in the number of larvae they produced. Therefore, no effect of heterosis or inbreeding was witnessed on female fecundity. We supposed that other factors affect egg laying apart from the ones mentioned. In addition to the overnight mating, the mating was performed in large-group cages, and we were not able to examine the individual male investments.

Overall, the amount of sperm number transferred did not correlate with sperm activity, nor was there any correlation between spermathecae size and sperm activities. Nevertheless, there was an isolated correlation found between the heterotic supermale mated with Kil female, the sperm number transferred showed a significant correlation to spermatheca size but this was not found in other mating combination. Unfortunately, we

are not able to explain why. It would have been interesting to compare the present subject of sperm activity in *An. gambiae* in vivo with closely associated species.

Conclusively, in this work, we looked into the sperm activity and quantity in out-bred hybrid supermales and compared it with the inbred males, inbreeding significantly reduces sperm activity but not sperm amount. The sperm transferred by heterotic supermales were more active compared to the sperm transferred by the inbred males. No effect of heterosis was witnessed in the sperm amount transferred. The difference observed in the spermathecae size of the two female strains served a reproductive function of storing more sperm. There was no correlation; all the variables were independent of each other. We detected MAGs in the form of the *Plugin and Transglutaminase* from the tip abdomen of freshly mated females in all the mating combinations; however, no significant difference was found in the quantity of the *Plugin*. In *Transglutaminase,* the quantity transferred differs significantly between groups, Mopti mated with Mopti transferred higher amount of *Transglutaminase.* This high level of *Transglutaminase* transferred had no effect on to the male reproductive success. The average number of eggs laid and the number of larvae hatched were the same across the four mating combinations. Finally, in the course of the research we found that some sperm cells were still not active after 24hr, and we don't why. Therefore, there is a need for further research on sperm activation in *An. gambiae*.

5.3 Section B: Individual reproductive success of hybrid supermales of

An. gambiae s.s in small mating cages.

5.3.1 Introduction

In section A of this chapter, we had evidence that the heterotic supermales transfer sperm that are more vigorous than their inbred parental strain. However, no significant difference was found between the reproductive success of the heterotic supermale and the two inbred parental strains in that large-group mating cage experiment. Here we designed another experiment to assess individual male investments of the heterozygote supermales and their inbred parental strains in small lab-mating cages. Males were combined individually with 20 females in small lab-cages for 3 mating nights in 4 separate treatment groups. We measured the insemination rate of individual male per cage in each treatment group, counted the number of eggs laid and larvae produced by mated females, and compared them across treatment groups. The total number of offspring (hatched larvae) produced by each male was used to determine individual male reproductive success. The mean sperm numbers transferred by hybrid supermale was quantified and compared against that of their inbred parental strains. Being able to quantify sperm investments of individual males will assist in defining the reproductive fitness of males, thereby determining their reproductive potential and mating success. Knowledge of reproductive biology of *An. gambiae* particularly male mating investments can help to design new tools for implementation of programmes aimed at managing mosquito-borne diseases.

5.3.2 Methods

5.3.2.1 Individual cage set up and experimental design

The male and female individuals used in all the mating combinations were generated as described in sections 2.2.6 and 2.2.7 of chapter 2. For the individual mating cages, 20 Kil males were transferred into a small polystyrene cup covered with a clean mosquito net, fastened properly with an elastic band and masking tape. They were provided with 5% glucose solution. These males were referred to as 'swarming males' (not involved in the mating). The cup was placed into a bigger cup (710ml) tied as above.
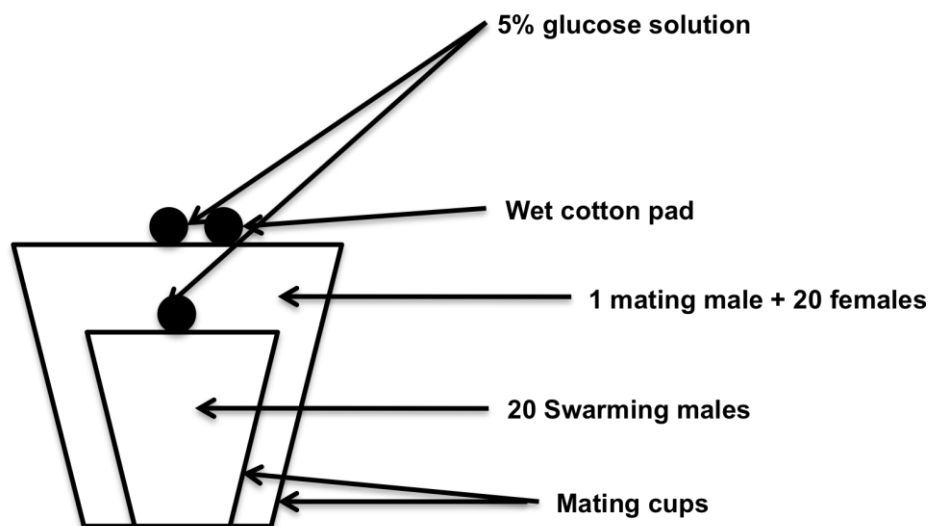
Fig 5.10 Individual male mating cage.

Using mouth aspirator 20 virgin Kil females were transferred into the 710ml cup. They were allowed to settle down. One hour before swarming time (usually about 7pm in the insectary), One virgin Kil male was introduced into the cup for mating with the 20 virgin Kil females for 3 nights (Fig 5.10). Similar mating cages were set for Mopti and hybrid supermale. For the hybrid supermale, one cage was combined with Kil females and the second cage was combined with Mopti females. Thus, the mating cages appear like the

following; 1Kil male x 20Kil females +20 swarming males and 1Mopti male x 20Mopti females + 20 swarming males, 1supermale x 20Kil females + 20 swarming males, 1supermale x 20Mopti females + 20 swarming males. One hundred cages were set up for four experimental groups, 25 mating cages for each treatment group (Fig 5.11). All the mating cages were provided with 5%, glucose solution on a cotton pad and a second cotton pad soaked with deionised water placed on top. Both cotton pads were checked and moistened daily. After 3 nights of mating, the males were removed using mouth aspirator and stored in 70% ethanol for winglength measurement. The surviving females were carefully transferred to another cage and blood fed, while the dead ones were removed, stored in 70% ethanol for determination of mating status, the swarming males were discarded. Each treatment group was uniquely labelled for easy identification.
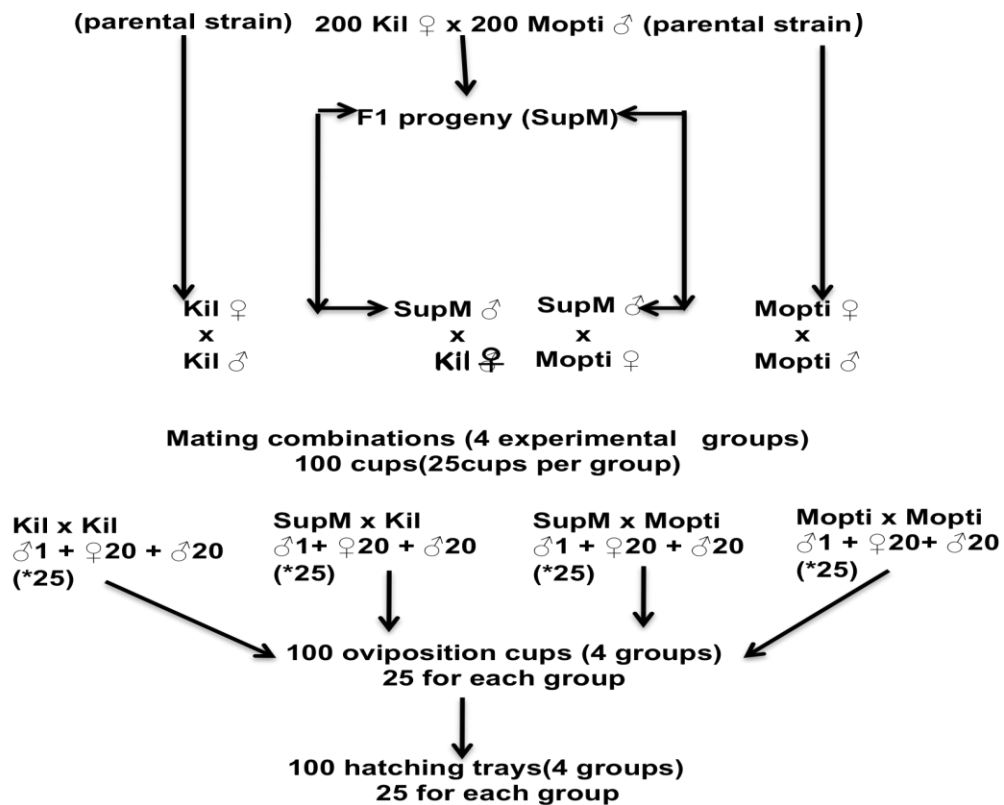
Fig 5.11: Experimental design and mating combinations in the individual-male reproductive success. Sample size for each treatment group was 25 cages. The mating combinations for the supermales were done with Kil and Mopti females in two separate treatment groups respectively.

5.3.2.2 Blood feeding

Following 3 nights of mating the females was blood fed with defibrinated horse blood (TCS Biosciences Ltd Ref.HB034) using an artificial membrane feeder (Hemotek membrane feeding system, discovery workshops, UK) (See chapter 2). Two days post blood meals, the small oviposition cups (lined with white filter paper) within the blood-fed cages were inverted to face up, and half filled with water using a plastic water dropper for egg laying.

5.3.2.3 Egg counting

After three nights of egg laying the females were removed using mouth aspirator and store in 70% ethanol. The number of eggs laid by the females in each treatment cup was counted. To count the eggs, the white filter paper with eggs and some first larval instars (some of the eggs laid on the first night were already hatching) was carefully placed on a clean petri dish that was clearly divided into 10 rows with permanent marker. They were altogether placed under a dissecting microscope. The eggs (plus the newly hatched larvae) were then counted manually using a tally counter. To make sure that all the eggs were counted, each dish was moved up and down and counted row by row. The counting was done twice and the average taken and recorded as the number of eggs laid by females per cage in each of the treatment groups. At the end of the counting, the eggs were washed into a small transparent plastic snack box with water dropper and extra 250ml of water was added. The plastic box with counted eggs were placed gently on the insectary shelve and covered with perspex lid

5.3.2.4 Hatching rate

The eggs of *An. gambiae* when maintained under of 22-27ºC moist conditions for about 1-3 days will hatch (Impoinvil *et al.* 2007). To count the hatched larvae, the transparent plastic snack box containing the newly hatched larvae was removed from the shelf and placed gently put on a white positioned directly under the bright fluorescent light in the insectary. The bowl was allowed to settle down and when no noticeable movement was detected, the larvae were manually counted, with the aid of a hand magnifying glass (Rolson hand tools UK). Using a tally counter, a click was made for each larva counted starting from one corner of the plastic snack box to the other end without moving the plastic snack box. All hatched larvae were removed from the box using 0.3ml plastic pipette before placing the box back on the shelf. The counting continued daily for 3 days and by the morning of day 4, unhatched eggs were recorded as infertile and discarded. All the average for each day was added and divided by 4 to give the hatching rate per group.

5.3.2.5 Insemination rate

Five cups from each treatment group were randomly selected after egg laying and the female spermathecae were dissected for sperm bundle and subsequent sperm quantification (See Chapter 2). Mated females were identified by the presence of coagulated sperm bundle (effect of ethanol on sperm cells) in their spermathecae, and absence of sperm bundle suggests unmated status. The sperm bundles were transferred to 500µl of lysis mix with 5µl of proteinase K for overnight incubation at 55ºC and subsequent gDNA extraction used for sperm amount quantification. The female carcasses of both mated and unmated females were individually stored in the -20ºC freezer for winglength measurement.

5.3.2.6 Statistical Analysis

All analyses were carried out using JMP 10 software (SAS Institute, Inc. USA). The data were examined for heterogeneity of variances and normality in distribution and using Shapiro-Wilkinson goodness of fit test. ANOVA was used to compare means of data that were normally distributed and were displayed as bar plots at 95% confidence interval. Tukey test was used for post-hoc pairwise comparisons with a significant difference. Log estimated means of sperm numbers were displayed as bar plots at 95% confidence interval. Frequency distribution was calculated with Chi-Square and expressed in percentage. Non-parametric tests Kruskal-Wallis was used to analyze data that deviated from normal distribution which were also displayed using medians, and interquantile range. In these graphs the lower whiskers were constructed as 25th percentile -1.5* interquantile range and upper whiskers as 75th percentile + 1.5* interquantile range.

5.3.3 Results

5.3.3.1 Individual male insemination rates

The mean number of females inseminated by individual males over 3 nights was examined in all the mating combinations. The mean number of mated females in the Kil x Kil mating combination was 10.13 (95%CIs= 9.43-10.84), Mopti x Mopti mating combinations were 7.95 (7.32-8.58), SupM x Kil mating combination was 8.60 (7.89-9.31), and SupM x Mopti mating combination was 8.24 (7.57-8.90). Statistical analysis revealed a significant difference in the mean number of females inseminated in 3 nights (ANOVA: n= 66, $F_{3,65}$= 7.983, $P < 0.001$) (Fig 5.12). Post hoc Tukey test within mating combinations revealed that Kil x Kil mating combination had significantly more mated females than SupM x Kil, SupM x Mopti and Mopti x Mopti mating combinations (Tukey: $P < 0.017$ in all cases). Individual mating cages in each treatment were examined for eggs laid two days post blood

meal and a significant difference was found (Chi-square: n= 66, df= 3, X^2= 14.447, P= 0.002). The result revealed that 4 of the individual male mating cages in the Kil x Kil mating combination had no eggs laid. Suggesting that ~ 26.7% of Kil males might be sterile.
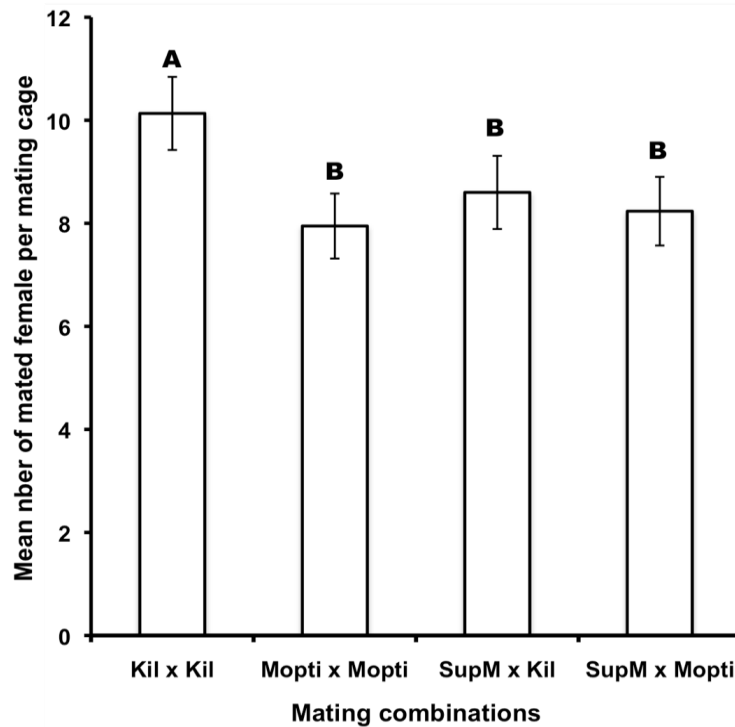
Fig 5.12: Mean number of inseminated females per individual mating cages in the Kil x Kil, SupM x Kil, SupM x Mopti and Mopti x Mopti mating combinations. Each error bar was constructed at 95% confidence interval.

5.3.3.2 Mean number of eggs per female per mating cage

5.3.3.2.1 Cages with eggs

The mean number of eggs per mated female per mating cage in individual male mating cages was 4.81 (95%CIs= -3.97-13-58) in the Kil x Kil mating combination, Mopti x Mopti mating combination was 22.60 (15.95-29.31) SupM x Kil mating combination was 15.49 (7.97-23.00) and SupM x Mopti mating combination was 29.33 (22.27-36.39) (Fig 5.13).
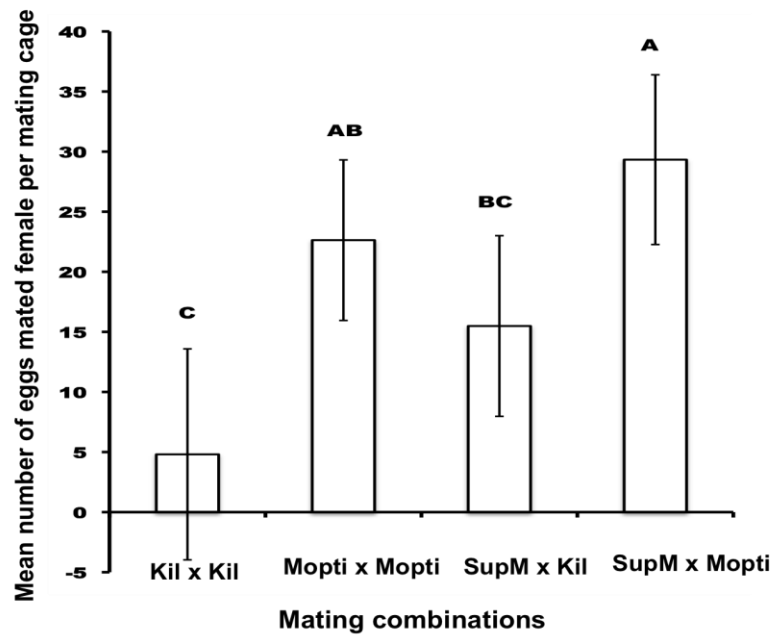
Fig 5.13: Mean number of eggs per mated female in individual male cages in the four mating combinations of Kil x Kil, SupM x Kil, SupM x Mopti, and Mopti x Mopti. Each error bar was constructed at 95% confidence interval. Significant differences are represented by different letters.

There was a significant difference in the mean number of eggs per mated female per cage in the four mating combination (ANOVA: n= 62, $F_{3,61}$= 7.006, P = 0.004). Post hoc Tukey test within the mating combinations showed that SupM x Mopti combination mating cages had significantly more number of eggs per mated female than Kil x Kil combination mating cages and SupM x Kil combination mating cages (Table 5.3).

5.3.3.2.1 All cages including those with zero eggs

A significant difference was observed in the mean number of eggs per mated female in all cages including those with zero eggs (ANOVA: n= 66, $F_{3,65.}$= 9.747, P < 0.001). Tukey post hoc tests showed that SupM x Mopti mating combination had less number of mated females with zero eggs laid than Kil x Kil mating combination and SupM x Kil mating combination (Table 5.4).

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Table 5.3: Post hoc Tukey test for mean number of eggs per mated female per cage in the mating combinations of Kil x Kil, SupM x Kil, SupM x Mopti, and Mopti x Mopti. Values with * shows significant difference.

Mating combinations	Mating combinations	CIs (95%)	P-Value
SupM x Mopti	Kil x Kil	9.638-39.408	0.004*
Mopti x Mopti	Kil x Kil	3.250-32.399	0.011*
SupM x Mopti	SupM x Kil	0.216-27.470	0.045*
SupM x Kil	Kil x Kil	-4.589-25.950	0.261
Mopti x Mopti	SupM x Kil	-6.142-20.431	0.491
SupM x Mopti	Mopti x Mopti	-6.144-19.541	0.517

Table 5.4: Post hoc Tukey test of mean number of mated female per cage with zero in the mating combinations of Kil x Kil, SupM x Kil, SupM x Mopti, and Mopti x Mopti. Values with * shows significant difference.

Mating combination	Mating combination	CIs (95%)	P-Value
SupM x Mopti	Kil x Kil	15.817-35.793	< 0.001*
Mopti x Mopti	Kil x Kil	9.368-28.845	0.002*
SupM x Mopti	SupM x Kil	3.855-23.831	0.007*
SupM x Kil	Kil x Kil	1.667-22.257	0.024*
Mopti x Mopti	SupM x Kil	-2.594-16.883	0.148
SupM x Mopti	Mopti x Mopti	-2.714-16.112	0.160

5.3.3.3 Egg hatching rate per cage

The frequency of egg hatching was examined in all cages where eggs were laid in the four experimental groups and no significant difference was found (Chi-Square: n= 62, df= 3, X^2= 1.229, P= 0.746). However, the Mopti x Mopti mating combination achieved the lowest egg-hatching rate of 68%. Old inbred Kil x Kil mating combination achieved 75%

egg hatching rate and the two heterotic supermale mating combinations (SupM x Mopti and SupM x Kil) achieved 83% and 73% respectively.

5.3.3.4 Mean number of larvae per mated females per mating cage

5.3.3.4.1 Cages with larvae

The overall mean numbers of larvae per mated female was 3.78 (95%CIs= -2.81-10.39) in Kil x Kil mating combination, 13.09 (7.38.32-18.81) in Mopti x Mopti mating combination, 13.45 (7.48-19-42) in SupM x Kil mating combination, and 11.08 (5.79-16.37) in SupM x Mopti mating combinations. There was no significant difference in the mean number of larvae per mated female in each mating combination (ANOVA: n= 46, $F_{3,45}$= 2.021, $P = 0.126$).

5.3.3.4.2 All cages including those with zero larvae

The overall mean numbers of larvae per mated female was 2.27 (95%CIs= -2.67-7.22) in Kil x Kil mating combination, 8.27 (3.88-12.66) in Mopti x Mopti mating combination, 9.86 (4.94-14.82) in SupM x Kil mating combination, and 9.12 (4.48-13.77) in SupM x Mopti mating combinations. There was no significant difference in the mean number of larvae per mated female in each mating combination (ANOVA: n= 66, $F_{3,65}$= 1.998, $P = 0.124$).

5.3.3.5 Sperm numbers transferred

The mean estimated sperm numbers in Kil x Kil mating combination was 916 (95% CIs= 756-1074), Mopti x Mopti mating combination was 1058 (871-1246) SupM x Kil mating combination was 1266 (1115-1418) and SupM x Mopti mating combination was 803 (560-1046) (Fig 5.14). The effects of treatment on sperm numbers invested by males in the four

experimental groups were analyzed, and a significant effect of treatment was found (ANOVA: n= 20, $F_{3,19}$= 5.585, P= 0.008). Tukey Post hoc tests on least square mean on showed that SupM x Kil mating combination had significantly more sperm number than SupM x Mopti mating combination (Tukey: n=10, P= 0.006).
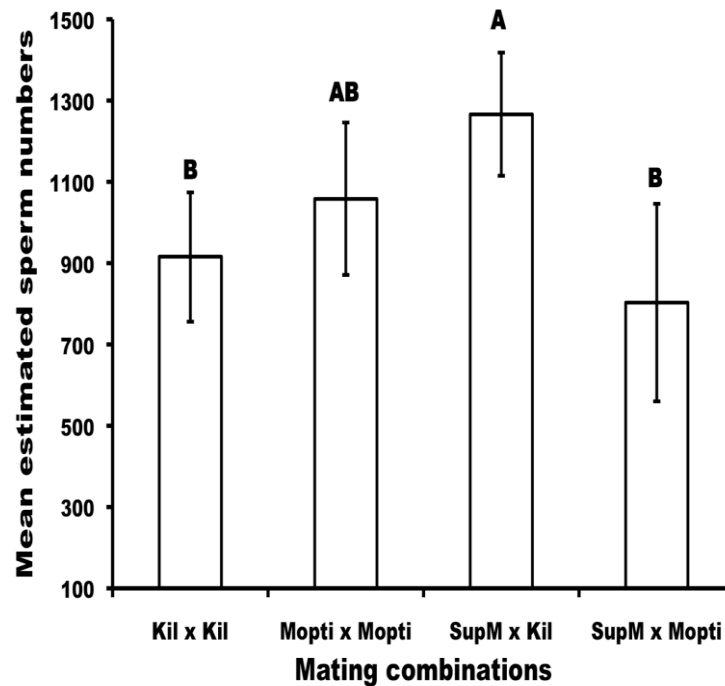
Fig 5.14: Mean estimated sperm numbers recovered in spermathecae of females from the four mating combinations. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.3.3.6 Male reproductive success

The male reproductive success was determined in all the mating cages. This includes those cages where mated females laid eggs, cages where eggs laid did not hatch and cages with hatched larvae. A significant difference was found in the male reproductive success in the four treatment groups (ANOVA: n= 66, $F_{3,65,}$= 4.380, P = 0.007). Overall, mean number of larvae per male in Kil x Kil mating combination was 23 (95%CIs= -13.51-61.11) in Mopti x Mopti mating combination it was 71.56 (38.43-104.73) in SupM x Kil mating combination it was 96.73 (59.42-134.04) and in SupM x Mopti mating combinations it was

111.15 (76.10-146.19) (Fig 5.15). Tukey Post hoc tests within the mating combination showed that SupM x Mopti and SupM x Kil mating combinations had significantly more number of larvae produced than Mopti x Mopti and Kil x Kil mating combination (Tukey: $P < 0.037$ in both cases).
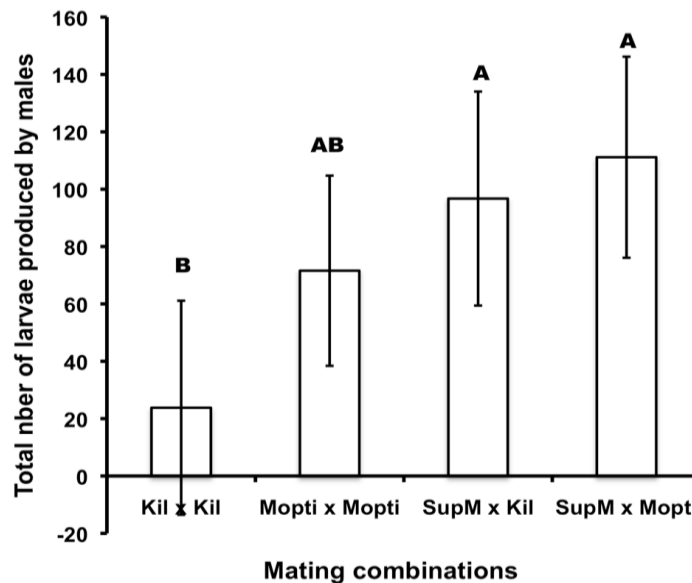
Fig 5.15: Overall male reproductive success per mating combination in the four experimental groups. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.3.3.7 Male reproductive success between heterotic supermales and inbred males

5.3.3.7.1 All cages with eggs laid, hatched eggs and larvae produced and unhatched eggs

The reproductive success of the heterotic supermales and their parental inbred male strains were compared in cages where mated females laid eggs, cages where eggs laid did not hatch and cages with hatched larvae. A significant difference was found (ANOVA: n= 66, $F_{1,65}= 8.889$, $P= 0.004$). The heterotic supermales achieved significantly higher reproductive success than the inbred males. The mean reproductive success for supermale was 104.39 (78.47-130.31) and the inbred male was 50.50 (25.35-75.65) (Fig 5.16). The overall frequency of reproductive success was 78.1% for supermales and 61.8% for the inbred males.
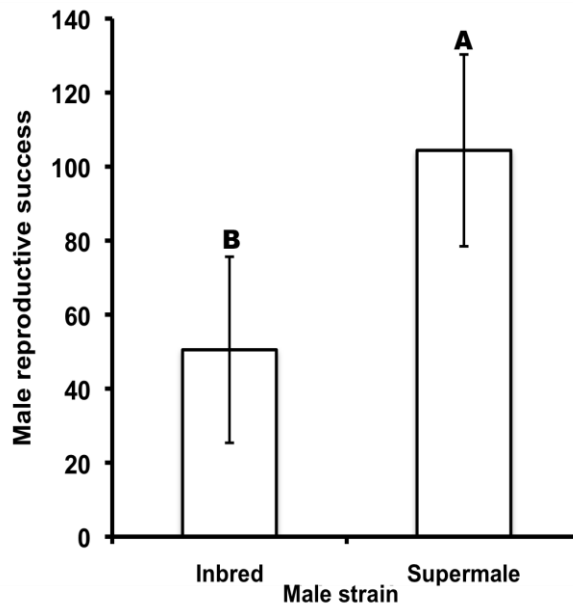
Fig 5.16: Overall male reproductive success between the heterotic supermales and the inbred males. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters

5.3.3.7.2 Number of mated females

There was no significant difference found in the mean number of mated females over 3 nights between the heterotic supermale and their inbred parental strain (ANOVA: n= 66, $F_{1,65}$= 1.701, P= 0.197). The mean number of females mated by heterotic supermale was 8.41(7.85-8.96) and the inbred male was 8.92 (8.37-9.45).

5.3.3.7.3 Male fertility

Egg laying frequency was compared between the two male strains and a significant difference was found (Chi-Square: n= 66, df= 1, X^2= 4.005, P= 0.045). The egg laying frequency was 100% for the heterotic supermale and 88% for the inbred males.

5.3.3.8 Total number of larvae produced

A significant difference was found in the total number of larvae produced between the heterotic supermale and their inbred parental strain (ANOVA: n= 46, $F_{1,45}$= 6.982, P= 0.011). The heterotic supermales produced significantly more number of larvae than the inbred males. The mean number of larvae for supermale was 133.62 (106.89-160.35) and the inbred male was 81.76 (52.60-110.92).

5.3.3.9 Female reproductive success

5.3.3.9.1 Mating success

The number of females inseminated over 3 nights was compared between the two females strains. The Kil females strain had significantly more females inseminated than the Mopti females strain (ANOVA: n= 66, $F_{1,65}$= 1.701, P= 0.007). On the average, Kil had 9.39 (8.83-9.89) females inseminated per cage and Mopti had 8.08 (7.60-8.60) females inseminated per cage (Fig 5.17).
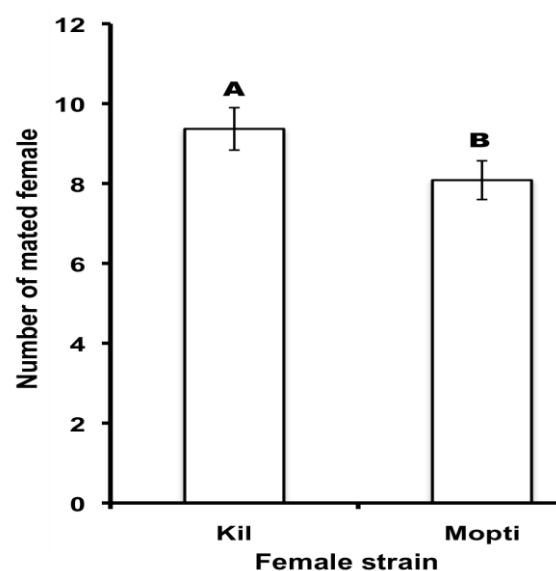
Fig 5.17: The mean number of mated females per strain over 3 nights. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.3.3.9.2 Egg laying rate per mated female

The mean number of eggs laid by Mopti females was 25.79 (20.81-30.78) and that of Kil females was 10.97(5.11-16.83) (Fig5.18). Overall, the Mopti female strain had significantly more eggs laid per mated female than the Kil females strain (ANOVA: n= 62, $F_{1,65}=$ 14.866, $P=$ 0.003).The Mopti females had 100% egg laying while Kil females had 86% egg laying.
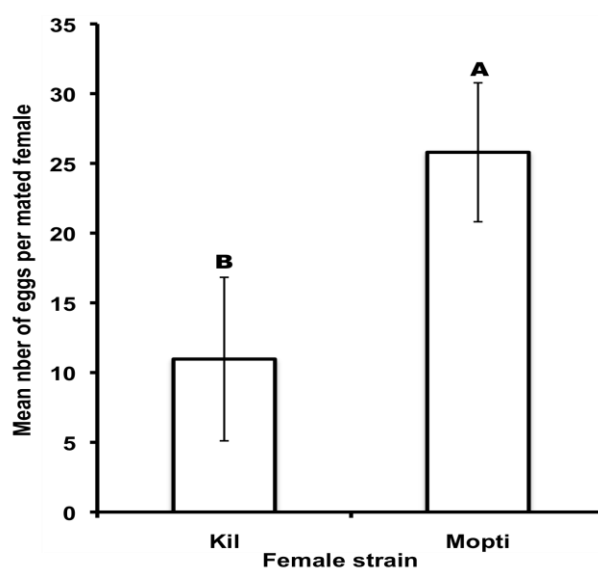
Fig 5.18: The mean number of eggs laid per mated female strain (Mopti and Kil). Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.3.3.9.3 Number of hatched larvae per mated female strain

There was no significant difference in the mean number of larvae that hatched in either of female strains (ANOVA: n= 46, $F_{1,45}=$ 0.926, $P=$ 0.341).The mean number of hatched larvae per mated Mopti females was 12.01(8.00-16.02) and that of Kil females was 9.10(4.53-13.68)

5.3.4 Discussion

In this study, we found that old inbred Kil males achieved significantly high insemination rate than Mopti males and the heterozygote supermales. Kil males were also thought to be frequently sterile due to inbreeding since some of the mating cages had no eggs laid by the females after 3 mating nights. Females mated by the heterotic supermales produced significantly more eggs than females mated with the inbred males, but no significant difference found in the egg-hatching rate in all the experimental groups. The sperm number recovered from the female spermathecae was significantly more in Supermale mated with Kil female than in the other experimental groups. There was a significant increase in the mean number of larvae fathered by the heterozygote supermale compared to the inbred Mopti and Kil male strains. The heterotic supermales achieved significantly higher male reproductive success than the inbred males. These hypotheses were tested by combining females from the inbred Kil and Mopti strain, with the heterotic supermales in two different cages and at the same combining the inbred females with their own inbred males in another two cages. We assumed that if two inbred strains are crossed, homozygous recessive deleterious alleles at the over dominant loci will be masked resulting in heterozygous advantage which will cause an increase in the reproductive success of the hybrid males. However, where this fails the number of offspring produced by both male strains would be comparable.

Reproductive activity is one of the most challenging characteristics to transfer successfully under laboratory condition (Baker 1964). In this study, it was observed that the male insemination rate was significantly higher in the very old inbred strain than the freshly colonised strain and the heterozygote hybrid supermales. Variations in traits are gradual process through years of colonisation. By founder drift and selection models, it was observed that colonised strains become homogenous and genetically distinct from the

field population Benedict *et al.* (2009). Norris *et al.* (2001) believed that this might not represent the entire picture of what is happening because there are additional counter forces, which must be considered. In most insects, fitness can be measured by various biological parameters like fertility, growth, and mating competitiveness (Massonnet-Bruneel *et al.* 2013). Ferguson *et al.* (2005) documented that insect rearing under laboratory condition can result in a decrease in mating competitiveness due to inbreeding depression and diversity loss due to genetic restrictions. The high mating frequency observed in the old inbred Kil strain suggests an evolved adaptation to the laboratory rearing overtime. Similar observations of adaptation to the laboratory rearing in *An. gambiae* have been documented by Baeshen *et al.* 2014. On the average, the Kil males could inseminate up to 3 females per night. This observation is compatible with the findings of Giglioli and Mason (1966), the males of Anopheline inseminate up to 5 males per night, but in contrast with the findings of Kuriwada *et al.* (2011) where male mating performance of West Indian sweet potato beetles *Euscepes postfasciatus* was reduced to 6.3% per night due to inbreeding,

The old inbred Kil had more of their female strain inseminated than Mopti strain. This we also believed to be an adaptation to lab rearing by the females due to years of colonisation. Baeshen *et al.* (2014) observed changes in the size of male reproductive organs in laboratory reared *An. gambiae* due to years of colonisation. The very old inbred Kil strains had bigger testes size and smaller male accessory gland. They explained that such changes were evolved due the peculiar mating environment associated with insectary rearing. They also suggested that these changes might impact on male fitness thereby effecting mating competitiveness.

Fixation of deleterious alleles occurs in populations because of bottlenecking in that population (Hedrick and Kalinowski 2000). Inbreeding has a greater effect on fitness

characteristics such as fecundity and rate of development (Roff 1998), and may lead to decrease in fitness of progeny (Futuyma 2009). In *Aedes geniculatus,* fertility was low under lab condition and than wild due to inbreeding (Armbruster *et al.* 2000). We proposed that reduction in egg laying frequency in females mated by the inbred strain was due to inbreeding effect. Egg laying frequency was higher in females mated with supermales than females mated by inbred males in the four experimental groups. Females mated by supermales had a 100% increase in egg laying compare those mated by the very old inbred Kil males which had a 88% increase in egg laying . This is consistent with another study in seed-feeding beetle *Stator limbatus* where egg laying frequency was reduced to 54% due to sibling mating, but increases to 80% in outbred males (Fox and Scheibly 2006).

Inbreeding affects fecundity greatly (Roff 1998) and this effect in inbreeding trait is a gradual process through years of colonisation (Benedict *et al.* 2009). Egg laying frequency between Mopti females and Kil females revealed that Mopti females had a higher egg laying frequency than Kil females. Though the two female strains are inbred, Mopti females (10yrs+) are younger in colonisation than Kil females (35yrs+). Therefore, Mopti females with their high egg laying frequency might retain such trait from the field and or might not have been strongly inbred at the time of this study. Inbreeding extends the growth period and lowers reproductive success (Kerstes and Wenger 2011). It has been reported that adults of *Stator limbatus* produced by inbred required about >5% extra development time and this may be due to recessive deleterious alleles which affects hatching of eggs (Fox and Scheibly 2006). Observations by Liu *et al.* (2014) pointed out that in cabbage beetle *Calaphellus bowringi* females mated by sibling males (SS) had a lower hatching success of 63% compared to the same females mated with non sibling males (NN) which have 71% hatching success. In our experiment, hatching success was high in all the groups and no significant difference was found. The genetic make up of

females are known to affect early development and age affects egg-to-adult survival in *Drosophila* (Fry *et al.* 1998; Pekkala *et al.* 2014). In this study, no difference was found in hatching between the very old inbred Kil and the younger inbred Mopti. Thus, there does not seem to be an effect of inbreeding on that trait (hatching rate). In the supermales vs inbred males, no differences were found in the hatching rate of two inbred females (Kil and Mopti) mated by the heterotic supermales and their own inbred males. Therefore, no positive effects of heterosis on the early development of the offspring were detected.

Although high insemination rate was observed in the Kil male due to adaptation to laboratory rearing conditions, some of the males were thought to be sterile. 4 of the individual male mating cages had no eggs laid in them. Reduced fertility in males owing to inbreeding depression has been reported in some insects. In *Drosophila simulans,* inbreeding has a negative effect on the males. Male fertility i.e. number of offspring an outbred male mated with unrelated female produced was considerably more than the ones produced by inbred males (Okada *et al.* 2010). In damselfly *Ischnura senegalensis,* Nakahara and Tsubaki (2007) found that the inbred males transferred more dead sperm to the female than wild males during mating accounting for a significantly lower fertility of the females they mate. These reports are consistent with our finding. Inbred males of Kil have high mating frequency, but they appeared to sometimes transfer only infertile sperm, which seems to be reflected in the lack of egg-laying in some females and could have possibly also affect the number of eggs laid per mated female.

Sperm numbers recovered from the female spermathecae were quantified. The result showed a significant decrease in sperm numbers recovered from Mopti females mated with the supermale than the Kil females mated by supermales. We think that probably since the Kil females have adapted to receiving partially sterile sperm, they might be taking up more sperm than Mopti upon mating to compensate for the sterile sperm and

hence suggesting a potential selection for this trait. Thus, the difference in the quantity of sperm recovered from their spermathecae.

It is expected that the effect of heterosis should be strong on our measurement per male reproductive success because this variable is the result of all the other variables (insemination rate, sterility, low egg hatching rate and lower female fecundity). A 3.4-fold increase was detected in the mean number of larvae fathered by heterotic supermales compared to the inbred Kil males in the four experimental groups These increases in the number of offspring produced by the heterotic supermales were as of result of hybrid vigor. Further analysis between male strains (supermale and inbred) revealed a 17% increase in male reproductive success of the heterotic supermale. Overall, the mean number of larvae fathered by the heterotic supermales was significantly more than the larvae produced by the inbred males. This is in agreement with another study, where heterozygote lines had 13% higher male fertility than the homozygote lines when male fertility was determined by the number of crosses producing offspring out of 10 single mating pairs (Fry *et al.* 1998). This implies that reduced male reproductive success witnessed due to inbreeding can be recovered through hybrid vigor and that male reproductive success maybe less dependant on male size and male insemination rate but on other factors such as male fertility.

In conclusion, inbreeding did not affect all reproductive traits. The high insemination rate in inbred males is an adaptation trait to laboratory rearing due to colonisation. Reproductive trait such as the egg laying frequency was grossly affected by inbreeding whereas the hatching rate was not affected at all. Inbreeding and laboratory rearing resulted in reduced male reproductive traits such as egg laying frequency and number of larvae produced. These traits were improved by heterosis in supermales, which resulted in increase in female fecundity and overall male reproductive success. The impact of

laboratory rearing may pose problems to the vector control projects. Through heterosis, out bred crossing may be one fast and easy way to solve the problem and produce males with high reproductive success particularly for the GMM programmes, where male offspring will be required to carry a gene construct to the next generation.

5.4 Section C: The effects of inbreeding on sex peptide transfer in experimental swarms and survival in heterotic supermale of *An. gambiae s.s*

5.4.1 Introduction

In section A of this chapter, we measured the quantity of mating plug transferred by inbred and supermale based on the two most abundant proteins *Plugin* and *Transglutaminase*. We found no difference in the quantity of *Plugin* in all the treatment groups but the less inbred Mopti males transferred more *Transglutaminase* than the heterotic supermales and the old inbred Kil males. No effect of heterosis was seen. Given that it was an overnight mating with many males, more so we were not able to determine if that was the first mating or second mating because *An. gambiae* males are capable of inseminating up to 5 females per night (Giglioli and Mason 1966). Therefore, we designed a method to capture individual mating couples in experimental swarms induced in large mating cages and measured the size (surface area) of the mating plug transferred by male strains (supermales and their two inbred parental strains) to females during mating. The plugs and sperm measured here were first mating to determine if sperm quantities are dependant on the plug size deposited. Sperm numbers transferred were estimated using qPCR as in previous sections. Limited information is available on mating plug investment by male *An. gambiae* and its consequences on male reproductive success. The awareness of inbreeding effects on mating plug size and sperm investments may produce some new facts on male reproductive success which may be important to the development of novel approaches for vector control, particularly now that recent evidence suggests that variation in ejaculate investment might force some females to go back to swarms to re-mate.

Evidence on the deleterious effects of inbreeding on lifespan in insects has been described in many studies, but not many have looked into effects of inbreeding and

heterosis on survival in *An. gambiae*. Here, we experimentally tested the effect of inbreeding on the survival of the heterotic supermales and their two inbred parental strains using two experimental groups. One group was subjected to desiccation stress in an incubator while the other group was kept back in the insectary under optimum condition. Both groups were monitored until death. It is intended that the result would provide knowledge on the survival fitness of the heterotic supermales.

5.4.2 Methods

5.4.2.1 Mating combinations

Adults used in creating the mating combinations were produced according to the rearing protocols referred to in the preceding sections (A and B). Four mating combinations were prepared. Kil and Mopti males were combined with their own female strains in the first two mating combinations. The heterotic supermales were then combined with Kil and Mopti females in two other separate mating combinations. Using mouth aspirator at about 3pm, 250 virgin males were transferred into 5liter mating cages. They were provided with water and 5% glucose solution and wet cotton wool pad on top of the cages. One hundred virgin females were transferred into small polystyrene cups covered with mosquito netting and tied up with an elastic band. They were provided with 5% glucose solution and water using cotton pads and placed on top of the cups. The entire set up was set-aside on the insectary shelf until about 7pm before swarming time in the insectary. Overall, 1000 virgin males and 400 virgin females were used to set up 4 different mating combinations per replicate. The entire experiments were done in four replicates over 12 weeks. Each mosquito cohort was reared under the same insectary environmental condition. For the hybrid supermales, a new F1 progeny was generated for each replicate and used. The

rearing of the parental strains in all the crosses were synchronized to fit into the general rearing regime for each replicate.

5.4.2.2 Capturing of mating pairs

At about 7pm, the polystyrene cups with 100 virgin females were released into the mating cages for swarming and mating. The releases of the females into the male cages were performed simultaneously in the four mating combinations with help from other researchers within the research group. The released females promptly recovered from the transfer within a couple of minutes. During the capturing of the mating pairs, the insectary door was left slightly open to create enough illumination to see mating pair and at the same time not disrupt the swarming. The first mating pairs where typically observed about 2min after introducing females. Each cage was watched closely by a member of staff to capture mating pair in copula (Fig 5.17) with mouth aspirator as the mosquitoes swarm. Once caught the couples were allowed to complete the mating process inside the aspirator to ensure complete sperm transfer and mating plugs. Mating was assumed completed once the couple disengage. Each cage was observed for 30min, couples caught within the 30min period were knocked down on ice, transferred to 1.5ml centrifuge tube with 200µl of protease inhibitor and stored at -20C freezer for dissection later.

http://www.toptenz.net/top-10-facts-that-may-surprise-you-about-mosquitoes.php

Fig 5.19: Mating pair in copula.

5.4.2.3 Dissection and measurement of mating plug size

The samples were dissected in subsets of 10 per mating combination. Each set was removed from -20 freezer and allowed to thaw gradually on ice. Once thawed, the female was picked up and placed on a clean slide with a drop of PBS solution. The dissection was carried out under a Lieca EZ4 binocular dissecting microscope (Lieca Microsystems GmbH, Solms, Germany). The mating plug is seen blocking the atrium of the female genitalia on the last abdominal segment (Fig C2). A gentle downward push was made with the dissecting needle starting from the third segment and firmly pressing down the thoracic part of the body with the second dissecting pin. The mating plug comes out with some other tissues and the spermatheca. The dissected plug was promptly transferred to a phase contrast microscope (Olympus phase contrast culture microscope CKX41), and quickly positioned in the center of the viewing field using 400x magnification. Then adjusted to x1000 magnification at 9612 resolutions, using phase contrast slider 1x2-SL and the image of the plug was viewed and captured on the laptop screen (MacBook Pro laptop computer (Apple Inc.). To determine the size of the mating plug, the surface area (Fig 5.18) of the plug was measured twice using the imageJ 1.44 software (http://en.softonic.com/s/imagej-1.44:mac 2013). The average measurement was taken and converted to the nearest 1mm.
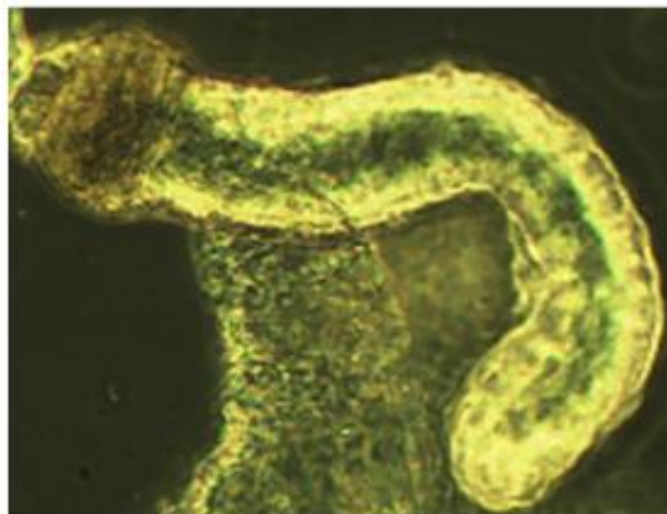
Fig 5.20: Mating plug from freshly mated female *An. gambiae s.s*

Due to the sensitive nature of the proteins that constitutes the mating plug (Rogers *et al.* 2009), as soon as the image of the plug was captured, the plug was carefully pickup and transferred into 1ml permanent storage with 200µl freshly prepared protease inhibitor and place back on the icebox till all the individuals in that set are dissected. The tubes with mating plugs were stored in -80 freezers for future analyses. Thereafter, the spermatheca was transferred into a 1.5ml centrifuge tube containing 500µl of lysis buffer and 5µl of protease K for overnight incubation on a heat block at 55ºC (Grants QBD2) and subsequent extraction of gDNA for sperm number estimation (See previous sections).

5.4.2.4 Survival of hybrid supermales

The survival of the heterotic supermales were explored and compared with their inbred parental male strains Kil and Mopti. The 3 types of adult males were kept under two contrasting environmental conditions (insectary and incubator). The heterotic supermales were generated as described in the previous sections by crossing 200 Kil females with 200 Mopti males for two nights of mating. The rearing and sexing was the same as in the adults used in experimental swarms. To ensure that all the adults used were of the same age the cups with the unmerged pupae were removed from the cages the next morning. The two experiments commenced 24h post emergence. Random samples of 120 males were taken from the three male types (Kil, Mopti, and supermale). They were transferred into a medium sized (296ml) polystyrene cups 20 individuals per cup. The cup was covered with mosquito net and fastened with an elastic band. For each male type three cups without water or sugar solution was placed in the incubator at 30ºC and 30% relative humidity and monitored every 3h until death. This group was referred to as the stressed group. The remaining 9 cups (3 cups for each male type) were kept in the insectary under optimum conditions (75-80% RH, 26±1ºC, 12:12 photoperiod). They were supplied with water and

5% glucose solution using cotton pads. They were referred to as the non-stressed group. The cups were reshuffled daily to avoid any confounding factor in the insectary. Every morning the cups were examined and dead mosquitoes were removed and kept in centrifuge tubes individually for winglength measurement.

5.4.2.5 Statistical analysis

The data sets were checked for normality in the distribution and heterogeneity using goodness of fit test. The plug sizes were compared using ANOVA. Tukey Post hoc tests was conducted on the effects with significant difference. Replicates and interactions were reported where significant. Non-parametric Wilcoxon sum rank tests were used to test the differences in survival between male types and treatments. Survival curve distributions were displayed by product-limited fit. Cox model proportional hazards analyses were used to test for effect of male types and body size on survival and Risk Ratios were used to compare survival hazard risk between significant effects.

5.4.3 Results

5.4.3.1 Mating plug size

Across all replicates and mating combinations, two hundred mating pairs were captured and 186 females were successfully dissected for mating plugs. The mean plug size for Kil mated with Kil females was 0.033 (0.032-0.034), Mopti mated with female Mopti, 0.036 (0.034-0.038), Supermale mated with Kil female 0.036 (0.034-0.038) and supermale mated with Mopti females was 0.037 (0.036-0.039). The size of plug transferred differed significantly between groups (ANOVA: n= 186, $F_{2,185}$= 4.70, P= 0.004). Post hoc Tukey test conducted on the mean revealed that the supermale mated with Mopti transferred

significantly larger mating plug than Kil mated with Kil (Tukey: n= 95, *P*= 0.003). Mopti mated with Mopti transferred significantly bigger mating plug than Kil mated with Kil (Tukey: n= 94, *P*= 0.049) (Fig5.21).
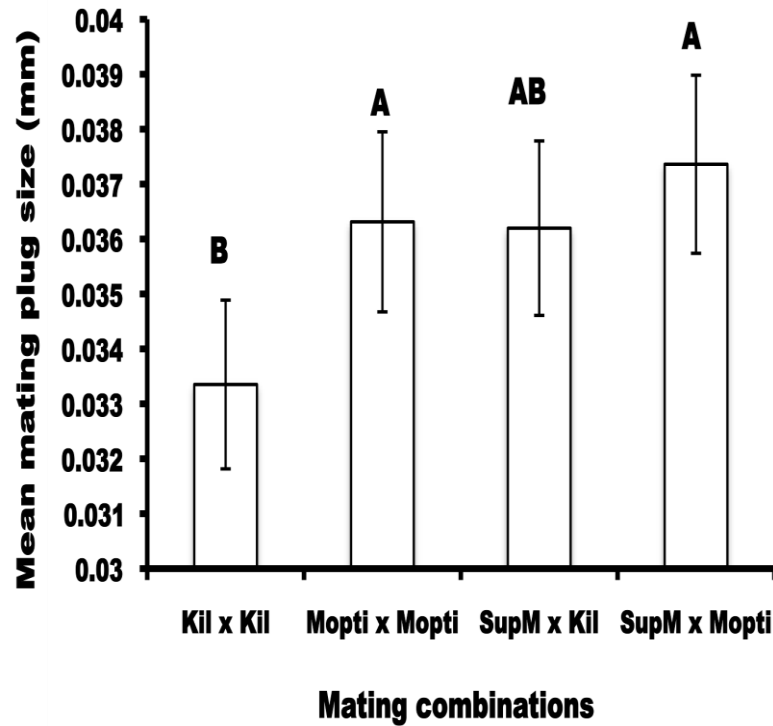
Fig 5.21: Mean mating plug size deposited by males in the four mating combinations (Kil x Kil, Mopti x Mopti, SupM x Kil and SupM x Mopti). Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.4.3.2 Mating plug size in supermale and inbred male

Mating plug size was compared between the supermales and inbred males and a significant difference was found (AONVA: n= 186, $F_{2,185}$= 6.192, *P*= 0.014). The supermales transferred significantly larger mating plug than inbred males. The mean plug size for supermale was 0.037mm (95% CIs= 0.035-0.038) and the mean plug size for inbred male was 0.035 (0.034-0.036) (Fig 5.22).

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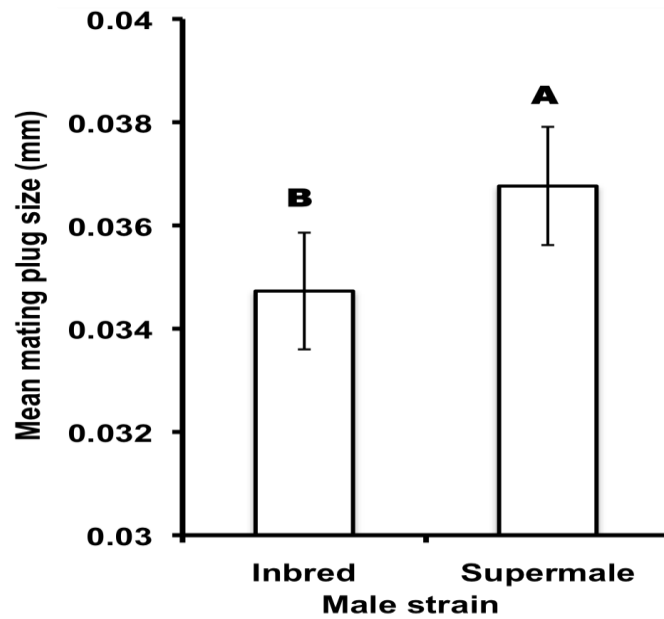
Fig 5.22: Mean mating plug size deposited by supermales and inbred males. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters.

5.4.3.3 Mating plug size in Kil and Mopti female

A significant difference was found in the mating plug size deposited in Kil females and Mopti females (ANOVA: n= 186, $F_{2,185}$= 6.370, P= 0.013). The Mopti females had bigger mating plugs than the Kil females. The mean plug size for Mopti female was 0.037mm (95% CIs= 0.035-0.038) and the mean plug size for Kil female was 0.035 (0.034-0.035) (Fig 5.23).
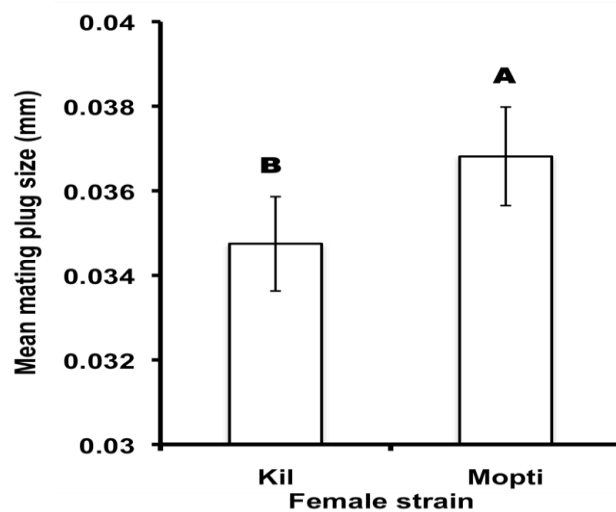
Fig 5.23:Mean mating plug size deposited in Kil and Mopti females. Error bars were constructed at 95% confidence interval. Significant differences are represented by different letters

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5.4.3.4 Double plugs

The frequency distribution of double mating plug observed in the experimental groups was examined. The frequency showed no significant difference (Chi-Square: n= 200, df= 3, X^2= 6.452, P= 0.092). Mopti mated with Mopti females had 12% double plugs and 88% single plug. The supermale mated with Mopti female had 10% double plug and 90% single plug and Supermale mated with Kil females had 6% double plug with 94% single plugs. The Kil mated with Kil female had no double plug. All the plugs deposited were 100% single plugs. Overall, the mean size of the double plug was 0.024 (95% CIs= 0.022-0.027). The second plug was generally smaller than the first plug (Fig 5.24).
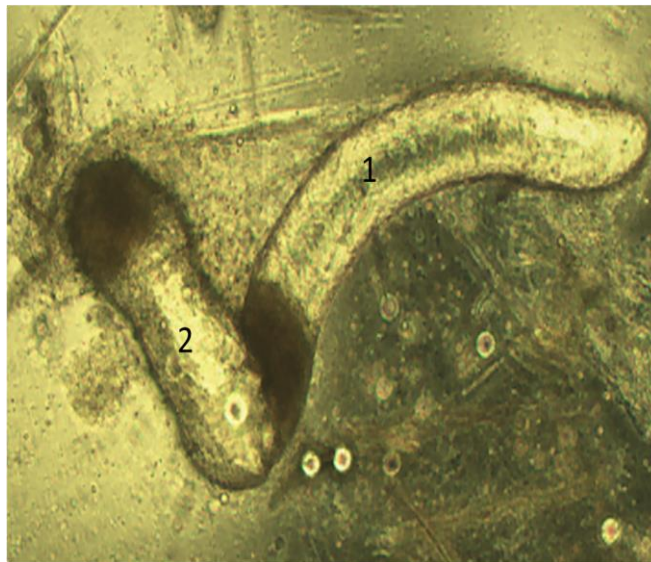
Fig 5.24: Double mating plug from *An. gambiae* female caught in experimental cage swarm.

5.4.3.5 Sperm quantification

There was no significant difference in sperm number transferred in the four mating combinations but the replicates were significantly different (ANOVA; treatment: n=182, $F_{3,181}$= 0.305, P= 0.822; replicate: n=182, $F_{4,181}$= 9.363, P< 0.001). The overall mean sperm number transferred was 28515 (95% CIs= 24390-432640) and mean sperm numbers transfer by Kil mated with Kil female was 24923 (1898-30875) Mopti mated by Mopti

32297 (20652-43394) supermale mated with Kil 34094 (24736-43450) and supermale mated with Mopti 23196 (17379-29013).

5.4.3.6 Overall correlations between plug size, sperm numbers, male and female body size

Pearson correlation was conducted excluding the mating pairs with double plugs. There were no correlations between mating plug, sperm numbers, male and female body size in the four treatment groups, (Pearson: n= 168, $P > 0.067$ in all cases). However, there was a significant positive correlation between male and female body size (Pearson: n= 171, $P < 0.001$). Within treatments, a significant correlation was observed between male and female body size in all the groups ($P < 0.034$ in three cases) except in the Mopti mated with Mopti treatment group (Pearson pairwise: n= 50, $P = 0.174$). However, linear regression conducted between male and female body size showed a very strong positive relationship overall and in between groups suggesting that bigger males tend to mate with bigger females (Linear regression: n= 171, $T = 4.30$, $P < 0.001$) (Fig 5.25).
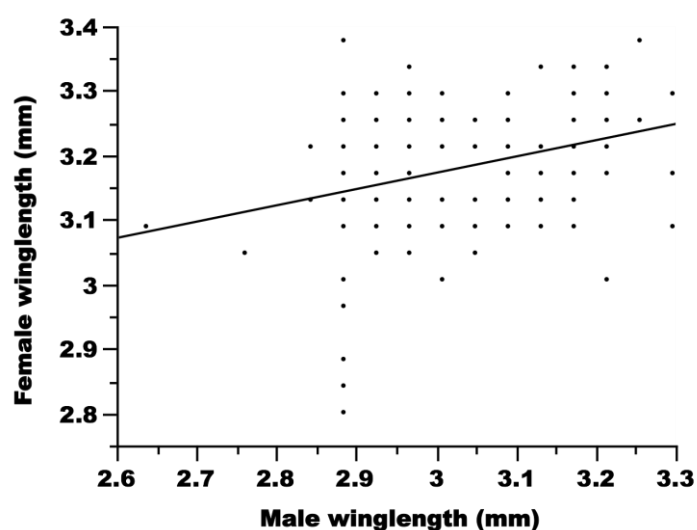
Fig 5.25: Linear regressions between the male and female body size (wing length) caught as mating pair in the four mating combinations.

5.4.3.7 Heterosis and male survival

5.4.3.7.1 Survival under desiccation stress

The difference in survival time between the heterotic supermale, Mopti and Kil male under stress was examined and a significant difference was found (Wilcoxon rank-sum test: n= 180, $df= 2$, $X^2= 61.37$, $P< 0.001$) (Fig 5.26). Overall, the mean survival time was 12.8h (95% CIs= 12.8-13.4h). Within groups, Kil had a mean survival time of 10.0h (9.2-10.8), Mopti had survival time of 15.0h (13.9-16.1) and supermale had a mean survival time of 13.3h (12.5-14.1).
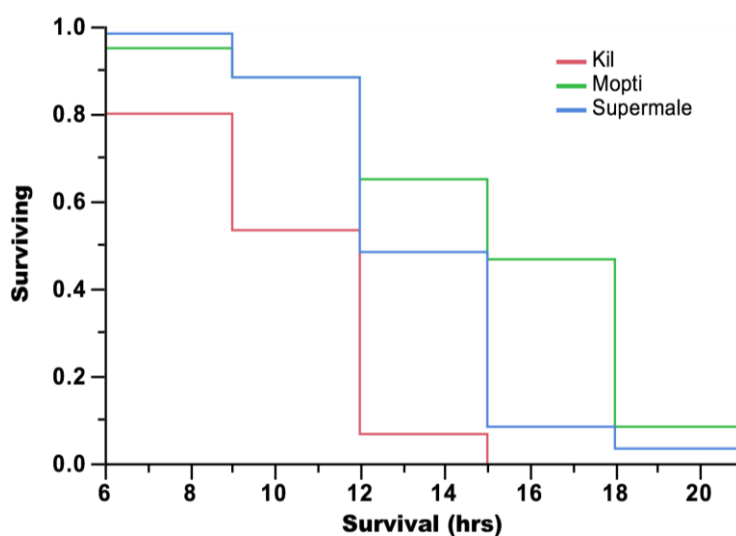
Fig 5.26: Limit survival plot of Kil, Mopti and hybrid supermales males exposed to hydric stress in incubator at 30°C, 30% RH till death.

The effect of survival male strain on was analyzed, and a significant effect was found (Cox Proportional Hazard: n= 180, $df= 2$, $X^2= 41.1$, $P< 0.001$). Risk Ratios for the male strain revealed that Mopti males survived significantly longer than Kil males (Proportional Hazard: Risk Ratios= 0.25, $P< 0.001$) and heterotic supermales survived significantly longer than Kil males (Risk Ratios= 0.43, $P< 0.001$) and Mopti males (Risk Ratios= 1.73, $P= 0.006$) (Table 5.5).

Table 5.5 :Risk Ratios for Kil, Mopti and heterotic supermales under stress in the incubator at 30°C, 30% RH.

Level 1	Level 2	Risk Ratio	P-value	CIs (95%)
Mopti	Kil	0.250	< 0.001	0.162-0.382
Supermale	Kil	0.433	< 0.001	0.295-0.634
Supermale	Mopti	1.733	0.006	1.175-2.561

5.4.3.7.2 Survival under insectary condition

Under optimum conditions in the insectary, the survival time between the three male strains was significantly different (Wilcoxon rank sum test: n= 180, *df*= 2, X^2= 15.3, *P<* 0.001). Overall, the mean survival time was 264h (250-279). The mean survival time for Kil was 251h (230-274), mean survival time for Mopti was 238h (214-262), and mean survival time for supermale was 308h (276-330) (Fig 5.27).
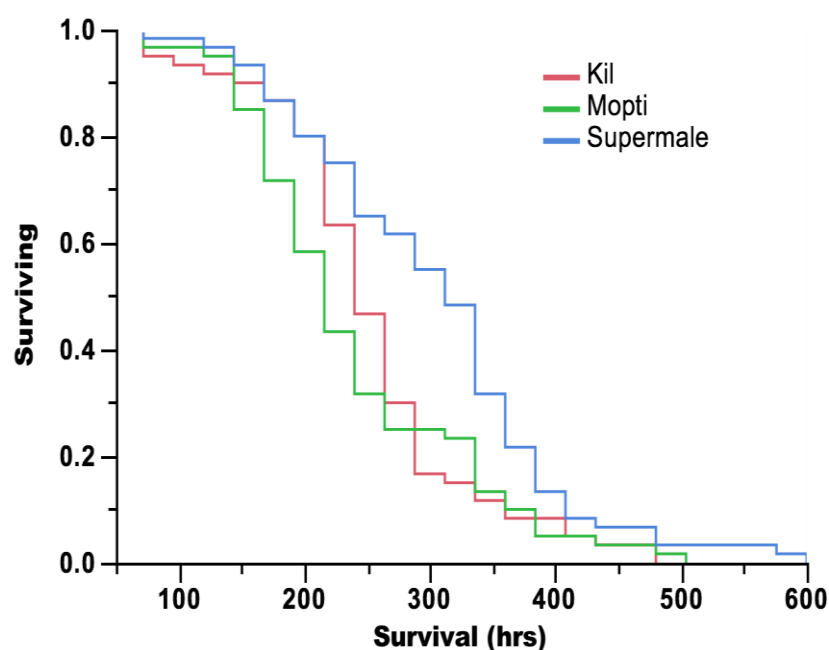
Fig 5.27: Limit survival plot of Kil, Mopti and heterotic supermales, under normal insectary condition at 80%RH, ± 26°C until death.

There was a significant effect of survival on male strain (Proportional Hazard likelihood: n= 180, df= 2, X^2= 10.8, P= 0.005). Risk Ratios for the male strain revealed that supermales survived significantly longer than Kil males (Risk Ratios= 0.63, P= 0.017, n= 120) and Mopti males (Risk Ratios= 0.56, P= 0.002, n= 120) (Table5.6). The two environmental conditions as expected gave rise to contrasted survival patterns (Fig 5.28).

Table 5.6: Risk Ratios for heterotic supermale, Mopti and Kil males under insectary conditions 80%RH, ± 26°C.

Level 1	Level 2	Risk Ratio	P-value	CIs (95%)
Mopti	Kil	1.135	0.495	0.789-1.636
Supermale	Kil	0.639	0.017	0.442-0.924
Supermale	Mopti	0.563	0.002	0.390-0.813
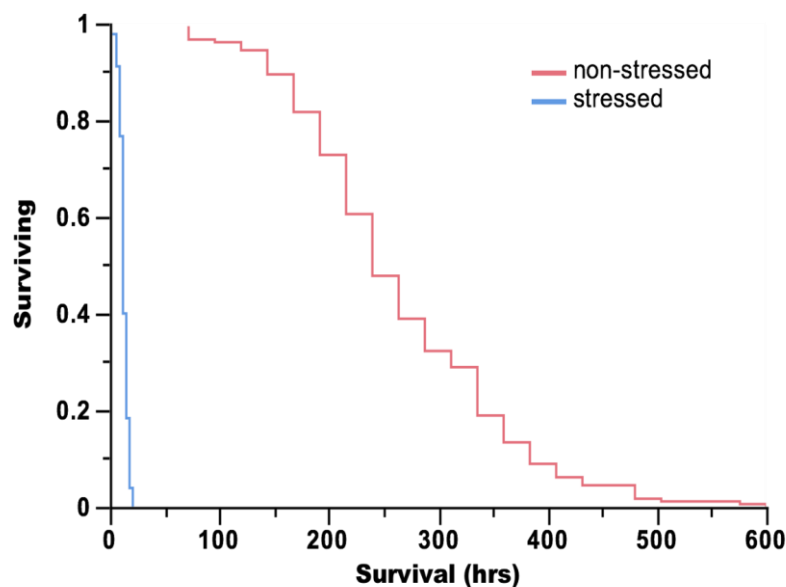
Fig 5.28: Product-Limit survival plot of Kil, Mopti and heterotic supermales in both experimental conditions. Stressed at 30°C, 30% RH and Non-stressed at 80%RH, ± 26°C (Wilcoxon sum rank test: P < 0.001)

5.4.3.8. Effect of stress and male body size on survival

The combined effect of stress and male body size on survival was examined by proportional hazard analyses in the two treatment groups. Treatment was significant, but male body size was not (Proportional Hazard: treatment; n= 356, $df= 1$, $X^2 = 407.1$, $P < 0.001$; Male body size; n= 356, $df= 1$, $X^2 = 2.30$, $P= 0.130$). The mean body size for the stressed male was 3.05mm (95%CIs= 3.04-3.07) and the mean body size for the non-stressed males was 3.06mm (3.04-3.07) (Table 5.7). Overall, there was no significant difference in male body size and within male strains on survival ($P > 0.180$ in all cases).

Table 5.7: The overall and within group mean body sizes of all the males used in the stressed and non-stressed treatment groups.

Treatment	No	Kil mean body size (mm)	Mopti mean body size (mm)	Supermale mean body size (mm)	Total mean body size (mm)
Non-stressed	180	3.05(3.02-3.08)	3.07(3.04-3.09)	3.06(3.04-3.09)	3.06(3.04-3.07)
Stressed	176	3.04(3.01-3.07)	3.07(3.04-3.09)	3.06(3.03-3.09)	3.05(3.04-3.07)
Mean body size (mm)	356	3.05(3.02-3.07)	3.07(3.04-3.09)	3.06(3.04-3.08)	3.06(3.05-3.07)

5.4.4 Discussion

This experiment could possibly explain the differences in male reproductive success observed in the previous section (Section B). First, we observed mating plug in the reproductive tracts of all the females caught as couples in the experimental swarm. Secondly, there was a significant difference in mating plug size deposited in the females across the four mating combinations. The heterotic supermales deposited significantly larger mating plugs than the inbred Kil and Mopti males and double mating plugs were detected in some mating combinations. Conversely, no significant difference was observed in the sperm numbers transferred in the mating combinations. The small plug size of inbred males could have resulted from inbreeding effect and consequently reduction in male reproductive success.

In the survival experiment, the very old inbred Kil males survived less well in both experimental conditions while the heterotic supermales survived better than Mopti and Kil inbred males. It is believed that inbred individuals could be more sensitive to environmental stress than outbred individuals, for the reason that stress enhances the manifestation of deleterious recessive alleles (Fox *et al.* 2011). More so, the degree of inbreeding depression coupled with the degree of environmental stress might have a profuse reproductive fitness consequence for vector control projects.

In the field populations, *An. gambiae* mosquitoes swarm to mate (Charlwood *et al.* 2002; Diabaté *et al.* 2009). Mating plugs were found in the reproductive tract of freshly mated females (Gillies 1956). The females in our experiment were caught as couples in lab-cage swarms, supporting the fact that *An. gambiae* mate in swarms, even in lab cages. All the females captured and dissected had mating plugs, confirms that mating plug is observed in freshly mated females (Gillies 1956; Roger *et al.* 2009). Our study revealed a significant difference in size of the mating plug deposited across the four groups. The

heterotic supermales deposited a significantly larger mating plug than their inbred parental strains Kil and Mopti males. Inbreeding effects on reproductive investments has been suggested to be more severe in males (Ala-Honkola *et al.* 2014). The male accessory gland produces mating plug, which serves as a barrier to the second mating (Tripet *et al.* 2005). In a recent study, it was shown that inbreeding has selective effects on the size of male accessory glands in *An. gambiae* and may affect male fitness (Baeshen *et al.* 2014). In section B of this study, we examined male reproductive success between supermales and inbred males and found that the supermales produced more offspring than the inbred males. Across the four experimental groups, Kil males deposited the smallest mating plug. Comparison between heterotic supermale and inbred males revealed that the inbred males deposit smaller plug sizes than the heterotic supermales. The bigger mating plug deposited by the heterotic supermale suggests that inbreeding could affect the production of the mating plug independent of the overall size of male accessory glands or perhaps the effect of inbreeding on accessory gland size worsened since this study was done 1-2yrs later hence reduction in plug size deposited by inbred Kil males.   Furthermore, if inbreeding affects the overall plug size, there is a likelihood the quantity of male-produced plug constituents could be affected too. The differences in male reproductive success observed in the previous section (Section B) could be because of insufficiency in the quantity of male-produced plug constituents, which could have affected the male fecundity thereby causing a decrease in male reproductive success of the inbred males.

On the other hand, Roger *et al.* (2009) reported that the mating plug is composed of six-protein from both male and female. The heterotic supermale when mated with Mopti females deposited large mating plugs but when mated with Kil females, smaller mating plug was were deposited. In view of the fact that it's been shown that inbreeding affects reproductive investments, Kil females (35yrs+) are more inbred than Mopti females so

their contribution towards the compositions of a mating plug might have been poor resulting in small mating plug. Whereas the bigger plug size in Mopti females (10yrs+) suggest a lesser inbreeding effect. Thus, the observed difference in mating plug size across the mating combinations can be explained as a product of inbreeding effect.   Mating plugs function as a physical block to subsequent insemination (Gillies 1956; Tripet *et al.* 2005). There were a few occurrences of double plugs in some mating combinations, suggesting a probable second insemination as proposed by Gillies (1956) and Tripet *et al.* (2005), and multiple mating in small size cages in *An. gambiae* has been discussed by Tripet *et al.* (2003). The double plugs observed could be due to the scrambling for female mate in the tight laboratory cages in which case the mating plug might not pre-empt re-mating as such. Surprisingly, no double plug was discovered in the Kil mated with Kil females, which from a previous study in section B recorded a very high mating frequency compared to Mopti. All mating plugs transferred by the Kil male were 100% single plugs, while Mopti males had 12% double plugs, twice the number that was recorded in heterotic supermales crossed with the Kil females (6%).Then, we suspect that the second plug might not have been because of second mating as they were generally smaller in size (0.024mm:CI= 0.022-0.027) than the initial one (0.036mm:CI=0.035-0.037). There is a strong possibility that the males depositing second plugs were not confident that the initial plug deposited was sufficient for proper sperm storage (Baldini *et al.* 2013) in which case depositing a second plug will be an added advantage for sure fertilization or maybe 'sneaky males' are of lesser quality, hence smaller plugs in general. Mating plugs contain sex peptide that helps in sperm storage (Rogers et al. 2008), and induce post-mating behaviours like refractoriness to subsequent mating host finding, and egg laying (Klowden 1999; Gillott 2003; Shut *et al.* 2010).

Across the four mating combinations, the quantity of sperm transferred showed no significant difference, but there was a huge variation in sperm numbers within the different groups. This observation was not different from what we saw in the previous chapters of this thesis. However, the different replicates revealed a difference in the quantity of sperm numbers. The male and female body size did not affect the sperm number transferred in all the experimental groups. The male and female body sizes across the groups were similar. However, there was a preference for the bigger males to mate with the bigger females. Similar observation between male and female body size is well documented across many mosquitoes (Ng'habi *et al.* 2005; Helinski and Knols 2009; Ponlawat and Harrington 2007, 2009). In the laboratory larger males of *An. gambiae* tends to mate with larger females when given the choice (Okanda et al. 2002). Cator *et al.* (2010) observed that in swarms the females of *An. gambiae* are able to match harmonics and associate it with larger males with adequate energy reserves thus respond to it more quickly in order to increase the possibility of mating with a larger individuals. This observation is consistent with our finding. In our experimental induced swarm, within 2mins mating couples were caught, and the larger females were paired with larger males.

Survival time was reduced in inbred males than in heterotic supermales regardless of the treatment given (stressed or not- stressed). Some fitness traits are affected by laboratory rearing and inbreeding in *An. gambiae* (Ferguson *et al.* 2005; Baeshen *et al.* 2014). Due to reduce fitness and inherent difference, inbred individuals are likely to show a decrease in survival and lifespan under most ecological conditions as compared with outbred individuals (Valtonen *et al.* 2011). The old inbred Kil surviving less well in stress and non-stress condition highlights another fitness trait that may probably be impacted by inbreeding. Inbred individuals are more susceptible to ecological stress than hybrid individuals, as stress increases the manifestation of deleterious recessive alleles (Fox *et al.*

2011). The extent of inbreeding depression together with the degree of environmental stress might have numerous fitness costs on vector control projects. The short survival time witnessed in the old Kil strain suggests that old colonised mosquitoes may not be suitable candidate for the vector control programmes, given that survival fitness amongst other reproductive quality is central to vector control methods relying on male releases. The M forms of *An. gambiae* aestivates during the long dry season in the Sahel to survive desiccation (Lehmann *et al.* 2010; Huestis *et al.* 2011, 2012). Mopti male, though inbred (10yrs+) but less inbred than Kil male (35yrs+) survived best under stressed condition. It could be that Mopti males (M form), retain some inversions that are critical for desiccation resistance and the effect of these inversions might be stronger than the benefits of outbreeding under our drastic desiccation stress conditions.

Hybridization of two genetically distinct strains can result in prolonged existence in adults (Menge *et al.* 2005; Pekkala *et al.* 2014). Heterozygote adults surviving better than inbred parental strain in the laboratory have been previously reported in *An. gambiae* (Menge *et al.* 2005). This is consistent with our results. The hybrid supermale had the longest surviving male in the non-stress condition and championed overall survival fitness in all treatment conditions. They could be better choice for vector control projects. There was no significant effect of body size on survival in either of the experiments, and this contradicts the observations of Fouet *et al.* (2012). One would explain the prolonged existence demonstrated by the supermales in both experiments to be the effect of heterosis.

Finally, we have shown that inbreeding affects the quantity of sex peptide transfer. We have evidence that inbreeding affects the size of mating plugs deposited, and we suspected that plug size might be dependent on the quantity of male producing sex peptide available, which as you expect, might have impacted on male reproductive success. We

have also demonstrated that the survival of inbred males was affected by inbreeding suggesting that they might not be right individuals for vector control programmes. We have also shown that through outbred crossing both traits could be restored, implying that the heterotic supermales might be a better choice for vector control programmes requiring high male fertility.

5. 5 General conclusion

Malaria has an overwhelming consequence on public health, and its control constitutes one of the major problems facing the world health (WHO 2013). The current methods of control over the past decades have been successful. However, the fact that the development of resistance to the current control measures by both parasite and vector is progressing and unavoidable, has called for needs to develop fresh functional tools and to improve existing control measures for vector control beyond the typical orthodox method of physical barriers and insecticides.   Although the SIT and GMM could hardly be referred to as new tools for vector controls, relying on them whether enhanced with modern techniques or the old chemo sterilization method, they are still genuinely promising options to vector control. However, there is number of problems that must be overcome before they can be used effectively. First, the success of any given SIT programme relies on performance of the sterile male and estimating male fitness components in the laboratory is the first stage before their release (Massonnet-Bruneel *et al.* 2013). Therefore, generating males with good reproductive quality is a primary factor for any SIT program. Secondly, hypothetical programmes developed for use in the genetically modified mosquitoes vector- control also required males that are fertile and vigorous. Ensuring that both sterilized and genetically modified mosquitoes are capable of performing the task required of them such as persisting to compete and or out compete their wild counterparts for mates is the foremost goal. Regrettably, little is understood of the male mosquito mating biology and male qualities needed for such vector control projects. Furthermore, there is even less information about male quality choice by wild female during mating (Perez-Staples *et al.* 2013). All these questions regarding male reproductive biology in relation to mating success are of interest to fitness of colonized males and mass releases of such males for vector control projects.

Therefore, we have conducted a series of investigations on the genetics and ecology of male reproductive investments to contribute to male mating success.

First, we developed new techniques based on already existing methods to determine activity and sperm investment of *An. gambiae* males. For sperm quantification, we developed a new method based on TaqMan qPCR quantitative assay and for sperm activity we developed a new in 'vivo' technique using a phase contrast microscope equipped with a motic images plus camera. The sperm quantification techniques used in other insects could only give approximation of the sperm numbers (Holman 2009). More so, in Anopheline mosquitoes because of the difficulties involved in the spermatheca dissection these methods have not been used (Helinski and Knols 2009). The amount of sperm in the male and female reproductive organ can contribute significantly to their reproductive success. Therefore, the understanding of what was invested and what was used and maintained by the female will contribute in appreciating the mosquito mating biology. Applying the newly developed technique to our investigations, we quantify sperm numbers in the spermathecae of field caught M and S molecular forms of Kela 2006. We observed huge variation in sperm number in the spermathecae of the females. It is not clear why there is this wide variation, though in nature, similar observations of variations in the number of sperm found in storage organs of different mosquitoes species have been documented (Ponlawat and Harrington 2007; Helinski and Knols 2009). These variations have also been be linked to biological factors such as male age and size.

We went further to look at the effect of hydric stress on male sperm investment because part of the challenge faced by adult mosquitoes as they emerge in the field is desiccation. Insufficient water in their tissues can result in stress, which may interfere in their physiological processes (Aboagye-Antwi and Tripet 2010). Reproductive success is one of such physiological processes that could be affected due to dehydration. The effect

of stress and how males release in the SIT or GMM project would react under stress is critical. This study may help to understand male mating success under field conditions. Studies of hydric stress will help and establish the importance of water in adult physiology and consequences of resistance to dehydration (Aboagye-Antwi and Tripet 2010). In the wild, seasonal changes affects reproductive success of the *An. gambiae* particularly those in the xeric zones. The M form of *An. gambiae* aestivates in dry season, females witness reduction in gonadotrophic cycle, both oviposition and egg laying drops. We supposed that the male under water stress might invest more in their current reproduction, because this could be the strategy that will maximize their lifetime reproductive success. We experimentally explored this hypothesis using the second technique. We determined sperm activity in stressed and non-stressed males and female and quantified sperm investment by male. We found that post mating hydric stress had no effect on sperm activity and sperm number maintenance in females all the treatment groups. However, hydric stress had a positive effect on sperm investment in pre-mating stressed males. There was a shift in the balance between current and future investment in reproduction towards investing more in the current reproduction. The stress condition induces the males to allocate more sperm to females to ensure future reproductive success. This is an adaptive response since the main fear is to overcome the threatening emergencies and so all reserve were channelled towards this.

In another investigation, we examined the male reproductive success in large-group cages. The biology of male mating in the Anopheline is poorly understood in comparison to female mosquitoes and the knowledge of male reproductive success is necessary for malaria vector control (Ferguson *et al.* 2005). Colonization and inbreeding can be a challenge to the mass rearing and male releases. One fast way to recover heterozygosity without the intricacies of back crossing is by hybridization of inbred strains. The impact of

heterosis and inbreeding on male reproductive investments in *An. gambiae s.s* has not been reported. With the assumption that hybridization of two inbred strains will restore heterozygosity in inbred lines, we generated heterotic supermales from two inbred strains and experimentally tested for the effects of heterosis on sperm and sex-peptide transfer by heterotic supermales in large-group mating experiments. We compared sperm activity and sperm numbers transferred by the hybrid supermales and the inbred males. We also compared the amount of sex peptides (*Plugin* and *Transglutaminase*) they transferred and finally we compared their reproductive success. As anticipated, the heterotic supermales transferred sperm that were more active than the inbred lines, but no difference was seen in the quantity of sperm transferred. The level of *Plugin* was similar for all groups but the level of *Transglutaminase* was more in one of the inbred line Mopti. The high level of *Transglutaminase* witnessed in Mopti suggests adaptation to lab-rearing i.e. Mopti strain being closer to the wild type might just be producing more *Transglutaminase* to adapt to lab-rearing. *Transglutaminase* has been reported to stimulate proper sperm storage rather than act as a physical barrier to the second mating (Roger *et al.* 2009). Therefore, a high amount of *Transglutaminase* transferred by the Mopti males was seen as an adaption to ensure proper sperm storage by the strain and increase female fertility to avoid inbreeding effects. No effect of heterosis was observed on the male reproductive success.

We further compared the effects of heterosis and inbreeding on reproductive success in individual males in small lab-cages. The result revealed that not all fitness qualities were affected by inbreeding. The very old inbred Kil males inseminated more females than the heterotic supermales and there was no difference in hatching rate of eggs laid by females mated by inbred males and heterotic supermales. However, the overall male reproductive success was impacted by heterosis. The heterotic supermales produced more eggs and fathered larvae per female they mated. This indicates that reduced male

reproductive success due to inbreeding can be recovered through hybrid vigor (Fry *et al.* 1998). Therefore, hybrid vigor might be one rapid and easy way to solve the problem of inbreeding and produce males with high reproductive success for the GMM projects requiring males with high reproductive success to carry a gene construct to the next generation.

In addition, we examined the size of the mating plug deposited by the heterotic supermales and the inbred males. Mating plugs contain sex-peptide that helps in sperm storage (Rogers et al. 2008), induces post-mating behaviours like refractoriness to subsequent mating host finding, and egg laying (Klowden 1999; Gillott 2003; Shut *et al.* 2010). Inbreeding effects, reproductive investment and its effect are more severe in males (Ala-Honkola *et al.* 2013). It has been reported that inbreeding affects the size of male accessory glands of *An. gambiae* (Baeshen *et al.* 2014). Our result showed that the very old inbred Kil male transferred smaller plug size than heterotic supermale. The heterotic supermale transferring large mating plug can be explained as a hybrid vigor effect. More so, Roger *et al.* (2009) reported that the formation of mating plug involves sex-protein from both male and female and it has been shown that inbreeding affects reproductive investments, thus old inbred Kil female (35yrs+) contribution to mating plug formation will be poor resulting in small mating plug formed. Whereas the bigger plug size was seen in, Mopti female (10yrs+) when mated with the supermales suggests that Mopti females were not strongly inbred as of the time of this study and could still be heterozygote to this trait.

Finally, the survival of inbred males and heterotic supermales was compared under two conditions, stress and non-stress. The old inbred Kil survived less well in both conditions than the heterotic supermales. This suggests that inbred old Kil strain may not be a suitable candidate for the vector control projects, since survival fitness amongst other

reproductive quality is central to vector control techniques relying on male releases. On the other hand, the heterotic supermales championed survival in both conditions. Hybridizations of two genetically distinct strains can result in prolonged existence in adults individuals (Menge *et al.* 2005; Pekkala *et al.* 2014). Heterozygote adults surviving better than inbred parental strain in the laboratory have been previously reported in *An. gambiae* (Menge *et al.* 2005). Thus, they might be the better choice for vector control projects.

In conclusion, several success stories have been associated with SIT projects across various insect taxa and field trials of GMM. In the *Anopheles*, the male mating competitiveness has been a huge hurdle to cross. Our investigations on male reproductive investment might serve to answer some of the questions regarding reproductive success and hence contribute towards finding solutions to the mating competitiveness. The generation of hybrid supermale from two inbred strains could be a very quick way to deal with effects of inbreeding and adaptation to lab-rearing problems in colonized strains. The heterotic supermales with high reproductive success would fit into SIT and GMM projects for the control of vector-borne diseases particularly malaria.

References

ABOAGYE-ANTWI, F., GUINDO, A., TRAORE, A. S., HURD, H., COULIBALY, M., TRAORE, S. & TRIPET, F. 2010. Hydric stress-dependent effects of *Plasmodium falciparum* infection on the survival of wild-caught *Anopheles gambiae* female mosquitoes. *Malaria Journal,* 9:243.

ABOAGYE-ANTWI, F. & TRIPET, F. 2010. Effects of larval growth condition and water availability on desiccation resistance and its physiological basis in adult *Anopheles gambiae sensu stricto*. *Malaria Journal,* 9:225

ABILIO, A. P., KLEINSCHMIDT, I., REHMAN, A.M., CUAMBA, N., RAMDEEN, V. & MTHEMBU, D. S. 2011. The emergence of insecticide resistance in central Mozambique and potential threat to the successful indoor residual spraying malaria control programme. *Malaria J*ournal.10: 110.

ACHUDUME, A. C., NWOHA, P. C. & IBE, J. N. 2009. Toxicity and Bioaccumulation of the Insecticide "Raid" in Wister Rats. *Environmental Toxicology,* 24**,** 357-361.

ALA-HONKOLA, O., HOSKEN, D. J., MANIER, M. K., LUPOLD, S., DROGE-YOUNG, E. M., BERBEN, K. S., COLLINS, W. F., BELOTE, J. M. & PITNICK, S. 2013. Inbreeding reveals mode of past selection on male reproductive characters in *Drosophila melanogaster*. *Ecology and Evolution,* 3**,** 2089-2102.

ALA-HONKOLA, O., MANIER, M. K., LUPOLD, S., DROGE-YOUNG, E. M., COLLINS, W. F., BELOTE, J. M. & PITNICK, S. 2014. No inbreeding depression in sperm storage ability or offspring viability in *Drosophila melanogaster* females. *Journal of Insect Physiology,* 60**,** 1-6.

ALPHEY, L. 2002. Re-engineering the sterile insect technique. *Insect Biochemistry and Molecular Biology,* 32**,** 1243-1247.

ALPHEY, L. 2010. Engineered sterile mosquitoes for dengue control - from lab to field. *American Journal of Tropical Medicine and Hygiene,* 83**,** 305-305.

ALPHEY, L. 2013. Control of *Aedes aegypti* by release of engineered sterile ('RIDL') males. *Pathogens and global health,* 107**,** 456-456.

ALPHEY, L. 2014. Genetic Control of Mosquitoes. *Annual Review of Entomology, Vol 59, 2014,* 59**,** 205-224.

ANOPHELES GAMBIAE | VECTORBASE
https://www.vectorbase.org/organisms/*anopheles-gambiae* (Accessed 11July 2011).

ANTHONY, T.G., HARBACH, R.E. & KITCHING, I. J. 1999. Phylogeny of the Pyretophorus Series of *Anopheles* subgenus *Cellia* (Diptera: Culicidae). *Systematic Entomology,* 24:193–205

APPLIEDBIOSYSTEM.2003. Creating standard curves with genomic DNA or. plasmid DNA templates for use in quantitative PCR. Overview genomic DNA (gDNA) *www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf* (Accessed 11 July 2011).

ARCHER, M. A., BRADLEY, T. J., MUELLER, L. D. & ROSE, M. R. 2007. Using Experimental Evolution to Study the Physiological Mechanisms of Desiccation Resistance in Drosophila melanogaster. Physiological & Biochemical Zoology, 80, 386-398.

ARIAS, L. N., SAMBUCETTI, P., SCANNAPIECO, A. C., LOESCHCKE, V. & NORRY, F. M. 2012. Survival of heat stress with and without heat hardening in *Drosophila melanogaster*: interactions with larval density. *Journal of Experimental Biology,* 215**,** 2220-2225.

ARMBRUSTER, P., BRADSHAW, W. E., RUEGG, K. & HOLZAPFEL, C. M. 2001. Geographic variation and the evolution of reproductive allocation in the pitcher-plant mosquito, *Myzomyia smithii. Evolution,* 55**,** 439-444.

ARMBRUSTER, P., HUTCHINSON, R. A. & LINVELL, T. 2000. Equivalent inbreeding depression under laboratory and field conditions in a tree-hole-breeding mosquito. *Proceedings of the Royal Society B-Biological Sciences,* 267**,** 1939-1945.

ARMBRUSTER, P. & REED, D. H. 2005. Inbreeding depression in being and stressful environments. *Heredity,* 95**,** 235-242.

ARRIVILLAGA, J. & BARRERA, R. 2004. Food as a limiting factor for *Aedes aegypti* in water-storage containers. *Journal of Vector Ecology,* 29**,** 11-20.

ASMAN, S. M., MCDONALD, P. T. & PROUT, T. 1981. Field studies of genetic-control systems for mosquitos. *Annual Review of Entomology,* 26**,** 289-318.

AULTMAN, K. S., GOTTLIEB, M., GIOVANNI, M. Y. & FAUCI, A. S. 2002. *Anopheles gambiae* genome: Completing the malaria triad. *Science,* 298**,** 13-13.

AYESHA, M. S., BREMAN, J.G, & MCKENZIE, F.E. 2010. Using the entomological inoculation rate to assess the impact of vector control on malaria parasite transmission and elimination. *Malaria Journal*,9: 122.

AZUCENA G, C., DE LOURDES, R. M., MARTHA, M. I., BERNARDINO V. J., IVAN, G. M., QUINTANILLA-VEGA, B., OST, R. P., ELENA, P. N. & ELIZABETH, R. A. 2010. Pattern of use and sale of pesticides in Nayarit, Mexico. *Revista Internacional De Contaminacion Ambiental,* 26**,** 221-228

BAER, B. & BOOMSMA, J. J. 2004. Male reproductive investment and queen mating-frequency in fungus-growing ants. *Behavioural Ecology,* 15**,** 426-432.

BAER, B., SCHMID-HEMPEL, P., HOEG, J. T. & BOOMSMA, J. J. 2003. Sperm length, sperm storage and mating system characteristics in bumblebees. *Insectes Sociaux,* 50**,** 101-108.

BAESHEN, R., EKECHUKWU, N. E., TOURE, M., PATON, D., COULIBALY, M., TRAORE, S. F. & TRIPET, F. 2014. Differential effects of inbreeding and selection on male reproductive phenotype associated with the colonization and laboratory maintenance of *Anopheles gambiae*. *Malaria Journal,* 13:19.

BAILEY, C., TILFORD, T., & LU, H. 2007. Reliability analysis for Power Electronics Modules. *30th International Spring Seminar on Electronics Technology*, 12-17.

BAKER, R. H. 1964. Mating problems as related to the establishment and maintenance of laboratory colonies of mosquitoes. *Bulletin of the World Health Organization,* 31**,** 467-8.

BALDINI, F., GABRIELI, P., ROGERS, D. W. & CATTERUCCIA, F. 2012. Function and composition of male accessory gland secretions in *Anopheles gambiae*: a comparison with other insect vectors of infectious diseases. *Pathogens and global health,* 106**,** 82-93.

BALDINI, F., GABRIELI, P., SOUTH, A., VALIM, C., MANCINI, F. & CATTERUCCIA, F. 2013. The Interaction between a Sexually Transferred Steroid Hormone and a female protein regulates Oogenesis in the malaria mosquito *Anopheles gambiae. PloS Biology,* 11:10.

BARBOSA, F. 1968. Sex identification 01-" Live mosquito pupae in the laboratory. *Mosquito news*, 28, 643-644.

BARILLAS-MURY, C. & KUMAR, S. 2005. Peroxidase mediate refractoriness to *Plasmodium* infection in *Anopheles gambiae. American Journal of Tropical Medicine and Hygiene,* 73**,** 2-2.

BARNES, A. I. & PARTRIDGE, L. 2003. Costing reproduction. *Animal Behaviour,* 66**,** 199-204.

BARTLETT, J. W. & FROST, C. 2008. Reliability, repeatability, and reproducibility: analysis of measurement errors in continuous variables. *Ultrasound in Obstetrics & Gynaecology,* 31**,** 466-475.

BARTLETT, **J** M. S & STIRLING, D. 2003. A Short History of the Polymerase Chain Reaction *Methods in Molecular Biology,* Vol: 226, Pg3-6, DOI: 10.1385/1-59259-384-4:3

BESANSKY, N J., LEHMANN T., FAHEY, G. T., FONTENILLE, D., BRAACK, L. E., HAWLEY, W.A. & COLLINS, F. H. 1997. Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae and An. arabiensis*, suggest extensive gene flow. *Genetics* , 147:1817-1828

BEIER, J. C., COPELAND, R., OYARO, C., MASINYA, A., ODAGO, W. O., ODUOR, S., KOECH, D. K. & ROBERTS, C. R. 1990. *Anopheles-gambiae* complex egg-stage survival in dry soil from larval development sites in western Kenya. *Journal of the American Mosquito Control Association,* 6**,** 105-109.

BENEDICT, M.Q. 2007. Appropriate sex-separation technology for anopheles mosquitoes.1Aug2007InternationalAtomicSource: http://www.anobase.org/embo_meeting/abstracts/transposons-transgenesis/Benedict.pdf.

BENEDICT, M. Q, KNOLS, B. G. J., BOSSIN, H. C., HOWELL, P. I., MIALHE, E., CACERES, C., & ROBINSON, A.S. 2009. Colonisation and mass rearing: learning from others, *Malaria Journal*, 8 (Suppl 2):S4

BENEDICT, M. Q. & ROBINSON, A. S. 2003. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends in Parasitology,* 19**,** 349-355.

BERNASCONI, G., HELLRIEGEL, B., HEYLAND, A. & WARD, P. I. 2002. Sperm survival in the female reproductive tract in the fly *Scathophaga stercoraria* (L.). *Journal of Insect Physiology,* 48**,** 197-203.

BIRCHLER, J A., AUGER, D. L., & RIDDLE, N C .2003. "In Search of the Molecular Basis of Heterosis". *Plant Cell* 15 (10): 2236–9.

BIRKHEAD, T. R., MARTINEZ, J. G., BURKE, T. & FROMAN, D. P. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proceedings of the Royal Society B-Biological Sciences,* 266**,** 1759-1764.

BLAY, S. & YUVAL, B. 1997. Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae) *Animal Behaviour*. 54:59–66.

BLACK, W.C., & LANZARO, G.C. 2001. Distribution of genetic variation among chromosomal forms of *Anopheles gambiae s.s*: introgressive hybridization, adaptive inversions, or recent reproductive isolation? *Insect Molecular Biology.* 10, 3–7.

BLOCH QAZI, M. C. & WOLFNER, M. F. 2003. An early role for the *Drosophila melanogaster* male seminal protein Acp36DE in female sperm storage. *The Journal of Experimental Biology,* 206**,** 3521-8.

BOAKE, C. R. B. 2000. Flying apart: Mating behavior and speciation. *Bioscience,* 50**,** 501-508.

BOECKMAN, F., HAMBY, K., & TAN, L . 2000. Real –Time PCR using the iCycler iQ Detection System and Intercalation Dyes. Bio-Rad Tech note 2684 (Amplification) Bio-Rad Laboratories, Inc. 1-2.

BOEETE, C., AGUSTO, F. B. & REEVES, R. G. 2014. Impact of mating behaviour on the success of malaria control through a single inundative release of transgenic mosquitoes. *Journal of Theoretical Biology,* 347**,** 33-43.

BUDIANSKY, S. 2002. How affluence could be good for the environment - Intensive farming means the United States uses less land to feed more people than ever. *Nature,* 416**,** 581-581.

BURT, A. 2014. Heritable strategies for controlling insect vectors of disease. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 369.

BUSTIN, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology,* 25**,** 169-193.

CABALLERO-MENDIETA, N. & CORDERO, C. 2013. Male mating costs in a butterfly that produces small ejaculates. *Physiological Entomology,* 38**,** 318-325.

CABRERA, M. & JAFFE, K. 2007. An aggregation pheromone modulates lekking behavior in the vector mosquito Aedes aegypti (Diptera: Culicidae). Journal of the American Mosquito Control Association 23(1):1-10. 2007

CALZETTA, M., SANTOLAMAZZA, F., CARRARA, G. C., CANI, P. J., FORTES, F., DI DECO, M. A., DELLA TORRE, A. & PETARCA, V. 2008. Distribution and chromosomal characterization of the *Anopheles gambiae* complex in Angola. *American Journal of Tropical Medicine and Hygiene,* 78, 169-175

CAPUTO, B., NWAKANMA, D., JAWARA, M., ADIAMOH, M., DIA, I., KONATE, L., PETRARCA, V., CONWAY, D. J. & DELLA TORRE, A. 2008. *Anopheles gambiae* complex along The Gambia river, with particular reference to the molecular forms of *An. gambiae s.s. Malaria Journal,* 7:182.

CATOR, L. J., NG'HABI, K. R., HOY, R. R. & HARRINGTON, L. C. 2010. Sizing up a mate: variation in production and response to acoustic signals in *Anopheles gambiae*. *Behavioural Ecology,* 21**,** 1033-1039.

CATTERACCIA, F. 2009**.** Meddling in mosquitoes sex live. www.imperial.ac.uk/../imperialcollege/.new_22-22-2009-10-57(Accessed 20 Dec.2011).

CATTERUCCIA, F., LEVASHINA, E. A., REIDHAAROLSON, J. F. & RONDINONE, 2009. RNAi in the Malaria Vector, *Anopheles gambiae*. *In:* C. M. (eds.) *Therapeutic Applications of Rnai: Methods and Protocols.*

CENTRE FOR DISEASE CONTROL AND PREVENTION (CDC 2010) Malaria. http://www.cdc.gov/malaria/about/biology/mosquitoes/index.html (Accessed 30 Nov. 2011)

CHARLESWORTH, B. & CHARLESWORTH, D. 1999. The genetic basis of inbreeding depression. *Genetical Research,* 74**,** 329-340.

CHARLESWORTH, D. & WILLIS, J. H. 2009. Fundamental concepts in genetics: The genetics of inbreeding depression. *Nature Reviews Genetics,* 10**,** 783-796.

CHARLWOOD, J. D. & JONES, M. D. R. 1979. Mating-behavior in the mosquito, *Anopheles-gambiae s.l* close range and contact-behavior. *Physiological Entomology,* 4**,** 111-120.

CHARLWOOD, J. D. & JONES, M. D. R. 1980. Mating in the mosquito, *Anopheles-gambiae s-1 .2.* swarming behavior. *Physiological Entomology,* 5**,** 315-320.

CHARLWOOD, J. D., PINTO, J., SOUSA, C. A., FERREIRA, C. & DO ROSARIO, V. E. 2002a. Male size does not affect mating success (of *Anopheles gambiae* in Sao Tome). *Medical and Veterinary Entomology,* 16**,** 109-111.

CHARLWOOD, J. D., PINTO, J., SOUSA, C. A., MADSEN, H., FERREIRA, C. & DO ROSARIO, V. E. 2002b. The swarming and mating behaviour of *Anopheles gambiae s.s.* (Diptera : Culicidae) from Sao Tome Island. *Journal of Vector Ecology,* 27**,** 178-183.

CHARLWOOD, J. D., THOMPSON, R. & MADSEN, H. 2003. Observations on the swarming and mating behaviour of *Anopheles funestus* from southern Mozambique. *Malaria Journal,* 2:2.

CIVETTA, A., ROSING, K. R. & FISHER, J. H. 2008. Differences in sperm competition and sperm competition avoidance in *Drosophila melanogaster. Animal Behaviour,* 75**,** 1739-1746.

CLEMENTS, A. N. 1999. The biology of mosquitoes; Sensory reception and behaviour. *The biology of mosquitoes; Sensory reception and behaviour***,** i-xv, 1-740.

COETZEE, M., CRAIG, M. & LE SUEUR, D. 2000. Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today,* 16**,** 74-77.

COETZEE, M., HUNT, R. H., WILKERSON, R., DELLA TORRE, A., COULIBALY, M. B. & BESANSKY, N. J. 2013. *Anopheles coluzzii* and *Anopheles amharicus*, new members of the Anopheles gambiae complex. *Zootaxa,* 3619**,** 246-274.

COLUZZI, M., PETRARCA, V. & DIDECO, M. A. 1985. Chromosomal inversion intergradation and incipient speciation in *Anopheles-gambiae. Bollettino Di Zoologia,* 52**,** 45-63.

COLUZZI, M., SABATINI, A., DELLA TORRE, A., DI DECO, M. A. & PETRARCA, V. 2002. A polyethene chromosome analysis of the *Anopheles gambiae* species complex. *Science,* 298**,** 1415-1418.

COLUZZI, M., SABATINI, A., PETRARCA, V. & DIDECO, M. A. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles-gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 73**,** 483-497.

CORDTS, R. & PARTRIDGE, L. 1996. Courtship reduces longevity of male *Drosophila melanogaster*. *Animal Behaviour,* 52**,** 269-278.

CROSS, C. (2004). Welcome Trust: Malaria. In The life cycle of *Anopheles* mosquitoes. http://malaria.wellcome.ac.uk/doc_WTD023872.html. (Accessed 20 Oct. 2011).

CRAIG, G. B., JR. 1967. Mosquitoes: female monogamy induced by male accessory gland substance. *Science (New York, N.Y.),* 156**,** 1499-501.

CRAMPTON, J. M., WARREN, A., LYCETT, G. J., HUGHES, M. A., COMLEY, I. P. & EGGLESTON, P. 1994. Genetic manipulation of insect vectors as a strategy for the control of vector-borne disease. *Annals of Tropical Medicine and Parasitology ,* 88**,** 3-12.

CREIGHTON, J. C., HEFLIN, N. D. & BELK, M. C. 2009. Cost of reproduction, resource quality, and terminal investment in a burying beetle. *American Naturalist,* 174**,** 673-684.

CURTIS, C. F. 1978. Genetic sex separation in *Anopheles arabiensis* and the production of sterile hybrids. Bulletin: World Health Organisation 45: 802-465.

DAHLGAARD, J. & HOFFMANN, A. A. 2000. Stress resistance and environmental dependency of inbreeding depression in *Drosophila melanogaster. Conservation Biology,* 14**,** 1187-1192.

DAO, A., KASSOGUE, Y., ADAMOU, A., DIALLO, M., YARO, A. S., TRAORE, S. F. & LEHMANN, T. 2010. Reproduction-Longevity Trade-Off in *Anopheles gambiae* (Diptera: Culicidae). *Journal of Medical Entomology,* 47**,** 769-777.

DAVIDSON, G., ODETOYIN.JA, COLUSSA, B. & COZ, J. 1970. A field attempt to assess mating competitiveness of sterile males produced by crossing 2 member species of *anopheles-gambiae* complex. *Bulletin of the World Health Organization,* 42**,** 55-&.

DAY, J. F. & CURTIS, G. A. 1989. Influence of rainfall on *Culex-nigripalpus* (Diptera, Culicidae) blood-feeding behaviour in Indian-river-county, Florida. *Annals of the Entomological Society of America,* 82**,** 32-37.

DE VALDEZ, M. R. W., NIMMO, D., BETZ, J., GONG, H.-F., JAMES, A. A., ALPHEY, L. & BLACK, W. C. 2011. Genetic elimination of dengue vector mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 4772-4775.

DELLA TORRE, A., FANELLO, C., AKOGBETO, M., DOSSOU-YOVO, J., FAVIA, G., PETRARCA, V. & COLUZZI, M. 2001. Molecular evidence of incipient speciation within *Anopheles gambiae s.s.* in West Africa. *Insect Molecular Biology,* 10**,** 9-18.

DELLA TORRE, A., COSTANTINI, C., BESANSKY, N. J., CACCONE, A., PETRARCA, V., POWELL, J. R. & COLUZZI, M. 2002. Speciation within *Anopheles gambiae* - the glass is half full. *Science,* 298**,** 115-117.

DIABATE, A., BALDET, T., BRENGUES, C., KENGNE, P., DABIRE, K. R., SIMARD, F., CHANDRE, F., HOUGARD, J. M., HEMINGWAY, J., OUEDRAOGO, J. B. & FONTENILLE, D. 2003. Natural swarming behaviour of the molecular M form of *Anopheles gambiae. Transactions of the Royal Society of Tropical Medicine and Hygiene,* 97**,** 713-716.

DIABATE, A., DABIRE, R., OUARI, A., MILLOGO, N. & LEHMANN, T. 2006. Analysis of reproductive barriers between the molecular forms of *Anopheles gambiae. American Journal of Tropical Medicine and Hygiene,* 75**,** 209-209.

DIABATE, A., DAO, A., YARO, A. S., ADAMOU, A., GONZALEZ, R., MANOUKIS, N. C., TRAORE, S. F., GWADZ, R. W. & LEHMANN, T. 2009. Spatial swarm

segregation and reproductive isolation between the molecular forms of *Anopheles gambiae*. *Proceedings of the Royal Society B-Biological Sciences,* 276**,** 4215-4222.

DIABATE, A., YARO, A. S., DAO, A., DIALLO, M., HUESTIS, D. L. & LEHMANN, T. 2011. Spatial distribution and male mating success of *Anopheles gambiae* swarms. *BMC Evolutionary Biology,* 11:184.

DMITRIEW, C. & ROWE, L. 2011. The Effects of Larval Nutrition on Reproductive Performance in a Food-Limited Adult Environment. *PloS One,* 6:£.

DYCK, V. A., FLORES, J. R., VREYSEN, M. J. B., FERNANDEZ, E. E. R., TERUYA, T., BARNES, B., RIERA, P. G., LINDQUIST, D. & LOOSJES, M. 2005. Management of area-wide integrated pest management programmes that integrate the sterile insect technique. *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management***,** 525-545.

EDVARDSSON, M. 2007. Female *Callosobruchus maculatus* mate when they are thirsty: resource-rich ejaculates as mating effort in a beetle. *Animal Behavior,* 74**,** 183-188.

EL-AKAD, A. S. & HUMPHREYS, J. G. 1990a. Effects of larval and adult diet plus mating-insemination upon ovarian development of laboratory-reared *Anopheles-pharoensis* in Egypt. *Journal of the American Mosquito Control Association,* 6**,** 96-98.

EL-AKAD, A. S. & HUMPHREYS, J. G. 1990b. Pre-mating blood feeding by anopheles-pharoensis (Diptera: Culicidae) and its effects on mating, longevity and egg-production. *Journal of Entomological Science,* 25**,** 57-63.

ENAYATI, A. & HEMINGWAY, J. 2010. Malaria Management: Past, Present, and Future. *Annual Review of Entomology. Vol. 55: 569-591.*

ENAYATI, A. A., LINES, J., MAHARAJ, R., & HEMINGWAY, J. 2009. Suppressing the vector In Shrinking the Malaria. Map a Prospectus on Malaria Elimination, ed. RGM Feachem, A.A Phillips, G.A Target, pp. 140–54. San Francisco: Global Health Group

FACCHINELLI, L., VALERIO, L., BOND, J. G., DE VALDEZ, M. R. W., HARRINGTON, L. C., RAMSEY, J. M., CASAS-MARTINEZ, M. & SCOTT, T. W. 2011. Development of a semi-field system for contained field trials with *Aedes aegypti* in Southern Mexico. *American Journal of Tropical Medicine and Hygiene,* 85**,** 248-256.

FANELLO, C., SANTOLAMAZZA, F. & DELLA TORRE, A. 2002. Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. *Medical and Veterinary Entomology,* 16**,** 461-464.

FARENHORST, M., MOUATCHO, J. C., KIKANKIE, C. K., BROOKE, B. D., HUNT, R. H., THOMAS, M. B., KOEKEMOER, L. L., KNOLS, B. G. J. & COETZEE, M. 2009. Fungal infection counters insecticide resistance in African malaria mosquitoes. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 17443-17447.

FAVIA, G., DELLA TORRE, A., BAGAYOKO, M., LANFRANCOTTI, A., SAGNON, N., TOURE, Y. T. & COLUZZI, M. 1997. Molecular identification of sympatric chromosomal forms of *Anopheles gambiae* and further evidence of their reproductive isolation. *Insect Molecular Biology,* 6**,** 377-383.

FAVIA, G. & LOUIS, C. 1999. Molecular identification of chromosomal forms of *Anopheles gambiae sensu stricto. Parasitologia (Rome),* 41**,** 115-118.

FAVIA, G., LANFRANCOTTI, A., SPANOS, L., SIDEN-KIAMOS, I. & LOUIS, C. 2001. Molecular characterization of ribosomal DNA polymorphisms discriminating among chromosomal forms of *Anopheles gambiae s.s. Insect Molecular Biology,* 10**,** 19-23.

FERGUSON, H. M., JOHN, B., NG'HABI, K. & KNOLS, B. G. J. 2005. Redressing the sex imbalance in knowledge of vector biology. *Trends in Ecology & Evolution,* 20**,** 202-209.

FISCHER, G. B., SARRIA, E. E., MADEIRO LEITE, A. J. & AMORIM DE BRITTOL, M. C. 2008. Parasitic lung infection and the paediatric lung. *Paediatric Respiratory Reviews,* 9**,** 57-66.

FLOORE, T. (2000). Mosquito information. *The American Mosquito Control Association.* Retrieved 19/02/00 from www.mosquito.org/mosquito.html.

FONTENILLE, D., LEPERS, J. P., CAMPBELL, G. H., COLUZZI, M., RAKOTOARIVONY, I., ANDRIANAIVOLAMBO, L. & COULANGES, P. 1993. Longitudinal study of malaria transmission and the biology of vectors in Sainte Marie Island, off the eastern coast of Madagascar, from 1988 to 1990. *Archives de l'Institut Pasteur de Madagascar,* 59**,** 99-125.

FONTENILLE, D. & LOCHOUARN, L. 1999. The complexity of the malaria vectorial system in Africa. *Parasitologia (Rome),* 41**,** 267-271.

FOUET, C., GRAY, E., BESANSKY, N. J. & COSTANTINI, C. 2012. Adaptation to aridity in the malaria mosquito *Anopheles gambiae:* Chromosomal inversion polymorphism and body size influence resistance to desiccation. *PloS One,* 7.

FOX, C. W. & REED, D. H. 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. *Evolution,* 65**,** 246-258.

FOX, C. W. & SCHEIBLY, K. L. 2006. Variation in inbreeding depression among populations of the seed beetle, *Stator limbatus. Entomologia Experimentalis Et Applicata,* 121**,** 137-144.

FOX, C. W., SCHEIBLY, K. L., SMITH, B. P. & WALLIN, W. G. 2007. Inbreeding depression in two seed-feeding beetles, *Callosobruchus maculatus* and *Stator limbatus* (Coleoptera : Chrysomelidae). *Bulletin of Entomological Research,* 97**,** 49-54.

FOX, C. W., STILLWELL, R. C., WALLIN, W. G., CURTIS, C. L. & REED, D. H. 2011. Inbreeding-environment interactions for fitness: complex relationships between inbreeding

depression and temperature stress in a seed-feeding beetle. *Evolutionary Ecology,* 25**,** 25-43.

FRANKE, K., HEITMANN, N., TOBNER, A. & FISCHER, K. 2014. Fitness costs associated with different frequencies and magnitudes of temperature change in the butterfly *Bicyclus anynana*. *Journal of Thermal Biology,* 41**,** 88-94.

FRANKHAM, R., BALLOU, J. D. & BRISCOE, D.A. 2002. Introduction to conservation genetics. Cambridge, UK: Cambridge University Press. p617

FRY, J. D., HEINSOHN, S. L. & MACKAY, T. F. C. 1998. Heterosis for viability, fecundity, and male fertility in *Drosophila melanogaster*: Comparison of mutational and standing variation. *Genetics,* 148**,** 1171-1188.

FU, G., LEES, R. S., NIMMO, D., AW, D., JIN, L., GRAYA., P., BERENDONK, T. U., WHITE-COOPER, H., SCAIFE, S., PHUC, H. K., MARINOTTI, O., JASINSKIENE, N., JAMES, A. A & ALPHEY, L. 2010. Female-specific flightless phenotype for mosquito control. *PNAS* vol. 107,10, 4550-4554

FUTUYMA, D., J. 2009. Evolution (Second edition) USA, Sinauer Associates, Sunderland, Massachusetts. ISBN 978-0-87893-223-8.

GERBERC, E. J. 1970. Manual for mosquito rearing and experimental techniques. AMCA Bulletin 5, 1-91.

GARCIA-GONZALEZ, F. & SIMMONS, L. W. 2005. Sperm viability matters in insect sperm competition. *Current Biology,* 15**,** 271-275.

GARY, R. E., (JR) CANNON, J. W., III & FOSTER, W. A. 2009. Effect of sugar on male *Anopheles gambiae* mating performance, as modified by temperature, space, and body size. *Parasites & Vectors,* 2:19.

GARY, R. E., (JR) & FOSTER, W. A. 2006. Diel timing and frequency of sugar feeding in the mosquito *Anopheles gambiae*, depending on sex, gonotrophic state and resource availability. *Medical and Veterinary Entomology,* 20**,** 308-316.

GENTILE, G., DELLA TORRE, A., MAEGGA, B., POWELL, J. R. & CACCONE, A. 2002. Genetic differentiation in the African malaria vector, *Anopheles gambiae s.s*, and the problem of taxonomic status. *Genetics,* 161**,** 1561-1578.

GIBBS A. G., 2002. Water balance in desert Drosophila: lessons from non-charismatic micro fauna. *Comparative Biochemistry and Physiology*. 133: 781–789.

GIBBS, A. G. & VANIER, C. H. 2004. Evolution of water balance and gene expression in desiccation-selected Drosophila melanogaster. *Integrative and Comparative Biology,* 44**,** 700-700.

GIGLIOLI, M. E. 1964. The female reproductive system of *Anopheles gambiae melas*. ii. the ovary. *Rivista di malariologia,* 43**,** 265-75.

GIGIOLI, M. E. C. & MASON, G. F. 1966. The mating plug in Anopheline mosquitoes. Proceedings of the Royal Entomological Society of London. Series A, *General Entomology,* 41, 123-129.

GILBERT, D. G. 1981. Studies of esterase-6 in *Drosophila-melanogaster* .8. ejaculate esterase-6 and initial sperm use by female *Drosophila-melanogaster. Journal of Insect Physiology,* 27**,** 641-650.

GILLIES, M. T. 1956. A new character for the recognition of nulliparous females of *Anopheles gambiae. Bulletin of the World Health Organization,* 15**,** 451-9.

GILLIES, M. T. & WILKES, T. J. 1965. A study of the age-composition of populations of *Anopheles gambiae Giles* and *A. funestus Giles* in North-Eastern Tanzania. *Bulletin of Entomological research,* 56**,** 237-62.

GILLOTT, C. 2003. Male accessory gland secretions: Modulators of female reproductive physiology and Behavior. *Annual Review of Entomology,* 48**,** 163-184.

GRAY, E. M. & BRADLEY, T. J. 2005. Physiology of desiccation resistance in *Anopheles gambiae* and *Anopheles arabiensis. American Journal of Tropical Medicine and Hygiene,* 73**,** 553-559.

GREENWOOD, B. & MUTABINGWA, T. 2002. Malaria in 2002. *Nature,* 415**,** 670-672.

GREENWOOD, B.M., FIDOCK, D.A., KYLE, D.E., KAPPE, S. H. I., ALONSO, P.L., COLLINS, F.H., & DUFFY, P.E. 2008. Malaria: progress, perils, and prospects for eradication. *Journal of Clinical Investigation.*118: 1266–1276.

HAEGER, E. J. D., BIDLINGMAYER, J. S., W. L., DOW, R. P., NAYAR, J. K., & PROVOST, M. W. 1972. Sexual Behaviour of Mosquitoes. Field Observations on Mating and Insemination of Marked Broods of *Aedes taeniorhynchus. Annals of the Entomological Society of America, Vol. 65, 4, 848-852(5).*

HARANO, T. 2011. Inbreeding depression in development, survival, and reproduction in the adzuki bean beetle (*Callosobruchus chinensis*). *Ecological Research,* 26**,** 327-332.

HARBACH, R. E. 1994. Review of the internal classification of the genus anopheles (Diptera, Culicidae) - the foundation for comparative systematics and phylogenetic research. *Bulletin of Entomological Research,* 84**,** 331-342.

HARBACH, R. E. 2004. The classification of genus Anopheles (Diptera : Culicidae): a working hypothesis of phylogenetic relationships. *Bulletin of Entomological Research,* 94**,** 537-553.

HARBACH, R. E. 2011. Classification within the cosmopolitan genus Culex (Diptera: Culicidae): The foundation for molecular systematics and phylogenetic research. *Acta Tropica,* 120**,** 1-14.

HARRIS, A. F., MCKEMEY, A. R., NIMMO, D., CURTIS, Z., BLACK, I., MORGAN, S. A., OVIEDO, M. N., LACROIX, R., NAISH, N., MORRISON, N. I., COLLADO, A.,

STEVENSON, J., SCAIFE, S., DAFA'ALLA, T., FU, G., PHILLIPS, C., MILES, A., RADUAN, N., KELLY, N., BEECH, C., DONNELLY, C. A., PETRIE, W. D. & ALPHEY, L. 2011. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nature Biotechnology,* 30**,** 828-830.

HASSAN, M. A. M., ZAIN, H. M., BASHEER, M. A., ELHAJ, H.-E. F. & EL-SAYED, B. B. 2014. Swarming and mating behavior of male *Anopheles arabiensis* Patton (Diptera: Culicidae) in an area of the Sterile Insect Technique Project in Angola, northern Sudan. *Acta Tropica,* 132**,** S64-S69.

HEDRICK, P. W. & KALINOWSKI, S. T. 2000. Inbreeding depression in conservation biology. *Annual Review of Ecology and Systematics* 31, 139–62.

HEID, C. A., STEVENS, J., LIVAK, K. J. & WILLIAMS, P. M. 1996. Real time quantitative PCR. *Genome Research,* 6**,** 986-994.

HELINSKI, M. E. H. & KNOLS, B. G. J. 2008. Mating competitiveness of male *Anopheles arabiensis* mosquitoes irradiated with a partially or fully sterilizing dose in small and large laboratory cages. *Journal of Medical Entomology,* 45**,** 698-705.

HELINSKI, M. E. H. & KNOLS, B. G. J. 2009. Sperm quantity and size variation in un-irradiated and irradiated males of the malaria mosquito *Anopheles arabiensis* Patton. *Acta Tropica,* 109**,** 64-69.

HOGG, J. C. & HURD, H. 1995. Malaria – induced reduction of fecundity during the first gonotrophic cycle of Anopheles stephensi mosquito. *Medical Veterinary Entomology*, 9,176-180.

HOGG, J. C., THOMSON, M. C. & HURD, H. 1996. Comparative fecundity and associated factors for two sibling species of the *Anopheles gambiae* complex occurring sympatrically in The Gambia. *Medical and Veterinary Entomology,* 10**,** 385-391.

HOLMAN, L. 2009. Sperm viability staining in ecology and evolution: potential pitfalls. *Behavioural Ecology and Sociobiology,* 63**,** 1679-1688.

HOWELL, P. I. & KNOLS, B. G. J. 2009. Male mating biology. *Malaria Journal,* 8. (Suppl 2).

HUESTIS, D. L., YARO, A. S., TRAORE, A. I., ADAMOU, A., KASSOGUE, Y., DIALLO, M., TIMBINE, S., DAO, A. & LEHMANN, T. 2011. Variation in metabolic rate of *Anopheles gambiae* and *A. arabiensis* in a Sahelian village. *Journal of Experimental Biology,* 214**,** 2345-2353.

HUESTIS, D. L., YARO, A. S., TRAORE, A. I., DIETER, K. L., NWAGBARA, J. I., BOWIE, A. C., ADAMOU, A., KASSOGUE, Y., DIALLO, M., TIMBINE, S., DAO, A. & LEHMANN, T. 2012. Seasonal variation in metabolic rate, flight activity and body size of *Anopheles gambiae* in the Sahel. *Journal of Experimental Biology,* 215**,** 2013-2021.

HUGHES, K.A. 1995. The evolutionary genetics of male life-history characters in *Drosophila melanogaster. Evolution*, 49, 521–537.

HUNTER, F. M. & BIRKHEAD, T. R. 2002. Sperm viability and sperm competition in insects. *Current Biology,* 12**,** 121-123.

IMAGEJ 1.44 SOFTWARE. http://en.softonic.com/s/imagej-1.44:mac (Accessed 30 July 2013).

IMPOINVIL, D. E., CARDENAS, G. A., GIHTURE, J. I., MBOGO, C. M. & BEIER, J. C. 2007. Constant temperature and time period effects on *Anopheles gambiae* egg hatching. *Journal of the American Mosquito Control Association*, 23, 124-130.

JIRAKANJANAKIT, N., LEEMINGSAWAT, S., THONGRUNGKIAT, S., APIWATHNASORN, C., SINGHANIYOM, S., BELLEC, C., & DUJARDIN, J. P., 2007. Influence of larval density or food variation on the geometry of the wing of *Aedes (Sethomyia) aegypti. Tropical Medicine and International Health* 12 (11), 1354–1360.

JOLY, D., CARIOU, M. L., LACHAISE, D. & DAVID, J. R. 1989. Variation of sperm length and heteromorphism in *Drosophila* species. *Genetics Selection Evolution,* 21**,** 283-293.

JOLY, D., KOROL, A. & NEVO, E. 2004. Sperm size evolution in *Drosophila*: inter- and intraspecific analysis. *Genetics,* 120**,** 233-244.

KAMAU, L., LEHMANN, T., HAWLEY, W. A., ORAGO, A. S. S. & COLLINS, F. H. 1998. Micro geographic genetic differentiation of *Anopheles gambiae* mosquitoes from Asembo Bay, Western Kenya: A comparison with Kilifi in coastal Kenya. *American Journal of Tropical Medicine and Hygiene,* 58**,** 64-69.

KELLER, L. & PASSERA, L. 1992. Mating system, optimal number of mating, and sperm transfer in the argentine ant *iridomyrmex-humilis. Behavioural Ecology and Sociobiology,* 31**,** 359-366.

KELLER, L. F. & WALLER, D. M. 2002. Inbreeding effects in wild populations. *Trends in Ecology and Evolution* 17:230-241.

KELLY, C. D. 2008. Sperm investment in relation to weapon size in a male trimorphic insect? *Behavioural Ecology,* 19**,** 1018-1024.

KERSTES, N. A. G. & WEGNER, K. M. 2011. The effect of inbreeding and out-crossing of *Tribolium castaneum* on resistance to the parasite *Nosema whitei. Evolutionary Ecology Research,* 13**,** 681-696.

KISZEWSKI, A., MELLINGER, A., SPIELMAN, A., MALANEY, P., SACHS, S. E. & SACHS, J. 2004. A global index representing the stability of malaria transmission. *American Journal of Tropical Medicine and Hygiene,* 70**,** 486-498.

KLASSEN, W. & CURTIS, C. F. 2005. History of the sterile insect technique. *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management***,** 3-36.

KLOWDEN, M. J. 1999. The check is in the male: Male mosquitoes affect female physiology and behavior. *Journal of the American Mosquito Control Association,* 15**,** 213-220.

KLOWDEN, M. J. 2001. Sexual receptivity in *Anopheles gambiae* mosquitoes: absence of control by male accessory gland substances. *Journal of Insect Physiology,* 47**,** 661-666.

KLOWDEN, M. J. 2006. Switchover to the mated state by spermathecal activation in female *Anopheles gambiae* mosquitoes. *Journal of Insect Physiology,* 52**,** 679-684.

KLOWDEN, M. J. & CHAMBERS, G. M. 2004. Production of polymorphic sperm by Anopheline mosquitoes and their fate within the female genital tract. *Journal of Insect Physiology,* 50**,** 1163-1170.

KNIPLING, E. F. 1959. Sterile-male method of population control. *Science (New York, N.Y.),* 130**,** 902-4.

KNOLS, B. G. J & TAKKEN, W. 1998. The wide-scale use of impregnated bed nets for malaria control in Africa: impact on mosquitoes. *Proceedings of Experimental and Applied Entomology*, N.E.V., Amsterdam 9, 15–22.

KNOP, N. F., ASMAN, S. M., REISEN, W. K. & MILBY, M. M. 1987. Changes in the biology of *Culex-tarsalis* (Diptera, Culicidae) associated with colonization under contrasting regimes. *Environmental Entomology,* 16**,** 405-414.

KOUDOU, B. G., KOFFI, A. A., MALONE, D. & HEMINGWAY, J. 2011. Efficacy of PermaNet (R) 2.0 and PermaNet (R) 3.0 against insecticide-resistant Anopheles gambiae in experimental huts in Cote d'Ivoire. *Malaria Journal,* 10:172

KOUTSOS, A. C., BLASS, C., MEISTER, S., SCHMIDT, S., MACCALLUM, R. M., SOARES, M. B., COLLINS, F. H., BENES, V., ZDOBNOV, E., KAFATOS, F. C. & CHRISTOPHIDES, G. K. 2007. Life cycle transcriptome of the malaria mosquito *Anopheles gambiae* and comparison with the fruit fly *Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 11304-11309.

KRAFSUR, E. S. 1998. Sterile insect technique for suppressing and eradicating insect populations: 55 years and counting. *Journal of Agricultural Entomology,* 15**,** 303-317.

KRZYWINSKI, J., SANGARE, D. & BESANSKY, N. J. 2005. Satellite DNA from the Y chromosome of the malaria vector Anopheles gambiae. *Genetics,* 169**,** 185-196.

KURIWADA, T., KUMANO, N., SHIROMOTO, K. & HARAGUCHI, D. 2011. The Effect of inbreeding on mating behaviour of West Indian sweet potato Weevil *Euscepes postfasciatu*s. *Ethnology.* 117**,** 822-828.

LACY, R. C. 1993. Impacts of inbreeding in natural and captive populations of vertebrates - implications for conservation. *Perspectives in Biology and Medicine,* 36**,** 480-496.

LANZARO, G. C. & TRIPET, F. 2003.Gene flow among populations of *Anopheles gambiae*: A critical review. In: Ecological Aspects for the Application of Genetically Modified Mosquitoes. (pp. 109-132) Eds. Takken, W. and Scott T.W. Frontis Press, Wageningen, The Netherlands.

LEE, Y., MENESES, C. R., FOFANA, A. & LANZARO, G. C. 2009. Desiccation Resistance Among Subpopulations of *Anopheles gambiae s.s.* From Selinkenyi, Mali. *Journal of Medical Entomology,* 46**,** 316-320.

LEHMANN, T., HAWLEY, W. A, KAMAU, L., FONTENILLE, D., SIMARD, F. & COLLINS, F.H.1996. Genetic differentiation of *Anopheles gambiae* populations from east and west Africa: comparison of microsatellite and allozyme loci. *Journal of Heredity.* 77(pt 2):192-200.

LEHMANN, T., BLACKSTON, C.R., BESANSKY, N.J., ESCALANTE, A. A., COLLINS, F. H., & HAWLEY W. A. 2000. The Rift Valley complex as a barrier to gene flow for *Anopheles gambiae* in Kenya: the mtDNA perspective. *Journal of Heredity.* 91:165-168.

LEHMANN, T., LICHT, M., ELISSA, N., MAEGA, B. T. A., CHIMUMBWA, J. M., WATSENGA, F. T., WONDJI, C. S., SIMARD, F. & HAWLEY, W. A. 2003. Population structure of *Anopheles gambiae* in Africa. *Journal of Heredity,* 94**,** 133-147.

LEHMANN, T., DAO, A., YARO, A. S., ADAMOU, A., KASSOGUE, Y., DIALLO, M., SEKOU, T. & COSCARON-ARIAS, C. 2010. Aestivation of the African malaria mosquito, *Anopheles gambiae* in the Sahel. *American Journal of Tropical Medicine and Hygiene,* 83**,** 601-606.

LEHMANN, T., DAO, A., YARO, A. S., DIALLO, M., TIMBINE, S., HUESTIS, D. L., ADAMOU, A., KASSOGUE, Y. & TRAORE, A. I. 2014. Seasonal variation in spatial distributions of *Anopheles gambiae* in a Sahelian village: Evidence for aestivation. *Journal of Medical Entomology,* 51**,** 27-38.

LENGELER, C. 2004. Insecticide-treated nets for malaria control: real gains. *Bulletin of the World Health Organization,* 82**,** 84-84.

LI, C. Y., MARRELLI, M. T., YAN, G. Y. & JACOBS-LORENA, M. 2008. Fitness of transgenic *Anopheles stephensi* mosquitoes expressing the SM1 peptide under the control of a vitellogene promoter. *Journal of Heredity,* 99**,** 275-282.

LINES, J. D. & CURTIS, C. F. 1985. Genetic sexing systems in *Anopheles arabiensis* Patton (Diptera :Culicidae). *Journal of Economic Entomology.* 78, 848-851.

LIU, X. P., TU, X. Y., HE, H. M., CHEN, C. & XUE, F. S. 2014. Evidence for inbreeding depression and pre-copulatory, but not post copulatory inbreeding avoidance in the cabbage beetle *Colaphellus bowringi*. *PloS One,* 9**,** 7.

LOUNIBOS, L. P., LIMA, D. C., LOURENCO-DE-OLIVEIRA, R., ESCHER, R. L. & NISHIMURA, N. 1998. Egg maturation in neotropical malaria vectors: One blood meal is usually enough. *Journal of Vector Ecology,* 23**,** 195-201.

LUKE, T. C. & HOFFMAN, S. L. 2003. Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated Plasmodium falciparum sporozoite vaccine. *Journal of Experimental Biology,* 206**,** 3803-3808.

MAHMOOD, F. & REISEN, W. K. 1982. A*nopheles-stephensi* (Diptera, Culicidae) -changes in male mating competence and reproductive-system morphology associated with aging and mating. *Journal of Medical Entomology,* 19**,** 573-588.

MAHMOOD, F. & REISEN, W. K. 1994. *Anopheles culicifacies* - Effects of age on the male reproductive-system and mating ability of virgin adult mosquitoes. *Medical and Veterinary Entomology,* 8**,** 31-37.

MAIGA, H., NIANG, A., SAWADOGO, S. P., DABIRE, R. K., LEES, R. S., GILLES, J. R. L., TRIPET, F. & DIABATE, A. 2014. Role of nutritional reserves and body size in *Anopheles gambiae* males mating success. *Acta Tropica,* 132**,** S102-S107.

MALARIA: STATISTICS.1996. http://archive.idrc.ca/books/reports/1996/01-07e.html (Accessed 15 Oct. 2011).

MANCINI, E., BALDINI, F., TAMMARO, F., CALZETTA1, M., SERRAO, A., GEORGE, P., MORLAIS I., MASIGA, D., SHARAKHOV, I. V., ROGERS, D. W., CATTERUCCIA, F. & DELLA TORRE, A. 2011.Molecular characterization and evolution of a gene family encoding male-specific reproductive proteins in the African malaria vector *Anopheles gambiae.   Evolutionary Biology*, 11: 292.

MANOUKIS, N. C, TOURE, M. B., SISSOKO, I., DOUMBIA, S., TRAORE, S. F., DIUK-WASSER, M. & TAYLOR, C. E. 2006. Is vector body size the key to reduce malaria transmission in the irrigated region of Niono, Mali? *Journal of Medical. Entomology.* 43, 820-827.

MANZAVA, A., CURTIS, C.F., MANGA, L. & TOURE, T.Y. 2006. Application of genetically modified mosquitoes in national vector control programmes; thoughts on integrated control. Bridging Laboratory and Field Research for Genetic control of Disease Vector. Edited by B. G. J. Knols and C. Louis. Wageningen UR Frontis Series. Springer.

MACK, P. D., HAMMOCK, B. A. & PROMISLOW. D. E. L. 2002. Sperm competitive ability and genetic relatedness in Drosophila melanogaster: similarity breeds contempt. *Evolution.* 56:1789–1795.

MARCHAND, R. P. 1984. Field observations on swarming and mating in *Anopheles-gambiae* mosquito in Tanzania. *Netherlands Journal of Zoology,* 34**,** 367-387.

MASSONNET-BRUNEEL, B., CORRE-CATELIN, N., LACROIX, R., LEES, R. S., KIM PHUC, H., NIMMO, D., ALPHEY, L. & REITER, P. 2013. Fitness of transgenic mosquito *Aedes aegypti* males carrying a dominant lethal genetic system. *PloS One,* 8:5.

MATTEY, S. N., STRUTT, L. & SMISETH, P. T. 2013. Intergenerational effects of inbreeding in *Nicrophorus vespilloides*: offspring suffer fitness costs when either they or their parents are inbred. *Journal of Evolutionary Biology,* 26**,** 843-853.

MENGE, D. M., GUDA, T., ZHONG, D. B., PAI, A., ZHOU, G. F., BEIER, J. C., GOUAGNA, L. & YAN, G. Y. 2005. Fitness consequences of *Anopheles gambiae* population hybridization. *Malaria Journal,* 4:44

METAPATHOGEN. http://www.metapathogen.com/mosquito/anopheles/(2011)(Accessed 20 Dec 2011).

MICHALCZYK, L., MARTIN, O. Y., MILLARD, A. L., EMERSON, B. C. & GAGE, M. J. G. 2010. Inbreeding depresses sperm competitiveness, but not fertilization or mating success in male *Tribolium castaneum. Proceedings of the Royal Society B-Biological Sciences,* 277**,** 3483-3491.

MINAKAWA, N., SONYE, G., MOGI, M., GITHEKO, A. & YAN, G. Y. 2002. The effects of climatic factors on the distribution and abundance of malaria vectors in Kenya. *Journal of Medical Entomology,* 39**,** 833-841.

MITCHELL, S. N., STEVENSON, B. J., MUELLER, P., WILDING, C. S., EGYIR-YAWSON, A., FIELD, S. G., HEMINGWAY, J., PAINE, M. J. I., RANSON, H. & DONNELLY, M. J. 2012. Identification and validation of a gene causing cross-resistance between insecticide classes in *Anopheles gambiae* from Ghana. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 6147-6152.

MOLINEAUX, L. & GRAMICCIA, G. 1980. The Garki Project: Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa. 311p. *WHO: Geneva, Switzerland. Illus. Maps. Paper***,** 311P-311P.

MOLINEAUX, L., STOREY, J., COHEN, T. E. & THOMAS, A. 1980. Longitudinal study of human Malaria in the West African Savanna in the absence of control measures. *American Journal of Medical Hygiene* 29(5) pp 725-737

MOORE, P. J., HARRIS, W. E., MONTROSE, V. T., LEVIN, D. & MOORE, A. J. 2004. Constraints on evolution and post copulatory sexual selection: Trade-offs among ejaculate characteristics. *Evolution,* 58**,** 1773-1780.

MPOFU, S. M., MASENDU, H. T., KANYIMO, K. H. & MTETWA, C. 1993. Laboratory colonization of *Anopheles-quadriannulatus* from sympatry with other sibling species of the anopheles-gambiae complex in Zimbabwe. *Medical and Veterinary Entomology,* 7**,** 122-126.

MUNSTERMANN, L. E. 1994. Unexpected genetic consequences of colonization and inbreeding - allozyme tracking in Culicidae (Diptera). *Annals of the Entomological Society of America,* 87**,** 157-164.

NAKAHARA, M. & TSUBAKI, Y. 2007. Function of multiple sperm-storage organs in female damselflies (*Ischnura senegalensis*): Difference in amount of ejaculate stored, sperm loss, and priority in fertilization. *Journal of Insect Physiology,* 53**,** 1046-1054.

NASCI, R. S., HARE, S. G. & WILLIS, F. S. 1989. Interspecific mating between Louisiana strains of *Aedes-albopictus* and *Aedes-aegypti* in the field and laboratory. *Journal of the American Mosquito Control Association,* 5**,** 416-421.

NG'HABI, K. R., JOHN, B., NKWENGULILA, G., KNOLS, B. G. J., KILLEEN, G. F. & FERGUSON, H. M. 2005. Effect of larval crowding on mating competitiveness of *Anopheles gambiae* mosquitoes. *Malaria Journal,* 4:49.

NG'HABI, K., JOHN, B., NKWENGULILA, G., KILLEEN, G., KNOLS, B. G. & FERGUSON, H. 2006. Male *Anopheles gambiae* mating success in a swarm: 'May the best man lose'. *American Journal of Tropical Medicine and Hygiene,* 75**,** 305-305.

NG'HABI, K. R., HORTON, A., KNOLS, B. G. J. & LANZARO, G. C. 2007. A new robust diagnostic polymerase chain reaction (PCR) for determining the mating status of female Anopheles gambiae mosquitoes. *American Journal of Tropical Medicine and Hygiene,* 77**,** 293-293.

NG'HABI, K. R., MWASHESHI, D. , KNOLS, B. G. J. & FERGUSON H. M. 2010. Establishment of a self-propagating population of the African malaria vector *Anopheles arabiensis* under semi-field conditions. *Malaria Journal,* 9:356.

NG'HABI, K. R., KNOLS, B. G. J., LEE, Y., FERGUSON, H. M. & LANZARO, G. C. 2011. Population genetic structure of *Anopheles arabiensis* and *Anopheles gambiae* in a malaria endemic region of southern Tanzania. *Malaria Journal,* 10:289.

NIKOLAEVA, N. V. 1976. The swarming of mosquitoes of the genus *Aedes Culicidae* in the forest tundra. *Parazitologiya (St. Petersburg),* 10**,** 310-314.

NORRIS, D. E., SHURTLEFF, A. C., TOURE, Y. T. & LANZARO, G. C. 2001. Microsatellite DNA polymorphism and heterozygosity among field and laboratory populations of *Anopheles gambiae s.s.* (Diptera : Culicidae). *Journal of Medical Entomology,* 38**,** 336-340.

O'DONNELLY, D. & ARMBRUSTER, P. 2010. Inbreeding depression affects life-history traits but not infection by *Plasmodium gallinaceum* in the Asian tiger mosquito, *Aedes albopictus. Infection Genetics and Evolution,* 10**,** 669-677.

OKANDA, F. M., DAO, A., NJIRU, B. N., ARIJA, J., AKELO, H. A., TOURE, Y., ODULAJA, A., BEIER, J. C., GITHURE, J. I., YAN, G., GOUAGNA, L. C., KNOLS, B. G. J. & KILLEEN, G. F. 2002. Behavioural determinants of gene flow in malaria vector populations: *Anopheles gambiae* males select large females as mates. *Malaria journal,* 1:10.

OKADA, K., BLOUNT, J. D., SHARMA, M. D., SNOOK, R. R. & HOSKEN, D. J. 2011. Male attractiveness, fertility and susceptibility to oxidative stress are influenced by inbreeding in *Drosophila simulans. Journal of Evolutionary Biology,* 24**,** 363-371.

ONIFADE, A.K., AKANNI, E.O & MEWOYEKA, O.O. 2007. Incidence of Malaria Infection among Human Immune deficiency Virus Patients in Ondo State, Nigeria. *Middle-East Journal of Scientific Research, 2,2: 48-53.*

PAPATHANOS, P. A., BOSSIN, H. C., BENEDICT, M. Q., CATTERUCCIA, F. MALCOLM, C. A., ALPHEY, L. & ANDREA CRISANTI, A. 2009. Sex separation strategies: past experience and new approaches. *Malaria Journal,* 8(Suppl 2):S5 doi:10.1186/1475- 2875-8-S2-S5

PARKER, G. A. 1970. Sperm competition and its evolutionary consequences in insects. *Biological Reviews of the Cambridge Philosophical Society,* 45**,** 525

PARTRIDGE, L., MACKAY, T. F. C. & AITKEN, S. 1985. Male mating success and fertility in *Drosophila-melanogaster. Genetical Research,* 46**,** 279-285.

PASINI, M. E. 2010. *Drosophila subobscura* short sperm have no biochemical incompatibilities with fertilization. *Open Entomology Journal,* 4**,** 25-29.

PATES, H. & CURTIS, C. 2005. Mosquito behavior and vector control. *Annual Review of Entomology.*

PEKKALA, N., KNOTT, K. E., KOTIAHO, J. S., NISSINEN, K. & PUURTINEN, M. 2014. The effect of inbreeding rate on fitness, inbreeding depression and heterosis over a range of inbreeding coefficients. *Evolutionary Applications,* 7**,** 1107-1119.

PEREZ-STAPLES, D. & ALUJA, M. 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. *Journal of Insect Physiology,* 52**,** 839-845.

PEREZ-STAPLES, D., SHELLY, T. E. & YUVAL, B. 2013. Female mating failure and the failure of 'mating' in sterile insect programs. *Entomologia Experimentalis Et Applicata,* 146**,** 66-78.

PETRIE, A. & SABIN, C. 2005. Medical statistics at a glance, Second Edition. Massachusetts, USA: Blackwell Publishing ISBN: 1405127805.

PHUC, H. K., ANDREASEN, M. H., BURTON, R. S., VASS, C., EPTON, M. J., PAPE, G., FU, G., CONDON, K. C., SCAIFE, S., DONNELLY, C. A., COLEMAN, P. G., WHITE-COOPER, H. & ALPHEY, L. 2007. Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology,* 5:11.

PLUESS, B., TANSER, F.C., LENGELER, C. AND SHARP, B. L. 2010. Indoor residual Spraying for preventing malaria. Cochrane Database System. Rev 14(4) CD006657.

PONDEVILLE, E., MARIA, A., JACQUES, J.-C., BOURGOUIN, C. & DAUPHIN-VILLEMANT, C. 2008. *Anopheles gambiae* males produce and transfer the vitellogenic steroid hormone 20-hydroxyecdysone to females during mating. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 19631-19636.

PONLAWAT, A. & HARRINGTON, L. C. 2007. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera : Culicidae). *Journal of Medical Entomology,* 44**,** 422-426.

PONLAWAT, A. & HARRINGTON, L. C. 2009. Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti. American Journal of Tropical Medicine and Hygiene,* 80**,** 395-400.

RADHAKRISHNAN, P., & FEDORKA, K.M. 2011. Influence of female age, sperm senescence and multiple mating on sperm viability in female *Drosophila melanogaster. Journal of Insect Physiology.* 10:017.

REICHARDT, A. K. & WHEELER, D. E. 1995. Estimation of sperm numbers in insects by fluorometry. *Insectes Sociaux,* 42**,** 449-452.

REINHOLD, K., KURTZ, J. & ENGQVIST, L. 2002. Cryptic male choice: sperm allocation strategies when female quality varies. *Journal of Evolutionary Biology,* 15**,** 201-209.

REISEN, W. K. 2003. Lessons from the past: an overview of studies by the University of Maryland and the University of California, Berkeley. *Ecological Aspects for Application of Genetically Modified Mosquitoes,* 2**,** 25-32.

REISEN, W. K., BAKER, R. H., SAKAI, R. K., MAHMOOD, F., RATHOR, H. R., RAANA, K. & TOQIR, G. 1981a. *Anopheles-culicifacies giles* - (Diptera, Culicidae) mating-behavior and competitiveness in nature of chemo-sterilized males carrying a genetic sexing system. *Annals of the Entomological Society of America,* 74**,** 395-401.

REISEN, W. K., AZRA, K. & MAHMOOD, F. 1982. *Anopheles-culicifacies* (Diptera, Culicidae) - horizontal and vertical estimates of immature development and survivorship in rural Punjab-province, Pakistan. *Journal of Medical Entomology,* 19**,** 413-422.

REZAVAL, C., PAVLOU, H. J., DORNAN, A.J., CHAN, Y.B., KRAVITZ, E.A., & GOODWIN, S.F. 2012. Neural circuitry underlying *Drosophila* female post mating behavioural responses. *Current Biology.* 22(13): 1155-1165.

ROBINSON, A. S., CAYOL, J. P. & HENDRICHS, J. 2002. Recent findings on medfly sexual behavior: Implications for sit. *Florida Entomologist,* 85**,** 171-181.

ROBERT, G. & COLLINS, F. H.1996. Anopheline mosquitoes and the agents they transmit. The biology of disease vectors (Barry. J. Beaty and Williams. C. Marquardt, Eds) University Press of Colorado.

ROFF, D. A. 1998. Effects of inbreeding on morphological and life history traits of the sand cricket, *Gryllus firmus. Heredity,* 81**,** 28-37.

ROGERS, D. W., BALDINI, F., BATTAGLIA, F., PANICO, M., DELL, A., MORRIS, H. R. & CATTERUCCIA, F. 2009. *Transglutaminase*-Mediated semen coagulation controls sperm storage in the malaria mosquito. *PloS Biology,* 7:12.

ROGERS, D. W., WHITTEN, M. M. A., THAILAYIL, J., SOICHOT, J., LEVASHINA, E. A. & CATTERUCCIA, F. 2008. Molecular and cellular components of the mating machinery in *Anopheles gambiae* females. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 19390-19395.

ROLL BACK ON MALARIA.
http://www.rollbackmalaria.org.globaladvocacy/pr2005.html (Accessed Dec 2011).

ROWE, M. & PRUETT-JONES, S. 2011. Sperm competition selects for sperm quantity and quality in the Australian Maluridae. *PloS One,* 6:1.

SACCHERI, I. J., LLOYD, H. D., HELYAR, S. J. & BRAKEFIELD, P. M. 2005. Inbreeding uncovers fundamental differences in the genetic load affecting male and female fertility in a butterfly. *Proceedings of the Royal Society B-Biological Sciences,* 272**,** 39-46.

SAEED, I. E. & AHMED, E. S. 2003. Determinants of acquiring malaria among displaced people in Khartoum state, Sudan. *Eastern Mediterranean health journal = La revue de sante de la Mediterranee orientale = al-Majallah al-ṣiḥḥiyah li-sharq al-mutawassiṭ,* 9**,** 581-92.

SCHARF, I., PETER, F. & MARTIN, O. Y. 2013. Reproductive Trade-Offs and direct costs for males in arthropods. *Evolutionary Biology,* 40**,** 169-184.

SCOTT, J. A., BROGDON, W. G. & COLLINS, F. H. 1993. Identification of single specimens of the *anopheles-gambiae* complex by the polymerase chain-reaction. *American Journal of Tropical Medicine and Hygiene,* 49**,** 520-529.

SCOTT, T. W., MORRISON, A. C., LORENZ, L. H., CLARK, G. G., STRICKMAN, D., KITTAYAPONG, P., ZHOU, H. & EDMAN, J. D. 2000. Longitudinal studies of *Aedes aegypti* (Diptera : Culicidae) in Thailand and Puerto Rico: Population dynamics. *Journal of Medical Entomology,* 37**,** 77-88.

SEKO, T., MIYATAKE, T. & MIURA, K. 2012. Assessment of hybrid vigor between flightless lines to restore survival and reproductive characteristics in the ladybird beetle *Harmonia axyridis. BioControl,* 57**,** 85-93.

SELYE, H. 1975. Implications of stress concept. *New York State Journal of Medicine,* 75**,** 2139-2145.

SHARMA, V. P., PATTERSO.RS & FORD, H. R. 1972. Device for rapid separation of male and female mosquito pupae. *Bulletin of the World Health Organization,* 47**,** 429-432.

SHARMA, V. P., LABRECQU.GC & PATTERSO.RS 1974. Device for pupae separation of male from female mosquitoes in field. *Mosquito News,* 34**,** 9-11.

SHAW, W. R., TEODORI, E., MITCHELL, S. N., BALDINI, F., GABRIELI, P., ROGERS, D. W. & CATTERUCCIA, F. 2014. Mating activates the heme peroxidase HPX15 in the sperm storage organ to ensure fertility in *Anopheles gambiae. Proceedings of the National Academy of Sciences of the United States of America,* 111**,** 5854-5859.

SERVICE, M.W. 2004. Medical Entomology for Students. Third edition. Cambridge University Press.

SHIAO, S., IMMO, A. H., JINSONG, Z., DOUGLAS, H. S., & ALEXANDER, S. R. 2008. Juvenile hormone connects larval nutrition with target of rapamycin signalling in the mosquito *Aedes aegypti*. *Journal of Insect Physiology*. 54(1): 231–239.

SHIFF, C. 2006. Malaria Epidemiology; John Hopkins University Bloomberg School of Public Health. www.jhsph.edu/course/malarialogy/PDFs/lecture 3.(Accessed Nov. 2011).

SHUTT, B., STABLES, L., ABOAGYE-ANTWI, F., MORAN, J. & TRIPET, F. 2010. Male accessory gland proteins induce female monogamy in Anopheline mosquitoes. *Medical and Veterinary Entomology,* 24**,** 91-94.

SIMMONS, L. W. & ROBERTS, B. 2005. Bacterial immunity traded for sperm viability in male crickets. *Science,* 309**,** 2031-2031.

SIMMONS, L. W. & BEVERIDGE, M. 2011. Seminal fluid affects sperm viability in a cricket. *PloS One,* 6:3.

SINGH, B., KIM, S.L., MATUSOP, A., RADHAKRISHNAN, A., SHAMSUL, S.S., COX-SINGH, J., THOMAS, A., & CONWAY, D. J. 2004. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet*. 363:1017–1024.

SIROT, L. K. & LAPOINTE, S. L. 2008. Patterns and consequences of mating behavior of the root weevil *Diaprepes abbreviatus* (Coleoptera : Curculionidae). *Florida Entomologist,* 91**,** 400-406.

STEVEN, A. J. 2007. Population Dynamics. *Journal of America Mosquito* Control Association. 23 (2 Suppl): 265–275.

SIVINSKI, J. 1980. Sexual selection and insect sperm. *Florida Entomologist,* 63**,** 99-111.

SMITS, A., ROELANTS, P., VAN BORTEL, W., & COOSEMANS, M. 1996. Enzyme polymorphisms in the *Anopheles gambiae* (Diptera: Culicidae) complex related to feeding and resting behavior in the Imbo Valley, Burundi. *Journal of Medical Entomol*ogy. 33:545–553

SNOOK, R. R. 2005. Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution,* 20**,** 46-53.

SNOW, R.W., GUERRA, C. A., NOOR, A. M., MYINT, H.Y. & HAY, S. I. 2005. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*. 434: 214–217.

SOUTH, S. H., STEINER, D. & ARNQVIST, G. 2009. Male mating costs in a polygamous mosquito with ornaments expressed in both sexes. *Proceedings of the Royal Society of British Biological Sciences,* 276**,** 3671-3678.

SPIELMAN, A., WATERMAN, R. E. & MELLER, S. M. 1974. Diversity in patterns of coital contact of mosquitoes - scanning electron-microscopic study. *Journal of Morphology,* 142**,** 187-203.

SUKOWATI, S. & BAIMAI, V. 1996. A standard cytogenetic map for *Anopheles Sundaicus* (Diptera: Culicidae) and evidence for chromosomal differentiation in populations from Thailand and Indonesia. *Genome,* 39**,** 165-173.

SULLIVAN, R. T. 1981. Insect swarming and mating. *Florida Entomologist,* 64**,** 44-65.

TABACHNICK, W. J. 2003. Reflections on the *Anopheles gambiae* genome sequence, transgenic mosquitoes and the prospect for controlling malaria and other vector borne diseases. *Journal of Medical Entomology,* 40**,** 597-606.

TAKKEN, W., KLOWDEN, M.J. & CHAMBERS, G. M.1998. Effect of body size on host seeking and blood meal utilization in *Anopheles gambiae sensu stricto*(Diptera: Culicidae the disadvantage of being small. *Journal of Medical Entomology*. 35, 639-645.

TAKKEN, W. & SCOTT, T. W. 2003. Ecological Aspects for Application of Genetically Modified Mosquitoes. Vol 2, ISBN: 978-1-4020-1585-4.

TAKKEN, W., CONSTANTINI, C., DOLO, G., HASSANALI, A., SAGNON, N. & OSIR, E. 2006. Mosquito mating behavior. In bridging laboratory and field research for genetic control of disease vectors edited by: Knols BGJ, Louis C. Netherlands: Springer :183-188

TAYLOR, C., TOURE, Y. T., CARNAHAN, J., NORRIS, D. E., DOLO, G., TRAORE, S. F., EDILLO, F. E. & LANZARO, G. C. 2001. Gene flow among populations of the malaria vector, *Anopheles gambiae,* in Mali, West Africa. *Genetics,* 157**,** 743-750.

THAILAYIL, J., MAGNUSSON, K., GODFRAY, H. C. J., CRISANTI, A., & CATTERUCCIA, F. 2011. Spermless males elicit large-scale female responses to mating in the malaria mosquito *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 13677-13681.

TOURE, Y.T., PETRARCA, V., TRAORE, S. F., COULIBALY, A.; MAIGA, H. M., SANKARE, O., SOW, M., DI DECO, M. A., & COLUZZI, M. 1994. Ecological genetic studies in the chromosomal form Mopti of *Anopheles gambiae s. s.* in Mali. West Africa, *Genetics*: 213-223.

TOURE, Y.T., TRAORE, S. F., SANKARE, O., SOW, M. Y., COULIBALY, A., ESPOSITO, F., & PETRARCA, V. 1996. Perennial transmission of malaria by the *Anopheles gambiae* complex in a north Sudan Savanna area of Mali. *Journal of Medical Veterinary Entomology,*10:197–199.

TOURE, Y. T., PETRARCA, V., TRAORE, S. F., COULIBALY, A., MAIGA, H. M., SANKARE, O., SOW, M., DI DECO, M. A. & COLUZZI, M. 1998. The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa. *Parasitologia (Rome),* 40**,** 477-511.

TRAM, U. & WOLFNER, M. F. 1999. Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*. *Genetics,* 153**,** 837-844.

TRAPE, J. F & ROGIER, C. 1996. Combating Malaria Morbidity and Mortality by Reducing Transmission. *Tropical Medical and Hygiene. 81* (SUPPI.2) 10-18

TRAPE, J. F. & ZOULANI, A. 1987. Malaria and urbanization in Central-Africa - the example of Brazzaville .3. relationships between urbanization and the intensity of malaria transmission. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 81**,** 19-25.

TRAPE, J. F. 1987. Malaria and urbanization in Central-Africa - the example of Brazzaville .1. Description of the town and review of previous surveys. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 81**,** 1-9.

TRIPET, F., DOLO, G. & LANZARO, G. C. 2005a. Multilevel analyses of genetic differentiation in *Anopheles gambiae s.s* reveal patterns of gene flow important for malaria-fighting mosquito projects. *Genetics,* 169**,** 313-324.

TRIPET, F., TOURE, Y. T., TAYLOR, C. E., NORRIS, D. E., DOLO, G. & LANZARO, G. C. 2001. DNA analysis of transferred sperm reveals significant levels of gene flow between molecular forms of *Anopheles gambiae. Molecular Ecology,* 10**,** 1725-1732.

TRIPET, F., TOURE, Y. T., DOLO, G. & LANZARO, G. C. 2003. Frequency of multiple inseminations in field-collected *Anopheles gambiae* females revealed by DNA analysis of transferred sperm. *American Journal of Tropical Medicine and Hygiene,* 68**,** 1-5.

TRIPET, F., THIEMANN, T. & LANZARO, G. C. 2005b. Effect of seminal fluids in mating between M and S forms of *Anopheles gambiae. Journal of Medical Entomology,* 42**,** 596-603.

TURNER, T. L., HAHN, M. W. & NUZHDIN, S. V. 2005. Genomic islands of speciation in *Anopheles gambiae. PloS Biology,* 3**,** 1572-1578.

TURNER, T. L, & HAHN, M.W. 2007. Locus and population-specific selection and between incipient species of *Anopheles gambiae. Molecular Biology Evolution.* 24:2132–2138

TWIG, E. & YUVAL, B. 2005. Function of multiple sperm storage organs in female Mediterranean fruit flies (*Ceratitis capitata*, Diptera: Tephritidae). *Journal of Insect Physiology,* 51**,** 67-74.

VAISERMAN, A. M., ZABUGA, O. G., KOLYADA, A. K., PISARUK, A. V. & KOZERETSKA, I. A. 2013. Reciprocal cross differences in *Drosophila melanogaster* longevity: an evidence for non-genomic effects in heterosis phenomenon? *Biogerontology,* 14**,** 153-163.

VALTONEN, T. M., ROFF, D. A. & RANTALA, M. J. 2011. Analysis of the effects of inbreeding on lifespan and starvation resistance in *Drosophila melanogaster. Genetics,* 139**,** 525-533.

VAN DEN HURK, A.F, HALL-MENDELIN, S., PYKE. A.T., FRENTIU, F.D., MCELROY, K., DAY, A., HIGGS. S., & O'NEILL, S.L. 2012. Impact of Wolbachia on

infection with Chikungunya and Yellow Fever viruses in the mosquito vector *Aedes aegypti*. *PloS Neglected Tropical Disease*. 6(11)1892.

VAN LIESHOUT, E., TOMKINS, J. L. & SIMMONS, L. W. 2013. Heat stress but not inbreeding affects offensive sperm competitiveness in *Callosobruchus maculatus*. *Ecology and Evolution,* 3**,** 2859-2866.

VAZ, S., FALKMER, T., PASSMORE, A. E., PARSONS, R. & ANDREOU, P. 2013. The case for using the repeatability coefficient when calculating test-retest reliability. *PloS One,* 8:9.

VERGEER, P., WAGEMAKER, N. & OUBORG, N. J. 2012. Evidence for an epigenetic role in inbreeding depression. *Biology Letters,* 8**,** 798-801.

VINCENT, M. A., MCCABE, M. P. & RICCIARDELLI, L. A. 1999. Factorial validity of the Bulimia Test-Revised in adolescent boys and girls. *Behaviour Research and Therapy,* 37**,** 1129-1140.

VOORDOUW, M. J., KOELLA, J. C. & HURD, H. 2008a. Comparison of male reproductive success in malaria-refractory and susceptible strains of *Anopheles gambiae. Malaria Journal,* 7:103

VOORDOUW, M. J., KOELLA, J. C. & HURD, H. 2008b. Intra-specific variation of sperm length in the malaria vector *Anopheles gambiae*: males with shorter sperm have higher reproductive success. *Malaria Journal,* 7:214

WASER, N. M. 1993. Sex, mating systems, inbreeding and out breeding. *The natural history of inbreeding and out breeding: Theoretical and empirical perspectives***,** 1-13.

WEDELL, N., GAGE, M. J. G. & PARKER, G. A. 2002. Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution,* 17**,** 313-320.

WEETMAN, D., CRAIG S., STEEN, K. W., PINTO, J. & DONNELLY, M .J. 2011. Gene Flow– Dependent Genomic Divergence Between *Anopheles gambiae* M and S Forms *Molecular Biology and Evolution* .Vol 28 (10).

WERNER, M. & SIMMONS, L. W. 2008. Insect sperm motility. *Biological Reviews,* 83**,** 191-208.

WERREN, J. H., BALDO, L. & CLARK, M. E. 2008. Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology,* 6**,** 741-751.

WHITE, G.B. 1985. *Anopheles bwambae* sp. A malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *An. gambiae* complex (Diptera: Culicidae). *Systematic Entomology 10*, 501–522.

WIGBY, S., SIROT, L. K., LINKLATER, J. R., BUEHNER, N., CALBOLI, F. C. F., BRETMAN, A., WOLFNER, M. F. & CHAPMAN, T. 2009. Seminal Fluid Protein Allocation and Male Reproductive Success. *Current Biology*, 19, 751-757

WHITLOCK, M. C. 2002. Selection, load and inbreeding depression in a large metapopulation. *Genetics,* 160**,** 1191-1202.

WHITTY, C. J. M., ROWLAND, M., SANDERSON, F. & MUTABINGWA, T. K. 2002. Science, medicine, and the future - Malaria. *British Medical Journal,* 325**,** 1221-1224.

WHO/TDR 2008.http://www.who.int/tdr/publications/disease/malaria/en/ (Accessed 20 Dec.2011).

WHO 2008. http://www.who.int/whr/2008/en/index.html (Accessed 20 Dec.2011).

WHO 2010. http://www.who.int/mediacentre/factsheets/fs094/en (Accessed 20 Dec.2011).

WHO 2011 http://malaria.who.int/docs/WHO-TRS-936s.pdf (Accessed 16 May 2014).

WHO 2012 World Malaria Report 2012. 1-249. http://www.who.int/malaria/publications/world_malaria_report_2012/report/en/(Accessed 16 May 2014).

WHO 2013. World Malaria Report 2013. http://www.who.int/malaria/publications/world_malaria_report/en/(Accessed 16 May 2014).

WHO 2014. Global Health Observatory (GHO) data http://www.who.int/gho/malaria/en/ (Accessed 21 May 2015).

WOLFNER, M. F. 1997. Tokens of love: Functions and regulation of *Drosophila* male accessory gland products. *Insect Biochemistry and Molecular Biology,* 27**,** 179-192.

WOLFNER, M. F. 2002. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity,* 88**,** 85-93.

WRBU 2011 http//www.wrbu.org,wrbu.si.edu.generapages/anopheles.html (Accessed 20 Dec. 2011).

YARO, A. S., TRAORE, A. I., HUESTIS, D. L., ADAMOU, A., TIMBINE, S., KASSOGUE, Y., DIALLO, M., DAO, A., TRAORE, S. F. & LEHMANN, T. 2012. Dry season reproductive depression of *Anopheles gambiae* in the Sahel. *Journal of Insect Physiology,* 58**,** 1050-1059.

YAWSON, A. E., MCCALL, P. J., WILSON, M. D. & DONNELLY, M. J. 2004. Species abundance and insecticide resistance of *Anopheles gambiae* in selected areas of Ghana and Burkina Faso. *Medical and Veterinary Entomology,* 18**,** 372-377.

YESHIWONDIM, A. K., GOPAL, S., HAILEMARIAM, A. T., DENGELA, D. O. & PATEL, H. P. 2009. Spatial analysis of malaria incidence at the village level in areas with unstable transmission in Ethiopia. *International Journal of Health Geographics,* 8:5

YUVAL, B. 2006. Mating systems of blood-feeding flies. *Annual Review of Entomology,* 51**,** 413-440.

YUVAL, B., BLAY, S. & KASPI, R. 1996. Sperm transfer and storage in the Mediterranean fruit fly (Diptera: Tephritidae). *Annals of the Entomological Society of America,* 89**,** 486-492.

YUVAL, B., FRITZ, G. N., VILLARREAL, C., RODRIGUEZ, M. H. & WASHINO, R. K. 1992. Multiple mating in Anophelines: Theoretical considerations and empirical evidence. *American Journal of Tropical Medicine and Hygiene,* 47**,** 253-253.

ZAJITSCHEK, S. R. K., LINDHOLM, A. K., EVANS, J. P. & BROOKS, R. C. 2009. Experimental evidence that high levels of inbreeding depress sperm competitiveness. *Journal of Evolutionary Biology,* 22**,** 1338-1345.