# Effects of genetic and environmental factors on reproductive success in the malaria mosquito *Anopheles gambiae* Sensu Stricto

Rowaidh Baeshen

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**SUBMISSION OF THESIS FOR A RESEARCH DEGREE**

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Name of Lead Supervisor: Dr. Frédéric Tripet

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(b) My research has been conducted ethically. Where relevant a letter from the approving body confirming that ethical approval has been given has been bound in the thesis as an Annex
(c) The data and results presented are the genuine data and results actually obtained by me during the conduct of the research
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(f) The greater portion of the work described in the thesis has been undertaken subsequent to my registration for the higher degree for which I am submitting for examination
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## Abstract

*Anopheles gambiae* is a major vector of malaria in sub-Saharan Africa. Given the widespread emergence of pesticide resistance in *Anopheles gambiae* populations, vector control programs involving the release of sterile males or genetically-modified male mosquitoes designed to diminish malaria transmission are eliciting renewed interest.

The success of such mosquito release projects depends on the ability of released males to effectively mate and transfer sperm to wild females. Here, firstly we investigated the interactive effect of the environmental factor hydric stress and adult mosquito phenotypic quality on male and female reproductive success. Secondly, we studied the impact of colonization and genetic modification on potential correlates of reproductive success by comparing sperm quality and sexual organs in the wild strains with transgenic and colonized strains. We then experimentally tested for the potential effects of inbreeding depression on reproductive traits by creating hybrid males from inbred colonized strains. The quality of these 'Super males' was assessed by comparing sperm quality, testes and accessory gland sizes, and estimating the quantity of Plugin and Transglutaminase proteins in their accessory glands. Finally, we assessed the reproductive performance of Super males by estimating the amount of accessory gland protein transferred to females after overnight mating and estimating female fecundity and number of larvae produced.

The results underline the importance of female phenotypic quality as determinant of mosquito reproductive success. Inbreeding associated with the colonization and/or genetic modification processes strongly affects sperm length. In contrast, offspring of field-collected females strain have smaller testes and larger accessory glands than older colonized strains. Super males had a good sperm quality. Importantly, the present study suggests that Super males could represent a way to improve male’s reproductive quality under laboratory conditions.

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# Chapter 1:

# Introduction:

## 1.1. General Introduction

Mosquitoes are of extreme medical importance to humanity. They not only cause pain by biting, but transmit dangerous diseases such as yellow fever, dengue fever and malaria. Human malaria is a disease caused by a protozoan parasite called *Plasmodium*. In 2009, 225 million malaria cases and 781000 malaria deaths were recorded in 106 countries worldwide (Fig. 1.1) (WHO, 2010) and US $1.8 billion was spent on international funding for a malaria control programs. Malaria control consists of three main control methods: anti-malarial medicines, indoor residual spraying (IRS) and insecticide treated mosquito nets (ITN). DDT is used for indoor residual spraying in 17 countries out of 71 (WHO, 2010). This method protects 10% of the population at risk of malaria in sub-Saharan Africa. Insecticide-treated mosquito nets were distributed to cover 42% of countries where there is a malaria risk. The malaria parasite has started to develop resistance to anti-malaria drugs as well as mosquitoes developing resistance to insecticide pyrethroids, which together constitute the current malaria control paradigm (WHO, 2010). However, the evolution of insecticide resistance in vectors of malaria indicates that these tools might become less effective for controlling mosquito numbers, and subsequently disease transmission (Catteruccia *et al*, 2009). Also, insecticides often have negative side effects on the environment (Crampton *et al.*, 1994). Thus, there is a strong need to find alternative means to control malaria mosquitoes, such as is offered by molecular biology and biotechnology (Crampton *et al*., 1994). Since 1962, sterile insect techniques (SIT) have been successfully used in a range of species (Knipling *et al*., 1968): these techniques are

based upon the concept of altering a population at the genetic level in order to control it (Knipling *et al*., 1968). Genetic modification may involve various methods, including meiotic drive, cytoplasmic incompatibility, distorted sex ratio, lethal factors, hybrid infertility and sterilization, either by chemical means or through the use of radiation (Knipling *et al.*, 1968). The general goal of these methods is to devise means of inducing reproductive failure in insects. In the simplest case, sterilization of male insects and their release in the natural populations leads to its decline (Knipling *et al*., 1968). The key factor in SIT programs is the quality of sterile males, as they must attract, copulate, inseminate successfully, and discourage females from further mating (Pérez-Staples *et al*, 2013). These authors' report that successful SIT programs depend on sterile males to mate effectively with wild females and induce female reproductive failure. This failure includes two steps namely: pre-copulatory and post-copulatory (Pérez-Staples *et al*, 2013). Before the females mate with the sterile males, there are several factors that could impact on female choice of partners such as the difference between sterile males and wild ones in terms of their pheromone production, dispersal and male courting success (Pérez-Staples *et al*, 2013). Other factors could impact on female reproductive failure in post –mating such as the ability of sterile males to allocate their seminal fluid and sperm that could affect further mating with females. Successful SIT programs release sterile males that compete with wild males to mate with wild females and this leads to a decline in the target species population (Pérez-Staples *et al*, 2013).

The Sterile Insect Technique (SIT) has been applied to the control of some insect species under laboratory and field conditions (Benedict and Robinson, 2003). SIT consists of the use of several techniques, including irradiation, aerial release and recombinant DNA technology (Alphey *et al.*, 2010). RIDL mosquitoes are genetically modified insects generated by using recombinant DNA technology carrying a repressable, dominant lethal

gene (Thomas *et al*, 2000), which is described by Alphey (2002), in this example using tetracycline-repressible expression system in the female fat body. RIDL males were fertile and viable however did not produce viable female progeny when they copulated with the field type females (Black IV *et al*, 2011). Released homozygous males carrying a dominant female–specific lethality gene were expected to suppress the targeted insect's wild population. Specifically the female progeny will decline, leading to overall population suppression (Black IV *et al*, 2011). The RIDL approach differs from SIT using different transgenes to induce sex-specific lethality and, crucially, late-acting lethal systems (Black IV *et al*, 2011). SIT stimulates early-acting (embryonic) lethality; transgenic methods potentially allow lethal genes to pass through female offspring, allowing a degree of self-sustaining population suppression (Phuc *et al*, 2007). In contrast, SIT depends on producing sterile males by using irradiation or chemo-sterilants to sterilize males' prior to mass-release to compete with wild males. However, there is a large body of evidence demonstrating that these production and sterilization methods impact negatively on male fitness components such as male mating competitiveness (Alphey, 2002; Andreasen and Curtis, 2005). For instance, *An. arabiensis* (Patton) sterile males in a SIT program in Sudan were created using several steps. Firstly, a dieldrin-resistant mutation treatment was used to create a genetic sexing strain (Damiens *et al*, 2013). Then the sexing strain's eggs were treated with dieldrin to produce males only. Finally, the pupae were exposed to a radiation dose of 70 GY to produce sterile males (Damiens *et al*, 2013). All these treatments were used separately or in combination and sperm production in sterile males was finally measured. It was found that irradiation alone stopped sperm production while dieldrin treatment had a radio-protectant effect on germinal cells (Damiens *et al*, 2013). Irradiation and dieldrin together or alone had a significant effect on sperm cell numbers in testes in 2 and 6 day old males (Damiens *et al*, 2013).

There are advantages to the use of SIT: for instance, SIT targets a specific insect species or species complex, and, as there is no requirement for insecticide treatment, has minimal environmental impact. Also, as only the male mosquito is released, there is no risk of increased transmission (Alphey *et al.*, 2010). However, transgenic technologies for SIT are currently still limited to preliminary research in the laboratory and, in a few cases, large cage experiments. Important research efforts are being made toward developing transgenic mosquitoes with special characteristics that would decrease their vectorial capacity but not suppress populations (Toure *et al*., 2004). However, until now all population replacement attempts have been carried out under laboratory conditions (Toure *et al*., 2004). So far, SIT programs have been largely unsuccessful in Anopheline mosquitoes (Catteruccia, 2007), except for a control project of *Anopheles albimanus* in El Salvador (Lofgren *et al*., 1974). The main problem with SIT in Anophelines is that sterilization negatively affects male competitiveness in comparison to wild males (Andreasen and Curtis, 2005). In addition, other difficulties for sterilization by radiation occur in finding the best radiation dose and developmental stage for intervention (Helinski *et al*, 2009). Also problematic is the cost and regulatory complexity of handling radioactive material, generating the huge numbers of irradiated insects needed for release (Helinski *et al*, 2009) and the relatively cryptic nature of male Anopheline mating behaviour (Townson, 2009). Any transgenic program involving population replacement or curbing strategies depends on successful mating and transfer of sperm to wild females during copulation (Helinski and Knols, 2008). However, several factors in *An. gambiae* s.l. such as mating barriers may cause male releases to fail (Davidson *et al.*, 1970). Generally, the reproductive biology in *Anopheles* mosquitoes is poorly understood (Ferguson *et al*., 2005). Therefore, the project work in the current thesis aims to highlight important genetic and environmental factors that relate to reproductive success in *An. gambiae*. The advances made here should improve the design and

implementation of malaria control strategies based on vector suppression or genetic modification.

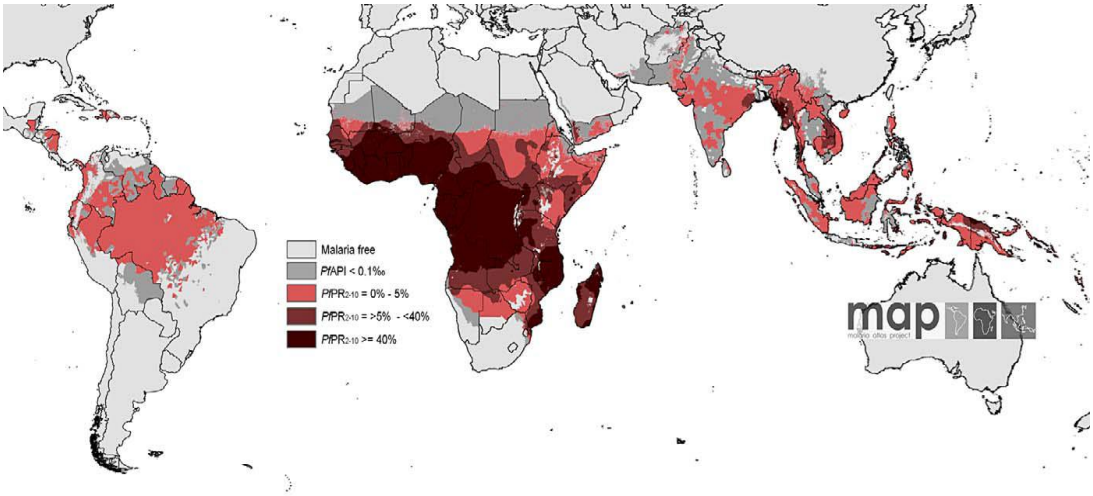


Fig. 1.1. *Plasmodium falciparum* malaria parasite rate in non-endemic and endemic countries (Hay *et al*, 2009).

### 1.1.1. Malaria

Human malaria is a dangerous disease which kills a large number of individuals annually and is caused by parasites in the genus *Plasmodium*. There are five known species of *Plasmodium* that cause malaria in *Homo sapiens*: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Cox - Singh and Singh, 2008). *P. falciparum* is responsible for the vast majority of global malaria mortality (Hay *et al*, 2009).

#### 1.1.1.1. Malaria life cycle

The parasite cycle starts when the mosquito takes an infected blood meal from a vertebrate host infected with malaria and carrying male and female gametocytes in the peripheral blood. Gametocytes are the sexual stages of the *Plasmodium* parasite (Fig. 1.2). Following ingestion by the mosquito, within 8-15 minutes male gametes (microgametes) produce eight flagella in a process called exflagellation (Knell, 1991; Sinden, 1984).

Within 60 minutes of intake of the blood meal, fertilization occurs between the male microgamete and female gametes (macrogametes), producing a zygote. After a further 10-25 hours, zygotes develop into motile ookinetes which penetrate the mid-gut epithelium and upon reaching the basal sidepenetrate the basal membrane (Sinden, 1984). Ookinetes pass through the cytoplasm of mid-gut epithelium cells, a process which induces the apoptosis and extrusion of damaged cells (Vlachou *et al*., 2006). Ookinetes cannot pass within the basal lamina (Sinden, 1984). When the ookinete reaches the basal side, they start transforming into a sessile oocyst by means of multiple meiotic divisions. The ookinete reaches the basal lamina 18 to 24 hours after infection (Sinden, 1978). The mean number of ookinetes per infected female in *An. gambiae* and *An. funestus* with *P. falciparum* is 5 to 12 (Beier *et al*., 1992). The young oocysts are covered by an amorphous capsule which decreases in thickness with maturity (Sinden, 1975). In mature oocysts, the capsule becomes thinner and stretches (Sinden and Strong, 1978). In the intercellular space between the basal lamina and mid-gut epithelium, the oocysts develop into thousands of sporozoites. These are released in the mosquito's hemolymph for a period of 7-12days (Sinden, 1984). The number of sporozoites in the salivary gland in *An.stephensi* is less than nine thousand (Rosenberg *et al*., 1990). Each oocyst wall has a weak area which allows the sporozoites to break through and pass to the haemocoel (Sinden and Strong, 1978). In the haemocoel, sporozoites disperse to different parts of the mosquito body and 1-2 days after their release reach the salivary glands and stay there, ready to be transferred to a new host through a mosquito bite (Vaughan *et al.*, 1992). The full development of *Plasmodium* takes 8-10 days in mosquitoes (Knell, 1991).

Following an infective bite, sporozoites enter the human blood stream to start the human part of their life-cycle (Simonetti, 1996; Vlachou *et al.*, 2006). They first travel to the liver cells to become hepatic trophozoites, and thereafter develop quickly into hepatic

schizonts, a stage of asexual multiplication which produces numerous exo-erythrocytic merozoites. One week from the start of the infection, mature merozoites move from the liver into the blood stream where they will initiate the asexual erythrocytic cycle. Merozoites penetrate erythrocytes and become ring stages, which themselves grow into trophozoites and within two to three days these grow into rosette-shaped shizonts. Shizonts release from 8 to 16 merozoites, which are released from red blood cells and re-infect red blood cells to continue the asexual erythrocytic life-cycle (Bannister and Mitchell, 2003; Knell, 1991). A small number of merozoites mature to male and female gametocytes and when a mosquito takes a blood meal from an infected human the gametocytes reach the mid-gut and will initiate a new life cycle (Anand and Puri, 2005).

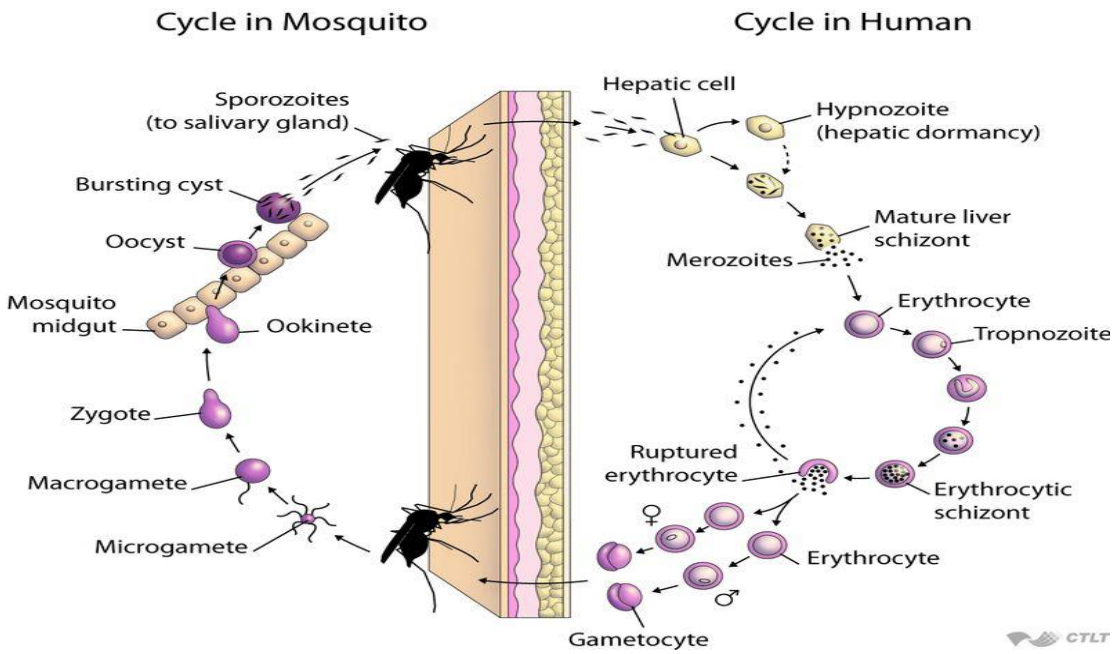


Fig. 1.2: Malaria life cycle in *Plasmodium falciparum* (Source:

http://www.malariasite.com/malaria/LifeCycle.htm

### 1.1.2. The vector of malaria

The Culicidae family consists of three subfamilies; Anophelinae, Toxorhynchitinae and Culicinae. The genus *Anopheles* contains about 476 species 70 of which recorded as vectors of human malaria (Service, 2012). The human malaria cycle requires the *Plasmodium* parasite, humans as a source of blood meal, and *Anopheles* mosquitoes as definitive host. *Anopheles* mosquitoes present variations in their behaviour and ecology as well as vectorial capacity and malaria transmission (Costantini *et al.,* 1999). For instance, the Forest form of *An. gambiae* s.s. in Southern Sierra Leone prefers temporary pools as a breeding site rather than rice fields or swamps (Bockarie *et al*., 1993). Meanwhile, *Anopheles darlingi*, a malaria vector in South and Central America and especially in Amazonian countries, prefers rivers, swamps and large lakes as oviposition sites. Also, adult mosquitoes are better adapted to humid climates than an arid (Hiwat and Bretas, 2011). In villages in Western Kenya, *An.gambiae* s.s. is adapted to wetter conditions, while *An. arabiensis* is better adapted to persist in an arid environment (Minakawa *et al*., 2002). In *An.gambiae* s.s., larval breeding sites influence adult distribution, while mosquito density depends on host availability (Minakawa *et al*., 2002). *Anopheles gambiae* s.s. prefers to bite humans in the specific body areas of the head and foot because of body temperature, moisture and smell in those two areas (Jong and Knols, 1995). In the Gambia, *Anopheles gambiae's* s.l. biting rate increases in the dry season, but the average malaria transmission is low due to high temperatures at that time which may impact upon parasite development (Lindsay *et al.,* 1991). The *An.gambiae* s.s. female is anthropophagic, preferring to feed on human rather than animal blood, whereas *An. arabiensis* prefers feeding on animals and is thus zoophagic (Minakawa *et al.,* 2002; Diatta *et al.*, 1998). Obala *et al* (2012) studied *Anopheles* host blood preferences in Western Kenya. It was observed that *An. gambiae* s.s preferred human blood with 97% found to have taken a

human blood meal (as determined by PCR on the gut contents) in comparison with *An. arabienesis* (75%). Another study by Mwangangi *et al* (2013) on the Kenyan cost reported that *An. gambiae* s.l. and *An. funestus* have moved from human towards animal blood feeding and coupled with a reduction in *An. gambiae* s.l. population density has significantly decreased malarial disease burden over time. The decline in mosquito density was hypothesized to be due to ecological disturbance (Mwangangi *et al*, 2013).

#### 1.1.2.1. Morphology and life cycle in *Anopheles gambiae*

Mosquitoes are different from other dipterans, in that they have abundant scales on the different parts of the body, especially on the posterior edge of the wings, as well as having a highly modified, elongated frontal proboscis (Service, 2012). In *Anopheles gambiae,* the wing veins display ordered blocks of black scales, while the maxillary palps differ between the male and female, being longer and darker in the female, and broader in the male in its final section. The typical adult resting position is at an angle of about 45º (Cox, 1993). The life cycle comprises four stages that are; eggs, larvae with four instars, pupae and adults.

##### 1.1.2.1.1. Eggs

The eggs of Anopheles mosquitoes may be recognized in comparison to other species by their being boat-shaped and having two floats filled with air (Fig. 1.3). They are laid by females one by one on the water's surface (Service, 2012). Two days post blood feeding; females lay eggs which then hatch within two days (Coetzee and Meiswinkel, 1979). In *An. gambiae,* egg-laying has been found not to be affected either by female age or by blood feeding before copulation (Chambers and Klowden, 2001). However, in a different study, the number of eggs and oviposition were affected in *An. pharoensis* by age and size of females, as well as by food quality (El-Akad and Humphreys, 1988). The eggs

need humidity as they are vulnerable to desiccation. In the absence of water, the eggs of *An. gambiae* Giles and *An. arabiensis* Patton were found to remain alive for no longer than twelve days, and the eggs to hatch within two to five days when water was available (Beier *et al.*, 1990). Anopheline species tend to be choosy with regard to preferred oviposition sites (Service, 2012). For example, *An. balabacensis*, a vector of malaria in Thailand, preferred moist sand and water rather than just water to lay eggs under laboratory conditions (Wilkinson *et al*., 1978). In another study, combining small amounts of cow urine with water for oviposition sites attracted *Anopheles gambiae* s.l. and *Culex quinquefasciatus* to lay more eggs there under both artificial and field conditions (Kweka *et al*, 2011). Furthermore, Anopheline species of mosquito preferred fresh cow urine to seven-day-old urine-laying more eggs where fresh was used (Kweka *et al*, 2011). Wachira *et al.* (2010) showed that *An. gambiae* females avoid laying eggs in oviposition sites occupied by immature larvae of *Cx. quinquefasciatus*. Yaro *et al*.(2006a) report that, in *An. gambiae* and *An. arabiensis*, a large variation in egg maturation speed and hatching time was found within the same batch of eggs by using different water conditions as oviposition sites (Yaro *et al*, 2006a). This study also found that, smaller adults were produced when eggs hatched more quickly (Yaro *et al*., 2006a). The number of eggs laid by mosquito females depends on the quality of oviposition sites, and in addition to this, the day-night cycle has a direct effect upon oviposition activity (Sumba *et al*, 2004).

In the mosquito, oviposition generally occurs within a short period after eggs mature (Coetzee and Meiswinkel, 1979). A number of factors can have an impact on oviposition behavior. For instance, in a study of mosquito oviposition behavior by Russell and Rao, (1942), *An. culicifacies* females were observed at oviposition sites a little after dusk, when swarming had ended. But oviposition took place at all times of night. The females hovered over the water and dropped eggs individually onto the water's surface (Russell and Rao,

1942). Numerous females flew away after dropping only one or two eggs, which may be because they did not like the oviposition sites (Russell and Rao, 1942). Oviposition and the survival of eggs laid depend upon the presence of moisture in anopheline mosquitoes and mosquitoes in general (Clements, 1999). Anopheline fitness in the field relies upon the ability for eggs to survive upon a moist surface, and eggs are also deposited on moist surfaces under laboratory conditions in several Anopheline mosquitoes (Clements, 1999). However, oviposition and egg production can be held up or stopped by low water temperature at the breeding site and by short hours of daylight (Washino, 1969); eggs not retained by the female due to unfavourable conditions or because they were unmated prior to blood feeding are stored by the female (El-Akad and Humphreys, 1988). Eggs are stored until the mosquito locates a site for oviposition (Day and Curtis, 1989). Temperature and access to water have greater effect upon numbers and distribution of mosquitoes than other biotic variables (Lindsay and Bayoh, 2004). This need for relative humidity stems from the necessity for mosquitoes to locate moist sites for oviposition and as such is unsurprising (Rowley and Graham, 1968; Dow and Gerrish, 1970; and Day *et al.*, 1990). This need for high relative humidity for oviposition is supported by experimental research with gravid, field-collected *Culex nigripalpus*, mosquitoes which made use of a wind tunnel. Findings suggest that oviposition in the field may be postponed in conditions of intermittent rainfall, lengthening lifespan as a consequence (Day *et al.*, 1990). Research conducted in the field found a link between average egg raft numbers and relative humidity in *Culex quinquefasciatus* (Chaves and Kitron, 2011).

#### 1.1.2.1.2. Larvae

The body of the Anopheline mosquito larva consists of a head, a circular, wide thorax with long hairs, and ten abdominal segments without legs, each one with a pair of palmate hairs (Fig. 1.3). *Anopheles* larvae do not have a siphon (in contrast to *Culex* or

*Aedes*), but have a pair of posterior dorsal spiracles, and body hairs assist the larvae in staying parallel to the surface of water for breathing. Larvae take one or two weeks to grow to the pupal stage (Service, 2012). Both man-made and naturally-occurring environments may serve as breeding grounds for the Anopeline mosquito, whether swamps, rock pools, rice fields, drains, ditches or water pooling in tracks left by vehicles (Service, 2012). *Anopheles arabiensis* and *Anopheles funestus* prefer human made habitats and have permanent breeding sites during the dry season in Kenyan villages (Mala *et al*, 2011).

Water temperature and relative humidity in aquatic habitat have a positive correlation with larvae distribution. In *An. dirus*, the abundance of larvae is affected by both the temperature of the water and by the relative humidity in their environment. This produces a greater density of larvae in sites with high humidity and cooler water temperature in the morning, with greater levels of dissolved oxygen (Kitthawee *et al*., 1995). *An. gambiae* and *An. arabiensis* prefer to lay eggs in clean (i.e. free of organic matter), sunny, small pools with a low density of larvae (Gimnig *et al*., 2001). Females preferred to lay eggs in habitats that have a low density of larvae rather than habitats with no larvae (or eggs) or where there is a high larval density (Sumba *et al*., 2008). This suggests that they may be capable of detecting odours produced by larvae (Sumba *et al.*, 2008). As in eggs, temperature strongly influences larval development. Temperatures in breeding water of over 30C$^{o}$ increased the mortality rate at aquatic stages in *An. gambiae* s.s. under laboratory conditions (Bayoh and Lindsay, 2004). Rice fields provide a cooler environment than puddles, but host a greater larval density, and it is notable that in both environments, *An. gambiae* with the S molecular form have a greater adult emergence rate than do those of the M form. (Diabaté *et al*, 2005). Temperature is not the only factor affecting larval mortality. The mortality rate of larval stages is also influenced by the water depth of the breeding site (Tuno *et al*., 2004). Experiments also show that diving behaviour

entails a survival cost, particularly in muddy water (Tuno *et al*., 2004), and that aquatic insect predators significantly affect larval survival (Diabaté *et al*., 2008).

#### 1.1.2.1.3. Pupae

Mosquito pupae have a cephalothorax, comprising head and thorax, followed by an abdomen which is formed of ten segments. The last two segments are combined together and feature two paddles with an oval shape, with the overall form of the pupa resembling a comma (Service, 2012) (Fig.1.3). In Anopheline pupae, spines can be seen along the edge of the second or third to seventh segments on the abdomen. An additional distinguishing feature consists of short trumpets for respiration (Service, 2012). Due to the cessation of trophic behavior, pupae mostly remain inactive at the water's surface, but if disturbed they will swim strongly towards the bottom. The pupal stage is a short period which lasts only one to three days depending on environmental conditions, mainly temperature (Service, 2012). Mutuku *et al*., (2006) estimated the numbers of adult emergence from field habitats by comparing the density of pupal and larval stages, and showed that pupal density is a useful predictor of adult population density, thus making it a good tool in *An.gambiae* control programmes.

#### 1.1.2.1.4. Adults

The head, thorax and abdomen form the three component sections of the adult mosquito. The head is attached to the thorax by a short neck and carries a pair of compound eyes and antennae (Fig. 1.3). Males can be distinguished from females by their plumose (feather-like) antennae, while females have pilose (fewer and shorter hair-like structure) antennae (Fig. 1.4); male maxillary palps are as long as the proboscis as in females but their end is swollen. The male has a narrow and long abdomen, and also the

last male abdominal segment carries a pair of claspers. By contrast the, female has a wide abdomen and the last abdominal segment is pointed (Cook and Zumla, 2003). Mosquito females live on average between one to four weeks and males die before females (Service, 2012). Mating behaviour takes place from 24 hours post emergence. Females usually mate once in their life and after mating, sperm is stored in their spermatheca. After that, females obtain blood meals and egg maturation is started in the ovaries (Service, 2012).

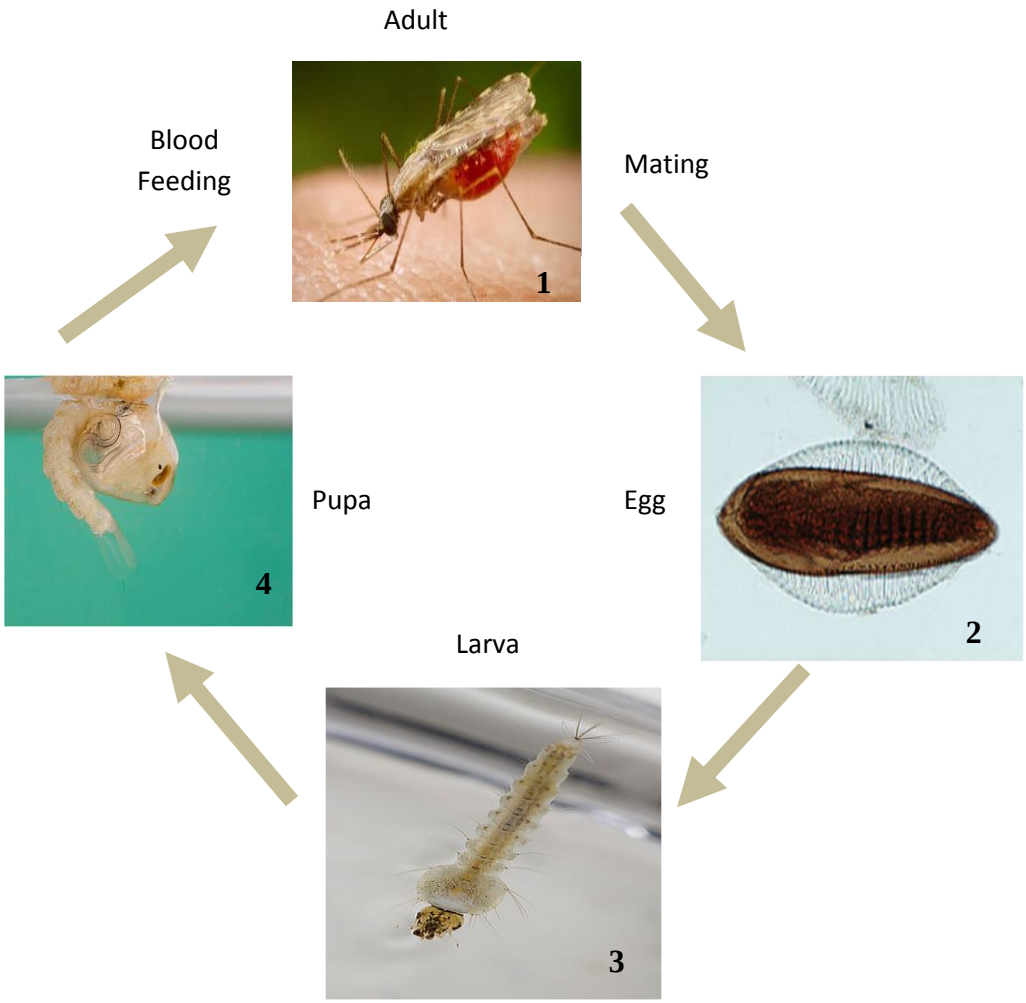


Fig.1.3**.** *Anopheles gambiae* life cycle

**Picture sources:**

**No.1**:
http://www.genetics.org/content/190/4/F1.medium.gif

**No.2**:
http://impact-malaria.com/cp/medias/4F5CA090-C3C5-490B-BB9B- 052A089A7401.jpg

**No.3:**
http://www.raywilsonbirdphotography.co.uk/Galleries/Invertebrates/000_invert_images/vector_images/2009-05-29_JS8Q0514_LSTM-Edit.jpg

**No.4:**
http://www.raywilsonbirdphotography.co.uk/Diary/Images/2009/June/2009-06-23_JS8Q1557_Anopheles_gambiae-Edit.jpg


Fig. 1.4. Male (♂) and female (♀) antenna in *An. gambiae* s.s.

(http+stalemamezivot.blogspot.com+201009+ anopheles-gambiae)

### 1.1.3. *Anopheles gambiae* distribution and species complex

#### 1.1.3.1. *Anopheles gambiae* complex species

*An. gambiae* sensu lato is a species complex which includes the main malaria vectors in Africa. The situation in Africa contrasts with that of the Middle East and Europe, where there is little or no malaria transmission in many countries (Sinka *et al.*, 2010). Seven sibling species in the complex of *An. gambiae* sensu lato have been elucidated through crossing trials (Davidson, 1964; Davidson and Hunt, 1973). The PCR technique was used to distinguish different larval species in a mixed pool population of *An.gambiae* and *An. arabiensis* (Charlwood and Edoh, 1996). When looking for the presence or absence of one or two of the dominant members of the *Anopheles gambiae* species complex in artificial environments (including wheel tracks or empty cans and naturally occurring habitats such as streams), out of 46 African countries, Kenya recorded the largest number of vector sites, with a total of 757 sites in the studied area, compared with one recorded vector site in Togo (Kantindi) (Sinka *et al*, 2010). The sibling members of the complex are: *An. gambiae* sensu stricto, *An. arabiensis*, *An. quadriannulatus* A, *An. quadriannulatus* B; *An. melas* and *An. merus*; and *An. bwambae*. The two most widespread malaria vectors in Africa are

*An. gambiae* sensu stricto and *An. arabiensis. An. gambiae* s.s. are found in moist areas, while *An. arabiensis* are found in dry, and the larvae of both are found in fresh water as a breeding site. *An. melas* and *An. merus* are found in coastal regions and their larvae live in brackish water (Tsy *et al.,* 2003, Moreno *et al.,* 2004). *An. bwambae* are found in hot spring areas and their larvae are found in brackish water as a breeding site from geothermal springs (White, 1985).

*An. gambiae* s.s. has been subdivided into five chromosomal forms based on extensive Polytene chromosome studies (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985). These forms are Savanna, Bamako, Mopti, Forest and Bissau and each live in specific environmental zones (Toure *et al.*, 1998). *An. gambiae* sensu stricto is also subdivided into two molecular forms, M and S (Favia *et al.*, 1997; 2001) based on sequence divergence of the ribosomal DNA region on the X chromosome (Favia *et al.*, 1997: 2001).

#### 1.1.3.2. *Anopheles gambiae* distribution in West Africa

High longevity, a preference for human blood, short larval development time and larval habitats related to human activities such as water in rice fields together mean that *An. gambiae* Giles is one of the most efficient vectors of malaria in the world (Catteruccia, 2007; Sinka *et al*, 2010). Adult females of *An. gambiae* s.s. along the Gambia river are of both S and M forms. (Caputo *et al.*, 2008). In Angola, the M form is found in dry regions while the S form is observed in humid locations (Calzetta *et al*., 2008). In Burkina Faso and Mali the M form can be mapped to the Mopti chromosomal form, which tolerates dry conditions, while the Savanna or Bamako chromosomal forms, which require humidity, are mapped to the S form (Favia *et al.*, 1997, della Torre *et al*., 2002) (Fig. 1.5). The M form also corresponds to the Bissau and certain Forest and Savanna populations in outside Mali and Burkina Faso (della Torre *et al*., 2002). Population density of the two molecular forms

of *An.gambiae* adults in Ghana depends on the area temperature. For instance, the M form is distributed in costal savanna and northern regions with a temperature range of between ~25 to >27C◦ whereas, the S form is distributed in the central area with a 24.9 to 26.8 C° temperature range (de Souza *et al*, 2010).

*An. arabiensis* is generally found in larger numbers in drier areas such as for example desert areas north of the Niger river in Mali, and the Nile river in Sudan. In fact, it is the river systems which allow *An. arabiensis* to inhabit such areas. (Coetzee *et al.*, 2000). In Tanzania, *An. arabiensis* is found in dry regions while *An.gambiae* lives in habitats closer to the sea (Mnzava and Kilama, 1986). *An. bwambae* is found in Uganda (White, 1985). *An. quadriannulatus* species A is found in South Africa and *An. quadriannulatus* species B in Ethiopia (Coetzee, 2004).

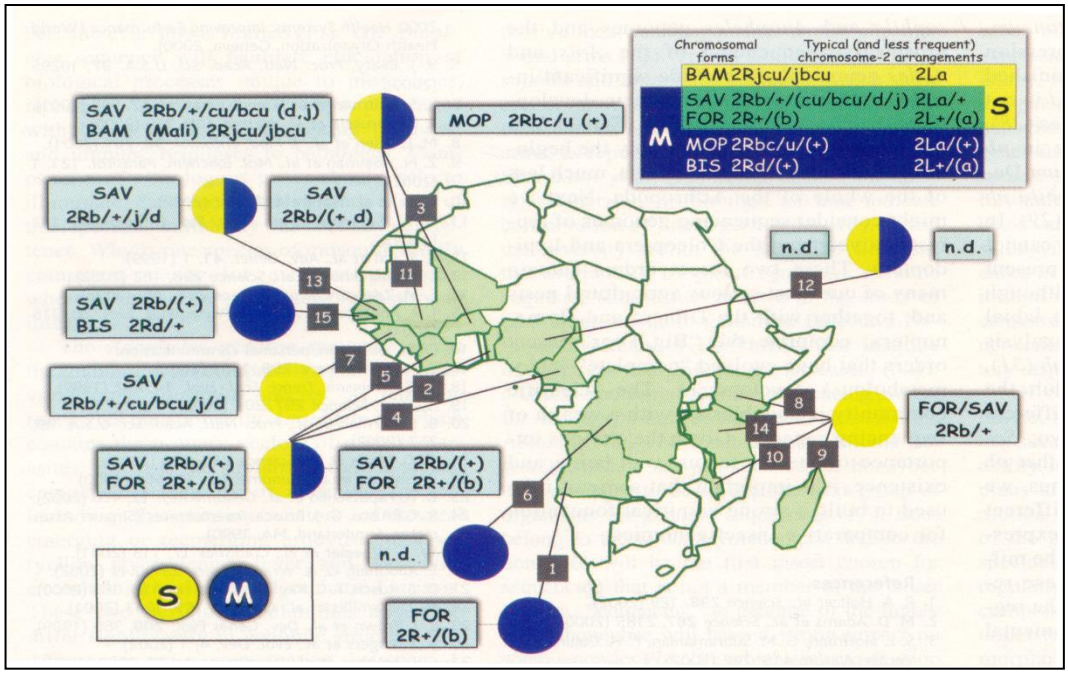


Fig. 1.5: Molecular and chromosomal forms distribution of *Anopheles gambiae* in Sub-Saharan Africa. In countries: 1- Angola, 2- Benin, 3-Burkina Faso, 4-Cameroon, 5-Ivory Coast, 6-Rep. of Congo, 7-Guinea, 8-Kenya, 9-Madagascar, 10- Malawi, 11-Mali, 12-Nigeria, 13-Senegal, 14-Tanzania, 15-Gambia (della Torre *et al.*, 2002).

### 1.2. Reproduction physiology and fitness

Reproduction is the means used by living organisms to maintain their populations. Reproduction is regulated by internal physiological factors, the neuro-endocrine system and external environmental factors. Studies on reproductive success in the main malaria vector in sub-Saharan Africa, *Anopheles gambiae,* have not covered all parts of reproductive biology. Studies of reproduction in mosquitoes have paid attention to the structure of organs (e.g. Christophers, 1911; Huho *et al*, 2006) mating behaviour and physiology (e.g.; Takken *et al.*, 1998; Klowden and Chamber 2004; Diabaté *et al*, 2011)

and function (Clements, 1992). The effect of infection on fecundity and fertility has been investigated (e.g. Hogg and Hurd 1995), including study of the potential costs of resistance to *Plasmodium* by Voordouw and Koella (2007). The effects of genetic background on mating have also been studied by different authors (Tripet *et al*., 2001; Diabaté *et al.*, 2003; and Ng'habi *et al.*, 2011), as well as the influence of environmental factors, for example, by Ng'habi *et al* (2005) and Manoukis *et al* (2009).

Adult male mosquitoes are unable to mate with females upon emergence as they need to go through a sexual maturation process. By 12-36h from emergence, male mosquitoes have inverted their terminalia through 180° rotation of the eighth to tenth abdominal segments, and their antennal fibrillae are capable of temporary erection (Clements, 1990). In a recent study using Dongola laboratory and Sudanese field-caught strains of *Anopheles arabiensis,* Oliva *et al.* (2011) explored the effects of colonization on the period within which the rotation of genitalia took place. A three-hour difference was found, with laboratory and wild males having rotated their genitalia and thus become able to copulate by 17h and 20h post-emergence respectively. An explanation for this may lie in temperature differences between the laboratory and field conditions (Oliva *et al.*, 2011).
Female mosquitoes respond to males within the first 30-60h after emergence. Sperm is transferred from males to females through contact of their genitalia following copulation, which is usually initiated in flight (Clements, 1999).

### 1.2.1. Morphology of female and male reproductive system

#### 1.2.1.1. Structure of female reproductive system

The female reproductive system comprises a pair of ovaries that are located dorsally in the posterior abdominal segments (Fig. 1.6). Ovarioles are functional units in ovaries which hold ovarian follicles and the ovary has a central chamber called the calyx

(Clements, 1992). The first part of the genital ducts is a pair of oviducts which come together to form a common oviduct. This is a large median tube and in *Anopheles* mosquitoes a gonopore leads through to an atrium with a sphincter formed from strong circular muscles. The passage of mature eggs upon ovulation is controlled by this sphincter, which allows the eggs from the ovarioles through to the lumen of the calyx. There are two additional organs important for reproduction: the spermatheca and the accessory gland. The ducts from the spermatheca and accessory gland open into the common oviduct. The female accessory gland is larger during the ovarian cycle and reduced in size at oviposition. The vagina is located at the end of the common oviduct (Giglioli, 1964; Detinova, 1962; Goma, 1966; Clements, 1999). In the *Anopheles* female, there is a single spermatheca, the diameter of which is 100-120 µm (Fig. 1.7) (Ndaye *et al*., 1997).

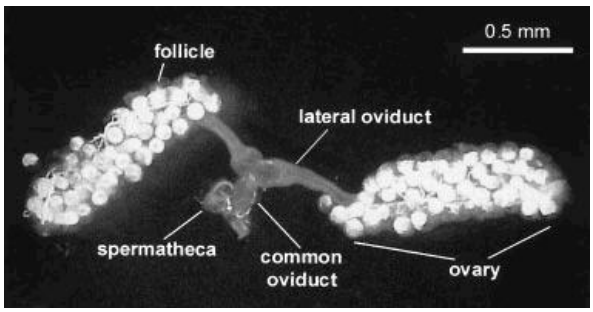


Fig. 1.6. Female reproductive system in *An. gambiae* s.s.(Tripet *et al*., 2001)

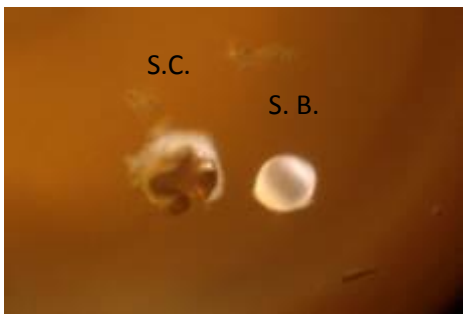


Fig.1.7. Spermatheca capsule (S.C.) and sperm bundle (S.B.) in *An. gambiae* s.s. (Tripet *et al*., 2001)

### 1.2.1.2. Structure of male reproductive system

The male reproductive system comprises a pair of testes where spermatazoa are stored, which are located in the $5^{th}$ and $6^{th}$ abdominal segments. The first part of the genital duct is a pair of vasa efferentia, which are narrow, thin–walled tubes, then a pair of seminal vesicles which come together to form a common ejaculatory duct, and a pair of accessory glands which open into the ejaculatory duct (Goma, 1966; Clements, 1999) (Fig. 1.8). Immature spermatozoa are stored in spermatocysts in the testes, while mature spermatozoa are held in a reservoir. Mature spermatocysts are observable in the posterior part of the testis and they break down and release spermatozoa into the sperm reservoir, which is located in the anterior division of the testes (Huho *et al*., 2006). Mature spermatozoa run into the sperm reservoir during mating and move via the vasa efferentia to the seminal vesicle and to the ejaculatory duct before passing to the female. Three morphological features predict male age and mating status: these are the number of spermatocysts, the sperm reservoir, and the presence of an area which is translucent surrounding the accessory glands (Mahmood and Reisen, 1982; Huho *et al*., 2006). In the young male (less than 4 days old), the number of spermatocysts is from three to five, the proportion of testes occupied by the sperm reservoir ranges from 10% to 50%, and a clear area is present (Fig. 1.9). On the other hand, in old males (over 4 days) the number of spermatocysts range from 0 to 2 and the proportion of the testes occupied by the sperm reservoir ranges from 50 to 100%, in the absence of a clear area (Huho *et al*., 2006).

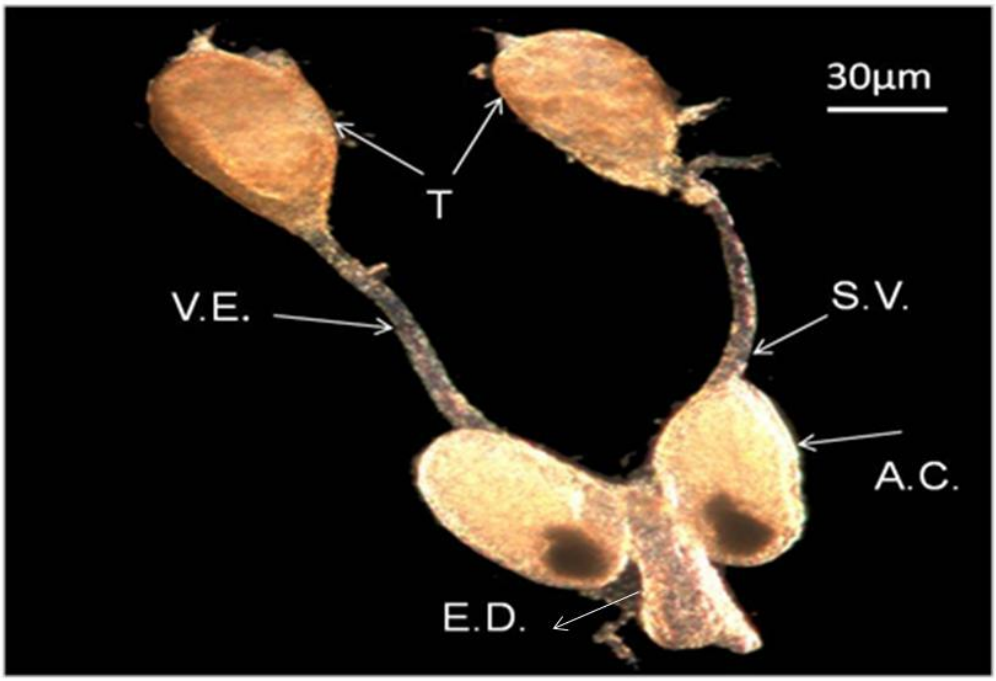


Fig. 1.8: The reproductive system of male *An. gambiae* s.s. The letters on the figure illustrate: Testes (T); Vas efferentia (V.E); Ejaculatory ducts (E.D); accessory Glands (A.C.); Seminal vesicle (S.V.).

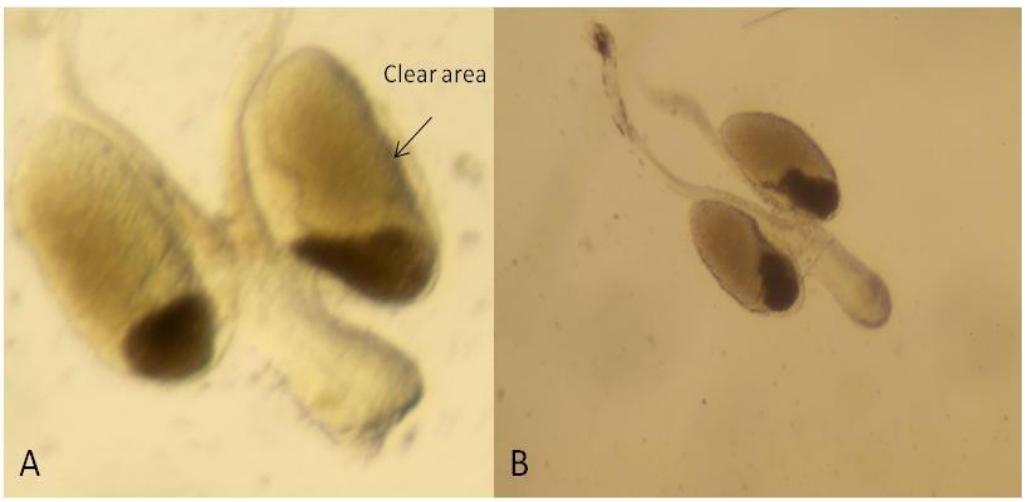


Fig. 1.9: Accessory glands, (A) with clear area, (B) without clear area

### 1.2.2. Fitness components

Fitness components in mosquitoes include; adult developmental time from egg to adult, lifespan, mating success, time to initiate oviposition, and fecundity / fertility of females. Fecundity has been defined by Lincoln *et al* (1998) as the extent of reproductive potential, as can be assessed through establishing how many gametes are produced per individual. By contrast, Lincoln describes fertility in terms of actual reproduction, measurable through the number of offspring. The fertilization rate in females can be estimated by observing the frequency of females with a mating plug (Gillies, 1956) or with sperm in the spermatheca (Ng´hab *et al.*, 2007). Laboratory studies have revealed that mosquito fecundity is affected by at least the following factors; body size, tenereal reserves (Briegel, 1990a; 1990b), blood meal source (Briegel, 1985), blood meal size (Lea *et al.*, 1978) and parasite infection (Hurd *et al.*, 1995; Hogg and Hurd, 1995).

#### 1.2.2.1. Factors affecting reproduction

It has been shown in several scientific reports on insect reproduction that a range of factors external and internal to the insect influences its fitness.

##### 1.2.2.1.1. Age

In mosquitoes, the age of both males and females affect reproductive success. When female *An. gambiae* were mated with males older than 6 days old, they laid fewer eggs than those with 2-day-old males (Chambers and Klowden, 2001). In *An. gambiae* s.s., the likelihood of oviposition was not affected by female age (Chambers and Klowden, 2001). In *Ae. aegypti*, Ponlawat and Harrington (2007) observed a positive correlation between the age of males and the number of spermatozoa, which increased significantly during the first 10 days from emergence in younger males and then remained steady for twenty days.

They also showed that fertility declined in older females (Ponlawat and Harrington, 2007). In *Aedes* spp, the same volume of blood meal was ingested by older and younger females, but the number of mature follicles was lower in older females (Volozina, 1967). Adult mosquito age can be predicted by using RTPCR technology to investigate the gene expression in *Anopheles* species of different ages (Sikulu *et al.*, 2011). Determination of gene expression levels can also be determined using microarrays. This has been carried out recently in *An. gambiae* females (Cook and Sinkins, 2010; Wang *et al.*, 2010).

#### 1.2.2.1.2. Nutritional requirements

Both the quantity and quality of larval food influence the fitness of adult mosquitoes in different ways. Rearing immature stages with different amounts and types of food per larva results in different sizes of females and males, which usually has important carry-over effects on adult physiology (El-Akad and Humphrey, 1990) and on ability for reproduction (Macdonald, 1956).

##### 1.2.2.1.2.1. Food quality

Sugar feeding is an ordinary element of the biology of both sexes. *An. gambiae* males differ from females in feeding pattern because they rely entirely on sugar feeding, and feed on average twice daily. In Western Burkina Faso, *An.gambiae* s.s. males from both molecular forms, S and M, prefer to stay close to favoured sugar sources such as certain flowers, and the odours of flowers stimulate male mosquitoes and determine their choice of food under both laboratory and field conditions (Gouagna *et al.*, 2010). On the other hand, females with daily access to both blood and oviposition sites fed on sugar only once every four days (Gary and Foster, 2006). In laboratory studies on *An. pharoensis* Theobald females, activation of ovaries and developing oocytes was stimulated by a blood meal which provided nutrients to initiate the gonotrophic cycle, and females often required

multiple blood meals in the course of a single gonotrophic cycle (El-Akad and Humphrey, 1990). Different vertebrate hosts' blood meals have a differential effect on egg production (Macdonald, 1956). In field studies, it was observed that *An. gambiae* prefers to feed on human blood and selects the host by olfactory attractants or host odours (Dekker *et al*., 2001). An abundance of males can result in females copulating before blood-feeding, but, when vertebrate hosts are available, females prefer to have a blood meal before mating (Onyabe *et al*., 1997). Guinea-pig blood containing high levels of nitrogen was seen to lead to notably greater numbers of oocytes and ovary maturation compared with human blood when given to *An. stephensi* females (Briegel, 1990b). Moreover, blood feeding alone leads to a reduced number of eggs in mosquitoes when compared to both sugar and blood meals. Energy for egg production taken from sugar means that less dietary protein is used for lipid synthesis (Clements, 1999). Lipids from the ovarian and extra ovarian compartment are used by gravid females, with lipids forming approximately half the dry weight of mature oocytes (Clements, 1999).

**1.2.2.1.2.2. Food quantity**

In some mosquito species, wild females require more than one blood meal to mature ovarian follicles (Macdonald, 1956). Notably, the main South American vector, *An. nyssorhynchus,* is an example of this (Lounibos *et al.,* 1998). Afrotropical malaria vectors are thought to be more strongly affected by the number of blood meals per ovarian cycle than are neotropical *An. nyssorhynchus* species (Lounibos *et al*., 1998). On the other hand, it was shown in field studies that the first blood meal of *An. gambiae* females is often used to increase metabolic reserves and, after that, to create eggs (Charlwood *et al.*, 2003). Studies of metabolite use have shown that *An. gambiae* uses blood protein in several ways. Less than 20% of blood meal protein content was built into ovaries, while four per cent was transformed to oocytes protein and fifteen per cent went into oocyte lipids. An

additional 33% of blood proteins were used as extra ovarian fuel, half of which was then degraded and used as energy during the subsequent gonotrophic cycle (Briegel, 1990a).

The positive correlation between adult body mass and the quantity of food supplied during larval stages was established early on by Nayar (1969) in *Aedes taeniorhynchus*. He found that adding more food during larval stages led to increased body size. Larvae growing under tougher environmental conditions in the field, such as in high density, produce small adults (Manoukis *et al.*, 2006). Similar patterns have been observed in *An. stephensi* adults emerging from high-density larval habitats, and these adults subsequently take smaller amounts of blood and have lower fertility (Reisen, 1975).

Variations in the amount of resources available to mature females also affect their reproductive ability. Small differences in the amount of food have a larger influence on egg production in small females that have small ovaries with a limited number of ovarioles, than on larger females (Briegel, 1990a). At the beginning of the ovarian cycle, the number of oocytes is limited by the number of ovarioles in the ovaries. For example in *Culex pipiens,* females have a variable number of ovarioles ranging from 340 to 430 depending on the conditions they were raised under (Hosoi, 1954- The subject reviewed by Clements, 1992). Variation in egg size is also observed. In *Aedes aegypti,* changes in egg length and width correlate with variation in female body size as well as blood meal size (Steinwascher, 1984, the subject reviewed by Clements, 1992). Both factors also affect the number of eggs produced, but egg size is more affected by blood meals than body mass (Steinwascher, 1984, the subject reviewed by Clements, 1992). The correlation between body size, blood meal volume and number of oocytes - as determined by quantification of haematin has been established for *An. albimanus, An. gambiae* and *An. stephensi* (Briegel, 1990a). For example, large *An. albimanus* females taking larger quantities of blood from vertebrate hosts hence developed more oocytes and had greater fertility (Briegel, 1990a).

#### 1.2.2.1.3. Temperature

Temperature is one of many factors that influence biological aspects in mosquitoes such as larval growth (Lyimo *et al.*, 1992), biting sites on the human body (Jong and Knols, 1995), blood feeding (Scott *et al.*, 2000), and geographic distribution (Kirby and Lindsay, 2004). *Aedes aegypti*, when reared under a wide range of temperatures (between 18 to 33C), were observed to have differences in adult dry weight, wet weight, size, and in the number of ovarioles produced; with both weight and the number of ovarioles being lower at higher temperatures (Van den Heuvel, 1963). *Culex nigripalpus* and *Ae. taeniorhynchus* seem less affected, as an increase in temperature to 28C° during larval stages still produced heavy weight adults (Nayar, 1968, 1969). In *Anopheles* species, changes in adult body size were observed during different seasons and were related to changes in fertility (Shannon and Hadjinicalao, 1941). Rua *et al*. (2005) found that both ovarian development and the length of the gonotrophic cycle in *An. albimanus* were reduced under higher temperature conditions (30°C as compared with 24°C). Armstrong and Bransby-Williams (1961) observed that the best temperature for rearing of *An.gambiae* was 26.5C° for adults and larvae and that increasing the temperature to 29.5°C reduced egg production. In *Aedes aegypti* it was found that under humid conditions (80%) and a lower temperature (25°C), females lived longer and oviposition was maximised, with both factors affecting both the proportion of females who laid eggs as well as the number of eggs produced (Costa *et al.*, 2010).

#### 1.2.2.1.4. Humidity

To calculate relative humidity, the water vapour content of the air must be measured and this figure divided by the same measurement taken from water-saturated air: both measurements must be taken under the same temperature conditions (Lincoln *et al*, 1998).

Day and Curtis (1989) found a positive correlation between feeding activity and rainfall in *Cx. nigripalpus*. It was proposed that rainfall raises humidity and that this would affect mosquito flight activity and host finding. Mattingly (1949) showed that large amounts of rain caused a slight reduction in the biting activity of *An. hargreavesi, Ae. africanus* and *An. gambiae.* It has been established through field surveys in Kenya that *An. gambiae* s.s. have multiple strategies for survival during the dry season: constant reproduction throughout the year, and embryo dormancy in moist soil for several days or more, and thus can maintain a large population size (Minakawa *et al*., 2001). Females prefer strongly moist soil as an oviposition site rather than dry soil under laboratory conditions (Minakawa *et al*., 2001).

**1.2.2.1.5. Infection**

New methods to control mosquitoes are required and these will rely on a better understanding of the interaction between the parasite and the vector. Malaria infection stimulates different changes in vector behaviour and physiology which subsequently affects reproductive fitness, particularly during the maturation and replication of parasite in the mosquito abdomen and sporozoite invasion of the salivary glands (Ahmad *et al.*, 1999; Hurd *et al.*, 1995). *Plasmodium* infections modify the survival, flight activity, blood feeding and reproduction of mosquitoes (Maier *et al.*, 1987; Kittayapong *et al.*, 1992; Hurd, 1993; 1994; 2003; Ferguson and Read, 2002). A reduction of *An. gambiae* s.s. fecundity was observed in field studies. Females infected with oocytes and/or sporozoites of *P. falciparum* produced fewer eggs than uninfected females and the decline in number of eggs could not be attributed to smaller female body mass (Hogg and Hurd, 1997). Blood meal mass did not correlate with lower survival of females (Hogg and Hurd, 1997), with larger females living longer in *An. maculatus* and thus have a higher vectorial capacity. However, body size was not seen to correlate with the number of oocysts or sporozoites in

infected females (Kittayapong *et al.*, 1992). Smaller size and smaller blood meals cannot be held responsible for reduction in fecundity: instead, this may be attributable to the interaction between mechanisms for vitellogensis and *Plasmodium* (Hogg and Hurd, 1997).

### 1.2.3. Genetic factors

The Scientific Group on Genetic and Insecticide Resistance (WHO 1964) defined genetic control as: "The use of any condition or treatment that can reduce the reproductive potential of harmful forms by changing or replacing the genetic material" (the subject is reviewed by Knipling *et al*., 1968). Genetic management can provide helpful tools to reduce vector populations, possibly without negative effects on the environment. But genetic vector control is only one aspect of the genetic factors which can affect mosquito fitness. Populations of *An. gambiae* are genetically complex and this natural complexity can result in fitness difference amongst sub-populations and individuals. Here, some genetic factors that affect reproductive success are highlighted.

#### 1.2.3.1. Molecular and chromosomal forms

Yaro *et al*. (2006b) measured the fecundity of *An. gambiae* M and S and *An. arabiensis* females and found that egg batch size correlated with body mass. They also measured egg quality by measuring the protein content of the 1$^{st}$ stage larvae. A similar correlation between size of female and number of eggs was noted for each group, although batch size was distinctive to each of the three populations. Eggs from M molecular forms had higher levels of protein when compared with S forms, which may reflect an adaptation

favouring greater investment in reproduction in less productive larval sites (Yaro *et al*., 2006b).

It has been shown that in Angola the M form of *An. gambiae s.s.* is distributed in tropical dry or sub-humid zones, whereas S forms sites with higher humidity (Calzetta *et al.*, 2008). In Madagascar meanwhile, *An. gambiae* complex distribution has remained constant (Tsy *et al*., 2003). For example, *An .arabiensis* is always found in sub-arid areas, whereas *An. gambiae* are seen in humid sites and *An. merus* is limited to coastal areas (Tsy *et al*., 2003). PCR targeting a sequence in the IGS of the rDNA in the heterochromatin of the X chromosome has been used to identify the species (Tsy *et al.*, 2003). The river Gambia offers a variety of contrasted larval breeding sites to members of the *An. gambiae* complex in the flooded alluvial zone bordering the river. *An. gambiae* s.s. does not use habitats with over 30% sea water, but *An. melas* is found in water with up to 72% sea water (Bogh *et al*., 2003). The M form is found with *An. melas* in the fresh water-retaining alluvial plain and plant cultivations along the Gambia river, and *An. arabiensis* and the S form of *An. gambiae* s.s. prefer free-draining soil covered with farmland. So, M forms adapted to semi-permanent larval habitats and S forms colonized temporary breeding sites (Caputo *et al.*, 2008).

**1.2.3.2. Laboratory colonization versus natural population:**

Colonies of insects established under laboratory conditions play an important part in scientific research because they can be used to increase the knowledge and understanding of the biology of insects. More than forty years ago, Armstrong and Bransby-WillIams (1961) succeeded in maintaining a colony of *An. gambiae* in a laboratory environment and they explained point by point how a colony is set up, and also recorded several observations on its life cycle. At that time, they had already observed that the number of

larvae was an important factor for larval development. However, colonization and laboratory rearing is associated with other density-dependent factors. For example, in *An. stephensi*, it has been shown that increased density of larvae also diminishes hatching rates (Timmermann and Briegel, 1993) and that a higher larval density increases male to female ratio (Reisen and Emory, 1977). Rearing of sibling species of *An. gambiae* under insectary conditions can be difficult as is the case for *An. quadriannulatus* (Mpofu *et al*., 1993), whereas, *Ae. japonicus japonicus,* a vector of the West Nile virus in the United States, was reared successfully under laboratory conditions for more than 35 generations over a five-year period (Hoshino *et al*., 2010). It has been found by using microsatellite polymorphism at nine loci located on chromosomal 3 that the mean number of microsatellite alleles and heterozygosities declined in two laboratory colonies of *An. gambiae* s.s .in comparison with a field population (Norris *et al*., 2001).

**1.2.3.3. Genetic modification**

The control of insects by genetic means is not a new tool. The release of sterile males to reduce wild populations of mosquitoes was used as early as the 1960's (Benedict and Robinson, 2003). This approach involves submitting males to radiation or chemicals which generate mutations in the DNA of germ cells resulting in non-viable gametes: in this case sperm (Knipling, 1955, 1959). When such males mate with normal females no offspring are produced, or females produce only a small number of viable eggs (Knipling *et al*., 1968). Genetic manipulation of insect vectors of disease can also involve the direct introduction of DNA into the germline of the insects. So-called transgenic methods are currently under development and would use gene constructs to spread genes affecting vectorial competence across populations (Collins and Jams, 1996), or even fully replace them with a modified mosquito which cannot transmit the parasites (Ito *et al*., 2002),

which does not reproduce so effectively and is weaker as a vector, or which can be more effectively targeted for control. This technique could possibly remove the problem of genome disruption encountered with crosses, and circumvent sub-population barriers. (Crampton *et al*., 1994).

Genetic manipulations can affect vector fitness in different ways. Males sterilized by irradiation and chemical sterilizants often suffer from low competitiveness in comparison to wild males and it is often difficult to design an effective mechanism to produce males only (Curtis, 1978; Lines and Curtis, 1985). Study by Li *et al* (2008) that used three lines of transgenic *An. stephensi*, VD35 & VD26 (single gene–insertion) and VD9 (double gene interstation) when mixed with a non-transgenic strain in cage-invasion experiments the transgene frequency declined over time indicating a fitness burden in transgenic individuals (Li *et al*, 2008). Also, a transgenic *An .stephensi* mosquitoes showed lower fitness than non-transgenic because fecundity in terms of the number of eggs declined and the mating success rate was lower. This decreased due to the reduced competitiveness of transgenic males compared to non-transgenic, meaning that females laid fewer eggs. Moreover, transgenic larval development was slow, with a resultant delay in adult sexual maturation (Li *et al.,* 2008). The authors concluded that this was due to a reduction in mating success and reproductive capacity caused by transformation and expression of the transgene (Li *et al.,* 2008). Release of sterile males produced by crossing two members of the *An. gambiae* complex was attempted in field experiments in Nigeria. *An. melas* and species B, a dieldrin-resistant mosquito reared in a colony in London for more than ten years. They found that such males did not mate with wild females due to a mating barrier which prevents mating between sterile males and wild females (Davidson *et al*., 1970).

Irvin *et al* (2004) assessed fitness costs for transgenic *Aedes aegypti* expressing a GFP marker and two transposase genes *Hermes* and *MOSI*. It was found that a significant

reduction in the fecundity, longevity, survivorship and fertility in transgenic strains in relative to a non-transgenic laboratory strain. For example, it was observed that transgenic EGFP-expressing females showed the highest sterility rate across four gonotrophic cycles. However, EGFP-expressing and non-transgenic (Orlando strain) mosquitoes lived longer than other transgenic stains. Fecundity was significantly reduced in all transgenic strains but it was very sharply reduced in the EGFP strain.

Transgenic *Anopheles stephensi* were used to study the role of two genes for malaria parasite blockage. These putative anti-malarial genes were, a tetramer of the SM1 dodecapeptide or the phospholipase A2 gene (PLA2) from honeybee venom. By estimating mosquito survival, fertility, fecundity, and by using population cage experiments it was found that there was no significant reduction in these fitness parameters in comparison with non transgenic strain, However the PLA2-gene proved to cause deleterious effects in mixed cage populations (Moreira *et al*, 2004). These results led to the conclusion that the fitness load of refractory genes is dependent on the effect of the transgenic protein produced in the mosquito (Moreira *et al*, 2004). Recently, transgenic OX513A (RIDL males carrying a tetracycline repressible lethal gene) of *Aedes aegypti* were used to study male mating fitness under laboratory conditions. It was found there was no decline in transgenic male's ability to inseminate several wild females in comparison with wild type strain (Rockefeller). When one male of each strain was caged with a ROCK female, the transgenic male displayed lower mating succes. When 10 males (with different ratio of ROCK/RIDL: 10/0, 8/2, 5/5, 2/8, 0/10) competed for 5 ROCK females, mating success and fertility were similar but the longevity of transgenic males was lower (Massonnet-Bruneel *et al*, 2013). The authors concluded that OX513A fitness was equivalent to that ROCK wild type males under laboratory conditions (Massonnet-Bruneel *et al*, 2013).

### 1.3. Mating behaviour

Understanding reproductive biology and particularly mating behaviour is often critical for assisting programmes aimed at controlling mosquito-borne diseases. Copulation in most Diptera is characterized by the ability to pair in flight (Downes, 1991). In *An. gambiae,* mating is initiated in flight and occurs in swarms, in which males may be able to locate and attract females through the sound produced by their wing-beat (Charlwood and Jones, 1979) (Fig. 1.10). There are relatively few investigations of mating performance in *An. gambiae,* and mating preferences and the exact mechanisms of mate choice are still inadequately understood (Dao *et al.*, 2008). Furthermore, understanding mating behaviour could also reveal the mechanisms of reproductive isolation between the chromosomal and molecular forms of *An. gambiae* s.s. as well as between sibling species of *An. gambiae* s.l. (Dao *et al.*, 2008).


Fig. 1.10: Mating behavior in *An.gambiae* s.s. (source: http://io9.com/anopheles-gambiae/)

### 1.3.1. Mating time

In the wild, mating swarms in *An. gambiae* s.s. take place during the first hour from dusk (Charlwood *et al*., 2002). When females enter the swarm, the antenna fibrillae in males becomes erect and they remain so until the end of swarming (Charlwood and Jones, 1979). It was observed that under field conditions in Anopheline males, swarming happened rarely before the second day after emergence (Reisen *et al*., 1981). Under laboratory conditions, in *An. gambiae* s.s this also occurred (Gary *et al.*, 2009).

### 1.3.2. Swarming and insemination

Males only start swarming when they are capable of erecting their antennal hair and tracking female wing-beats (Nuhout and Sheffield, 1979). Solitary males initiate zigzag flight patterns at recognized sites, in the early morning or evening, and are joined by other males (Russell and Rao, 1942; Reisen *et al.*, 1985). Within minutes of swarm formation the number of males increases, sometimes dramatically (Russell and Rao, 1942). In *An. maculipennis*, Var. *atroparvus,* the number of males in swarms is commonly twenty-five to fifty but sometimes reaches up to one thousand or more (Cambournac and Hill, 1940). The number of individuals in swarming is less than 500 males in *An.gambiae* s.s while in *An. melas* it is between 100 and 1500 males (Assogba *et al.*, 2010). In *An. gambiae*, larger swarm size is seen to positively influence mating outcomes, but without supporting the female preference model, as no advantage is given to males in terms of mating success from the larger swarm (Diabaté *et al*., 2011).

In *An. gambiae* s.s., swarming takes place only at dusk. Males start dancing over open ground within the female feeding site, and females arrive later in the swarms (Marchand, 1984). Females spend on average thirty seconds in swarms before copulating with a male, and moonlight can increase that time (Charlwood and Jones, 1980). In the field, swarming duration remained between ten and twenty minutes approximately in *An.*

*gambiae* s.s as well as *An. melas* (Assogba *et al*, 2010). The height of swarming in *An. gambiae* was lower than in *An. melas* (Assogba *et al*, 2010).

The shape of swarms is spherical (Marchand, 1984). Furthermore, large males in *An. freeborni* start swarming earlier than smaller ones and mate from eleven to twenty minutes after formation of the swarm (Yuval *et al.*, 1993). Insemination is a two-stage process whereby the orifice of the females' spermathecal duct is firstly pushed by the male's gonopore, before sperm and the secretions of accessory glands are transferred via insertion of the male aedeagus in the female's vagina (Spielman *et al*., 1974). The spermatozoa and accessory glands secretions determine the number of females which are inseminated by males. *An. gambiae* males can inseminate more than five females (Giglioli and Mason, 1966-reviewed by Clements, 1999). During sexual inactivity, seminal vesicles are refilled with sperm and the accessory glands topped up with secretions (Mahmood and Reisen, 1982).

### 1.3.3. Sperm quality, quantity and mating success

Sperm in mature males of *An.gambiae* are stored in the testes and move down to the seminal vesicles through the vasa deferentia during mating (Huho *et al.*, 2006). The two seminal vesicles are linked to the pair of accessory glands by a short duct that leads into an ejaculatory duct (Huho *et al.*, 2006). The sperm transferred to the female during copulation are stored in their spermatheca and remain there for days to months until oviposition. Only one spermatheca is found in *Anopheles* females (Clements, 1999).

In virgin males of *An.stephensi* the testes size (length and width) changed with age. For instance, the length of the testes at age 3 days was 354µm, while they had become shorter at age 7 days, at 327µm. Also, the width was 139 µm at age 3 days, becoming narrower by 7 days (121 µm). Mated males had a smaller testes size (Mahmood and Reisen, 1982). Testes size and sperm length were found to correlate significantly in eleven

*Drosophila* species: Males with testes forming 1-5% of dry body mass created short sperm, while those with testes occupying 8-11% of dry mass produced long sperm (Pitnick *et al.*, 1995). This was regardless of the fact that males of species with larger body size had longer sperm (Pitnick *et al.*, 1995). On the other hand, there was no correlation between testes size and sperm length among 83 mammalian species (Gage and Freckleton, 2003). The number of sperm in the testes of *An. arabiensis* males was found to increase with male age (Helinski and Knols, 2009). *An. gambiae* s.s. male testes were found to have a greater numbers of spermatocysts at emergence than at 8 days post emergence, dropping from an initial 3 to 5, to less than one (zero) (Huho *et al.*, 2006). Meanwhile however, the sperm reservoir was found to take up more of the testis with age, and this change occurred more quickly in virgins (Huho *et al.*, 2006). Among *Ae. aegypti* laboratory strains, greater variation in sperm number was found in testes and seminal vesicles in comparison with field collected mosquitoes. Field mosquitoes on average had larger sperm (Ponlawat and Harrington, 2007). The same authors observed that the number of sperm cells in laboratory strains ranged from around 500 to 1500, whereas in field mosquitoes there were 2000 to 5000 sperm cells (Ponlawat and Harrington, 2007). In unmated males of *Ae. aegypti*, a seminal vesicle contained approximately five thousand spermatozoa while approximately 930 were found in the seminal vesicle of force-mated males (Jones and Wheeler, 1965).

Virgin bumblebee males, *Bombus terrestris* had a higher sperm length variation than in those sperm stored in the queen`s spermatheca (Baer *et al.*, 2003). Sperm length was significantly correlated with body size in some species than in others in bumblebee males. This suggests that sperm length may be influenced by male genotype and also that there may be a genetic factor in cryptic female choice of sperm length (Baer *et al.*, 2003). Klowden and Chamber (2004) demonstrated that males of *An. gambiae* s.s. produce sperm which may differ widely in length, with an average of between 26 µm to above 500 µm

(Fig.1.11). In *D. subobscura* nucleated sperm is seen in two head and tail lengths (Bressac and Hauschteck-Jungen, 1996). The mean sperm length in *D. obscura* group was 0.113 and 20 mm in *D.littoralis* (Joly *et al.*, 1989). Sperm length in the field cricket, *Teleogryllus oceanicus* presented a significant intra –specific variation (Simmons *et al.*, 2003). In contrast, in *Ae. aegypti*, all sperm cells have very similar length (Klowden and Chamber, 2004). There was no impact of age on sperm length in *An.gambiae* (Voordouw *et al.*, 2008). But among forty two *Drosophila* species there was discovered a significant correlation between male age in adulthood and sperm length (Pitnick *et al.*, 1995).

Voordouw *et al.* (2008) suggest that short sperm in males of *An. gambiae* and *An. stephensi* had considerably higher reproductive success than long sperm and that this was not dependent on male body size. According to Klowden and Chamber however (2004), long sperm in *An.gambiae* s.s. had higher reproductive success, because the sperm found in the genital tract and spermatheca of females was longer than that sperm measured in male testes. In six species of *Drosophila obscura*, Snook and Karr (1998) found that eggs were only fertilized by long sperm, whereas in Pasini's (2010) opinion, both short and long sperm are active in egg fertilization in the *D. obscura* group. Nevertheless, sperm length did not control sperm journey from spermatophore to female spermatheca (Simmons *et al.*, 2003). In *Anopheles*, sperm quality and quantity are inadequately documented and there are limitations in the understanding the role of sperm length and male reproductive success.

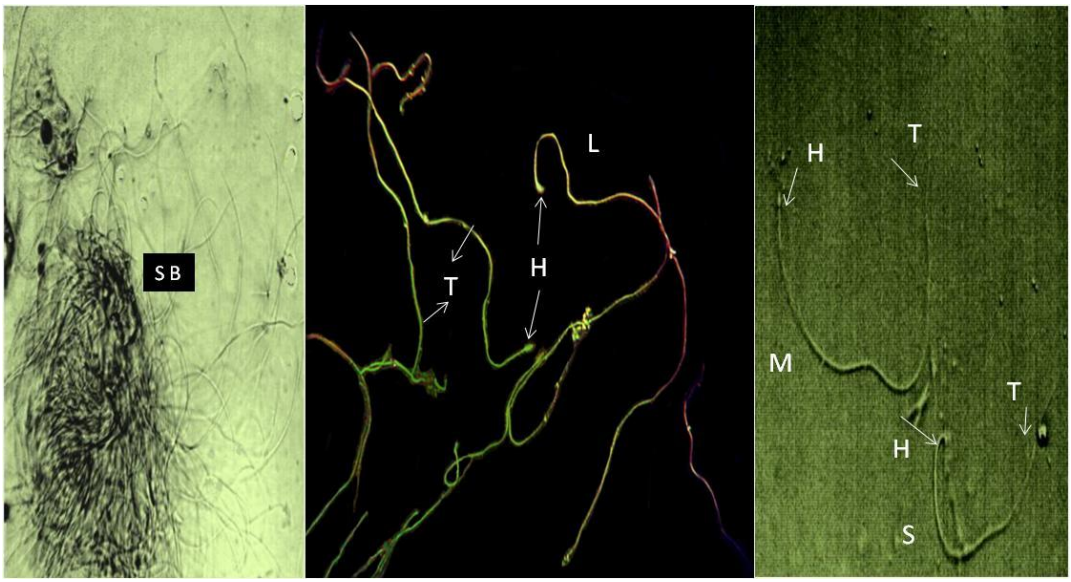


Fig.1.11. Sperm quality in *An. gambiae* s.s. the slides were examined under a Nomarski filter microscope. Note (H) the head and (T) the tail of sperm, and the difference in length (L) Long sperm; (M) Medium length sperm, (S) short sperm and (SB) sperm bundle.

### 1.3.4. Mating plug and accessory gland proteins

During the larval stage, the accessory glands develop in the ninth abdominal segment from imaginal disks and continue to grow throughout the pupal and adult stages (Clements, 1999). Among Culicids, accessory gland shapes vary, and can be pear-shaped, spherical or elongate (Clements, 1999). *An. albimanus*, *An. stephensi* and *An. culicifacies* have accessory glands with are egg-shaped and which are connected by a short duct to an ejaculatory pump (Mahmood and Reisen, 1982; 1994; Mahmood, 1997). The glands consist of two regions, an anterior part occupying three quarters of the volume, and a posterior region equivalent to a quarter of the gland, which contains different cells. The anterior part features a quantity of yellowish large granules while the other part has small white granules. The secretion in the first region was reduced in *An. culicifacies* after the male had mated once, then after further mating the same happened in the second part of the

glands (Mahmood and Reisen, 1982; 1994; Mahmood, 1997). In *Ae. aegypti* males were found to mate with up to 6 or 8 females until their accessory glands were depleted of secretions, and then to rest for 72 hours to replenish the glands (Dapples *et al.,* 1974; Ramalingham, 1983). As a result, mated males' accessory gland size (length and width) was smaller than virgins' at the same age (Dapples *et al.,* 1974; Mahmood and Reisen, 1982; Ramalingam, 1983).

Male accessory gland secretions are essential part of the seminal fluid transferred to the female during copulation, and plays an important role in regulating sperm storage, preventing further female mating, and stimulating egg-laying (Lung and Wolfner, 2001; Ram and Wolfner, 2007). Seminal fluid proteins send chemical signals to the female nervous system in order to change her behaviour and release reproductive hormones (Chapman, 2009). Lee and Klowden (1999) as well as Sirot *et al.* (2008) identified several sex peptides which were transferred to the female from the male during copulation and regulate mating, blood feeding and oviposition behaviour in *Ae. aegypti* females. The substance produced in the male *An. gambiae* accessory glands contains yellow gelatinous rods that are deposited in the female vagina and result in a mating plug (Giglioli and Mason, 1966). The mating plug is about 0.5mm long and dissolves by 24 hours after fertilization (Gillies, 1956). A recent study published by Roger *et al*. (2009) identified mating plug components and their function in *An.gambiae* s.s. They identified the proteins AGAP009368, or Plugin, and AGAP009099, or Transglutaminase, as key to the mating plug, acting on the seminal fluid proteins to connect, causing the semen to coagulate into a solid mass. Male accessory gland (MAGs) secretions contain several substances in small amounts, such as lipids, amino acids and carbohydrates, but have a huge quantity of different types of proteins (Gillott, 2003).

The quantity of sex peptide in *D. melanogaster* plays an important role in male reproductive success, in which males with bigger glands transfer more sperm to females (Wigby *et al.*, 2009). These peptides comprise: simple peptides, with fewer than 100 amino acids, signalling insemination and regulating female egg - laying; pro-hormone like peptides with between 200 and 400 amino acids, again regulating egg -laying; and glycoproteins, which act in the storage of sperm (Wolfiner, 1997). Secretions of the accessory glands have been shown to influence flight activity, host searching, egg laying, fertilization and ovarian development (Clements, 1999). Relatively little is known about male reproductive success in terms of factors and processes leading to sperm storage, oviposition, and prevention of further mating on the part of females.

### 1.3.5. Factors affecting mating success

Many factors can potentially affect mating behaviour and more specifically, mating success. Here, some environmental and genetic factors are discussed to study the effect of those factors on mating success in mosquitoes and particularly in *An. gambiae* s.s.

#### 1.3.5.1. Age

The age of both sexes in mosquitoes is known to influence mating success (Mahmood and Reisen, 2008). In the field, 10-day-old *Ae. aegypti* males transferred larger amounts of sperm to females during copulation than 1-day-old males (Ponlawat and Harrington, 2009). In addition to age; temperature, humidity and nutritional status influenced mating behaviour. The quantity of spermatozoa in male testes increased over time until males were 10 days old, and then remained steady until twenty days (Ponlawat and Harrington, 2009). Furthermore, Mahmood and Reisen (2008) observed that in *An. culicifacies* under laboratory conditions the number of mature spermatocysts decreased

with age. In addition, maximum mating activity took place when females were 5 to 12 days old and males 5 to 7 days old. Older males had a lower capacity for mating with females than the younger ones (Mahmood and Reisen, 2008).

#### 1.3.5.2. Environmental factors

Several environmental factors such as temperature and humidity, body size and condition influence the mosquito's behaviour. Currently very little is known of their effects on mating behaviour, but there follow some general effects of environmental factors on behaviour, while mating behaviour is discussed whenever literature is available.

##### 1.3.5.2.1. Temperature and Humidity

Mosquito behaviour is influenced by changes in temperature and humidity (Clements, 1999). In fact, it is difficult to separate the relation between level of activity and temperature or humidity. In the summer time *Cx nigripalpus* present a high level of flight activity due to the increase in temperature and humidity (Bidlingmayer, 1967). In addition, evening twilight is a notably more active time in the majority of mosquito species than is morning, considered to be due to lower morning temperatures (Bidlingmayer, 1967). Species differ in the minimum temperature required for activity. For example, *Ae. nigripes* stopped flying below 5°C (Corbet and Danks, 1973). High levels of humidity caused by heavy rains reduced the biting activity in *An. hargreavesi* and *An. gambiae* s.s (Mattingly, 1949). It is possible that these conditions would similarly affect mating behaviour such as swarming. Gingl *et al.* (2005) found that *Ae. aegypti* was able to detect fluctuations in temperature, both hot and cold, through the use of two thermoreceptive cells located in the tip of the antenna. Temperature was seen to influence swarming behaviour in *An.gambiae* s.s. males at 24h post emergence when deprived of sugar feeding, with such

males more able to swarm at 23°C than at 27°C. This effect disappeared however when males were observed at 72h post emergence (Gary *et al.,* 2009).

**1.3.5.2.2. Body size:**

The effect of body size on mating performance and other activities has been investigated in several species. Large-bodied females in *An. gambiae* stored more proteins, lipids and glycogen so lived longer and had higher biting activity and more rapid ovarian maturation compared with smaller females (Klowden and Chambers, 1988). In addition, large males in *An. freeborni* inseminated greater numbers of females in comparison with small ones (Yuval *et al*., 1993). Moreover, in *Ae. aegypti,* greater male size was linked to increased transfer of sperm during copulation (Ponlawat and Harrington, 2009). Furthermore, it has been observed that males showed a preference for mating with larger females in *An. gambiae* (Okanda *et al*., 2002). Voordouw and Koella (2007) however, studying the effects of genetic variation on reproductive success in the male using a quantitative genetic approach, did not find this effect, but did find significantly variations in ability to stimulate egg laying and hatching opportunities among full-sibling families of males In a study that used a full-sibling quantitative analysis to test genetic components in sperm length, males with shorter sperm had significantly higher reproductive success than those with longer sperm (Voordouw *et al.,* 2008). Sperm length was correlated with male body size (measured as wing length) (Voordouw *et al*., 2008).

Male body size plays an important role in swarming and mating activity in the *An. gambiae* mosquito. This was tested by feeding larvae three different amounts of food (10 mg, 20 mg and 40 mg), which produced dissimilar male body size. (Ng´hab *et al.*, 2008). Intermediate-sized males had more success mating during swarming than the smaller or larger males, but their average survival was fifteen per cent lower than that in the two other

groups. While indicating that larval feeding and subsequent body size plays an active role in mating success, the relationship between this and survival in different phenotypes is clearly complex (Ng´hab *et al*., 2008). Cator *et al* (2010) examined harmonic convergence behaviour in *An. gambiae*, and found that flight tone frequency varied with size, being notably higher in larger individuals. Shorter latency to higher frequency tones was shown in both males and females: this shows that both sexes use sound to identify size and therefore fecundity in potential mates.

**1.3.5.2.3. Body condition**

Lipids in mosquitoes are found as fat cells in the body wall and surrounding major organs. A good food supply during the larval stage stimulates accumulation of lipids, glycogen and protein in the fat body cells (Wigglesworth, 1942). Mosquitoes gain carbohydrates from nectar and other sugar solutions and consume these as energy for flight and survival, but a blood-meal is required by females for egg development. In *Ae. aegypti*, females fed first on a carbohydrate source and used this to synthesise considerable amounts of protein and lipid storage during the first gonotrophic cycle (Zhou *et al.*, 2004). In *Ae. aegypti*, oocytes involved more than thirty percent lipids (Troy *et al.*, 1975). The amounts of lipids in females differed depending on food supply and adult size (Briegel, 1990b). Egg lipids play an important role as a fuel for embryo growth. *Cx. quinquefasciatus* consumed ninety percent of lipids as energy during reproduction (Van Handel, 1993). In *Ae aegypti*, lipid synthesis was stimulated by feeding females sugar solution after they emerged and after blood feeding the lipids were found in the ovarian tissue. Declining lipid compounds in the ovaries led to decreases in the number of matured oocytes in *Ae. aegypti* (Ziegler and Ibrahim, 2001). In *Ae. aegypti*, reduction in the number of eggs was caused by decreased yolk lipids (Briegel *et al.*, 2002). Male fruit flies, *Ceratitis capitata,* when given food without protein, transferred fewer sperm during mating, thus affecting reproductive

success (Blay and Yuval, 1997). The reproductive fitness of *An.gambiae* s.l. males was measured in laboratory and field conditions by using body size and lipid reserves as a key to determined adult survival. They found the body size and lipid reserves in laboratory-reared male were less than wild male (Huho *et al*, 2007).

#### 1.3.5.3. Genetic factors

##### 1.3.5.3.1. Molecular and chromosomal forms

Mating studies can help in understanding reproductive isolation in relation to genetic polymorphism in different species (Coluzzi *et al.*, 1979). Thus, the study of mating behaviour in the malaria mosquito could give the means for understanding mechanisms of reproductive isolation between molecular forms of *An. gambiae* (Lanzaro and Tripet, 2003) and between the seven sibling species of *An. gambiae* s.l. (Marchand, 1984). Swarming initiation occurs in different ways in different species and at different sites (Yuval, 2006). Swarming in *An. maculipennis var. atroparvus* takes place outdoors and indoors in resting sites (Cambournac and Hill, 1940). In an investigation of mating strategies in *An. gambiae* s.s. and *An. arabiensis*, copulation was seen to occur mainly outdoors, but that this tendency was greater in M forms than S forms (Dao *et al.*, 2008). The authors also found a correlation between female body mass, molecular forms and multiple mating, with small females of M molecular form mating more frequently than the large ones in the same form (Dao *et al*., 2008). In the field, the two molecular forms have different swarming behaviour. Despite mixed populations of M and S forms in the rice field areas of Burkina Faso, numerous swarms were found with only M forms (Diabaté *et al.*, 2003). Sanford *et al.* (2011) suggested that wing beat frequency, as measured by wing length and width, plays a significant role in reproductive isolation between M and S

molecular forms of *An.gambiae*. The adult emergence rate of *An. gambiae* s.s. in S form in puddle and quarry habitats was higher than the M form in paddy fields (Diabaté *et al.*, 2005).

Female mosquitoes, after copulation and insemination, change their behaviour and physiology. To identify factors that change the behaviour of *An. gambiae* s.s. after copulation and mechanisms relevant to fertility Rogers *et al.* (2008) investigated mating machinery in the female at molecular and cellular level. Gene expression of tissue localization, when examined, revealed a subset influenced by mating in the lower reproductive tract, where the male seminal fluid is received, (Rogers *et al.*, 2008). Here upon copulation, molecular and structural components of the mating machinery were turned off, leaving the tissue incapable of subsequent insemination. A number of mating-responsive genes were found in the sperm storage organ, related to maintaining and using the sperm stored. (Rogers *et al*., 2008).

**1.3.5.3.2. Laboratory colonization versus natural population**

In an early study focusing on over twenty species of mosquito, Woodard and Chapman (1977) attempted to investigate the influence of post-emerging age on the insemination rate of colonized mosquitoes. They observed that females kept in narrow vials produced sterile eggs. In many species, mated females were unwilling to lay eggs. The highest rates of insemination occurred between 5 to 8 days of age in females in several species such as *An. albimanus* and *Ae. aegypti*, and declined in 11-day-old females. The process of colonization is often difficult because adequate conditions are not provided to ensure a normal mating process. For example, the colonization of *An. pseudopunctipennis*, one of the vectors of malaria in South America was difficult for many years. Finally, it was discovered that exposure of adults to blue stroboscopic light for a period of several nights

promoted mating behaviour (Lardeux *et al*., 2007). After several generations, mosquitoes were adapted to mating in the laboratory and difficulties were reduced (Lardeux *et al*., 2007). Similarly, providing low lighting levels to a colony of *An. subpictus* Grassi, a vector of malaria in the Maldive Islands, turned out to be the solution for colonizing this species which had refused to swarm and mate in the laboratory environment (Panicker and Bai, 1980). Sterile hybrid males produced from crossing between the five sibling species of *An.gambiae* complex under laboratory cage experiments could be mated with normal females which laid small numbers of eggs (Knipling *et al*., 1968). *Plasmodium*-refractory male *An. gambiae* in cage experiments preferred large females for mating rather than the smaller ones (Okanda *et al.*, 2002). The spread of malaria-refractory genes into the wild population could be limited by the fact that mate choice by males depends on female body size (Okanda *et al*., 2002). Thus female body size is a key component in reproductive fitness, affecting female reproductive competitiveness and egg laying (Okanda *et al.*, 2002).

A transgenic screwworm fly *Cochliomyia hominivorax* (Diptera: Calliphoridae) in laboratory culture presented lower larval productivity or fewer eggs in comparison with wild insects. The transgenic fly's fitness was less than the wild type as it suffered some problems related to transgene effect or genetic insertion site (Allen and Scholl, 2005). There was no discrete cost to the colonies of laboratory-reared *C. hominivorax* from the transgene used however (Allen and Scholl, 2005). An unknown factor which affected fitness in populations of transgenic *An. stephensi* declined the frequency of transgenic alleles over several generations (Catteruccia *et al.*, 2003). The decrease in allelelic frequency in transgenic *An. stephensi* resulted in reduced mating success and capacity for reproduction with time (Li *et al.*, 2008). This reduced the number of transgenic alleles

associated with allele expression or their insertion into the chromosome in the mosquito (Arca *et al*., 1999).

**1.3.5.3.3. Genetic modifications:**

Male reproductive success depends on the quantity and quality of sperm that is transferred to females during copulation. In a study conducted in relation to sterile male release programmes in *An. arabiensis*, it was shown that irradiation of male testes during the pupal stage had a notable effect in adulthood. The number and length of sperm of adult males were affected in irradiated testes, with fewer, shorter sperm. Older males had a greater number of sperm than smaller ones and body mass was not correlated with this (Helinski and Knols, 2009). Irradiated *An. arabiensis* males in Sudan transferred to field cage experiment resulted in a 60% insemination rate one to two days after mating with sterile males commenced. Also, those were males mating with females without choosing particular size and wing length, but small size insects had more mortality in the field cage. The number of eggs was low and the hatch rate was only 11% from females inseminated by irradiated males (Helinski *et al*., 2008). In another study using the genetically modified *Aedes aegypti* OX513A, (a lethal positive feedback system) and wild type mosquitoes to compare mating success in the male, it was found that mating in the genetically modified strain was reduced by almost 50% when compared with the wild type strain (Bargielowski *et al.*, 2011). Furthermore, the lifespan of the genetically modified males was notably reduced when they were kept with females, and this effect was seen to increase the greater the number of females in the cage (Bargielowski *et al.*, 2011). No notable variation in mating competitiveness between heterozygous transgenic *An. arabiensis* and wild–type males was uncovered in a study by Howell and Benedict (2009). They did however find a

difference in longevity, with the transgenic mosquitoes emerging more quickly and dying sooner.

From the literature it can be suggested that the effectiveness of methods of population suppression dependent upon the release into the wild of large numbers of sterile insects would be enhanced by combining transgenic approaches and sterile insect technique (SIT). However these methods entail a laborious process and the use of transgenesis to create strains of Anopheles suitable for SIT (Nolan *et al.,* 2011).

### 1.4. Aims and objectives of the study

#### 1.4.1. Objectives

1. To study the impact of environmental factors (food quality and water availability) on reproductive success in *An. gambiae* s.s. (Chapter 3).

2. To investigate the effect of colonization and genetic modification in *An. gambiae* s.s. on correlates of reproductive success by comparison of sperm quality in field versus colonized mosquito lines, by comparison of sperm quality in non-transgenic versus transgenic mosquito lines and by comparison of accessory glands and testes size, using several non-transgenic as well as transgenic strains (Chapter 4).

3. Test of inbreeding depression hypothesis by creating hybrids between two inbred non-related long-established strains to improve the quality of males (Chapter 4).

4. To estimate the amount of accessory gland proteins in virgin *An.gambiae* s.s. males in old-established strains versus hybrid super males (Chapter 5).

5. To estimate the quantity of accessory gland proteins transferred by Super males vs inbred males in female the reproductive tracts after transfer during copulation (Chapter 6).

6. To assess the quality of hybrid super males by studying their comparing their reproductive success to their old inbred parental strains (Chapter 6).

# Chapter 2

# General Materials and Methods

## 2.1. Mosquito colony

A colony of the Mopti laboratory strain (MRA-763) of *An.gambiae* s.s. was established in the Manson Insectary of the School of Life Sciences at Keele University in 18th July 2007. The source of the original live eggs of the colony was the Malaria Research and Reference Reagent Resource Centre (MR4). They originated in Mali, N'Gabacoro Droit, and were collected from there in 2003 by Dr. F. Tripet and Prof. Greg Lanzaro, University of California Davis. The colony is polymorphic for ″U″ inversions on the 2R chromosome, and display 2La/a and 2r+/+ chromosomal inversions.

Different Mopti strains were used in the current thesis. Both males and females were used in all experiments with the aim of expanding knowledge about male mating biology as a tool for controlling mosquito females and the transmission of malaria. KIL (the original non-modified strain) was an old strain originally from the LSHTM Marangu area in Tanzania since 1975) and reared at the Manson Keele Insectary for many generations since 1990. EE was KIL genetically –modified into a docking strain. VIDA was EE loaded with an antimicrobial peptide. Kisumu was brought from Kenya in 1975 to Liverpool school and had been reared at the Manson Keele Insectary since 2007. Mopti strains were of various ages (from 1 year to more than 35 years) and had been regularly crossed with field samples to refresh them (see section 4.2.1, chapter 4).

## 2.2. Maintenance of *Anopheles gambiae* s.s.

*An. gambiae* s.s. strains were established in the Manson Insectary of the School of Life Sciences, Keele University and kept under constant temperature (26 ± 1 ºC) and relative humidity (75 - 80 %), as well as a constant photoperiod (12h light and 12h dark). Mature mosquitoes were kept in cages (5 litre volume plastic buckets as shown in Figure 2.1); distilled water on moistened cotton wool was provided at all times and mosquitoes were fed on 5% glucose and 0.05% para-amino benzoic acid (PABA). Mature adult mosquitoes at 4-7days old were given a blood meal of horse defibrinated blood using an artificial feeder (Hemotek® membrane feeding system, Discovery workshops, UK) pre-warmed to 37ºC. Two days post blood feeding, mature female mosquitoes lay eggs into white polystyrene pots lined with filter paper and half filled with distilled water. After hatching out, 200 first instar larvae were located in trays (33 x 23 x 5 cm) including one litre distilled water (Fig. 2.1). Larvae go through four stages and a pupal stage within 6-9 days after hatching. First instar larvae were supplied with one drop per tray for a day, of liquifry (Interpet Ltd., Dorking, UK), then 40 mg to 80 mg of ground flakes baby fish food (Tetra werk, Melle, Germany) until they pupate. Pupae were continually removed from the larvae trays into pots filled with distilled water. The pots were placed into the adult mosquito cages into where they emerged and matured.


Fig. 2.1. Mosquito's culture in the insectary: (A) adult Plastic bucket cages, (B) larval trays. Different colours of marker were used on the labels of the trays and cages to make identification of the contents easier.

### 2.3. Variation in larval food availability

The aim of manipulation of the larval food regime was to create different body sizes in adult mosquitoes. Larvae were reared in plastic trays from newly hatched eggs using a pre-optimised feeding regime (Aboagye-Antwi and and Tripet, 2010). For each experiment, eight trays were assigned as a resource-poor group for which were provided 10mg of ground flakes of baby fish food per tray (Tetra werk, Mulle, Germany) once daily over the initial two days. After this and until pupation, 40mg of food was added to each tray once a day. A second batch of eight trays was then assigned as a high resource group. This food regime followed the previous one until the fourth day, and thereafter, 40mg of food was added twice a day (morning and evening) until pupation (at 6-10 days) (Table

2.1). In all, sixteen larval trays and two feeding regimes were established to produce two adult phenotypic qualities; poor (small adult size) and good (large adult size). Each group was divided by sex and the adults kept separately. Thereafter, 30 males and 30 females were selected randomly from each regime, and frozen, in order to kill them. Wing length was then measured in order to establish the impact on body size of each feeding regime.

Table 2.1 Feeding regimes at larval stage (Aboagye-Antwi and and Tripet, 2010) to create adults of differing body size. Ground fish food (Tetra werk, Mulle, Germany) was given to larvae kept in trays of 200 individuals.

| **Group1: Post-tray out of 1st instars –Good phenotype** | **Group 2: Post-tray out of 1st instars - Poor phenotype** |
|---|---|
| **Day 1**: 10 mg once a day | 10 mg once a day |
| **Day 2**: 10 mg once a day | 10 mg once a day |
| **Day 3**: 20 mg twice daily (morning & evening) in total, 40 mg | 20 mg once a day |
| **Day 4**:40 mg twice daily (morning & evening) in total, 80 mg fish food in total | 40 mg once a day |
| **Day 5**: 40 mg twice daily (morning & evening) in total, 80 mg fish food in total. | 40 mg once a day |
| **Day 6**: 40 mg twice daily (morning & evening) in total, 80 mg | 40 mg once a day |

## 2.4. Impact on adults of variation in water availability

Two water regimes were established by creating a variation in the length of time for which water was available (Aboagye-Antwi and and Tripet, 2010). A regime of hydric stress was established for one group, in which water availability was limited to 16 hours in each 24-hour period. For the other group, meanwhile, water availability was at an optimum, as mosquitoes had 24-hour access to the same amount of water. Mosquitoes were placed in groups into polystyrene pot cages of 568ml capacity, and water was made available by soaking cotton sheets initially with 30ml distilled water and providing these in each cage, and adding a further 15ml of water every 48 hours. Sugar meals were not provided in the water but as a sugar cube.

## 2.5. Mosquitoes blood feeding

*An.gambiae* s.s. mosquitoes were given a blood meal every week. The sugar solution was taken out from the mosquitoes' cages 16h before blood feeding and a cotton water bud was removed from the top of the cage just before blood feeding. In addition, tissue was put in the bottom of the cage to absorb any blood leaking. The artificial membrane feeder (Hemotek® membrane feeding system, Discovery workshops, UK- Fig. 2.2) (Nasirian and Ladonni, 2006) was used to feed mosquitoes by using a reservoir of 0.5ml, covered with a piece of parafilm and fixed by a black rubber ring (Fig. 2.3). The blood was added through two holes in the side of reservoir and the holes closed with plastic lids to keep the blood inside the reservoir (Fig. 2.3). The reservoir was then attached to a preheated probe to maintain temperature (37°C) during the mosquitoes' feeding. Mosquitoes were normally fed for 20 to 30 min or until spots of blood were seen in the bottom of the cage. A new blood bottle (horse blood defibrinated, Ref.HB034, 100ml, TCS Biosciences Ltd,

www.tcsbioscience.co.uk) and dropper were used to transfer the blood to the reservoir and these were used every week to minimize blood contamination.


Fig. 2.2. The artificial membrane feeder that used for the mosquitoes' blood feeding. The letters on the figure denote: (A) preheated probe, (B) adults' mosquito cages, (C). central unit of Hemotek® membrane feeding system.

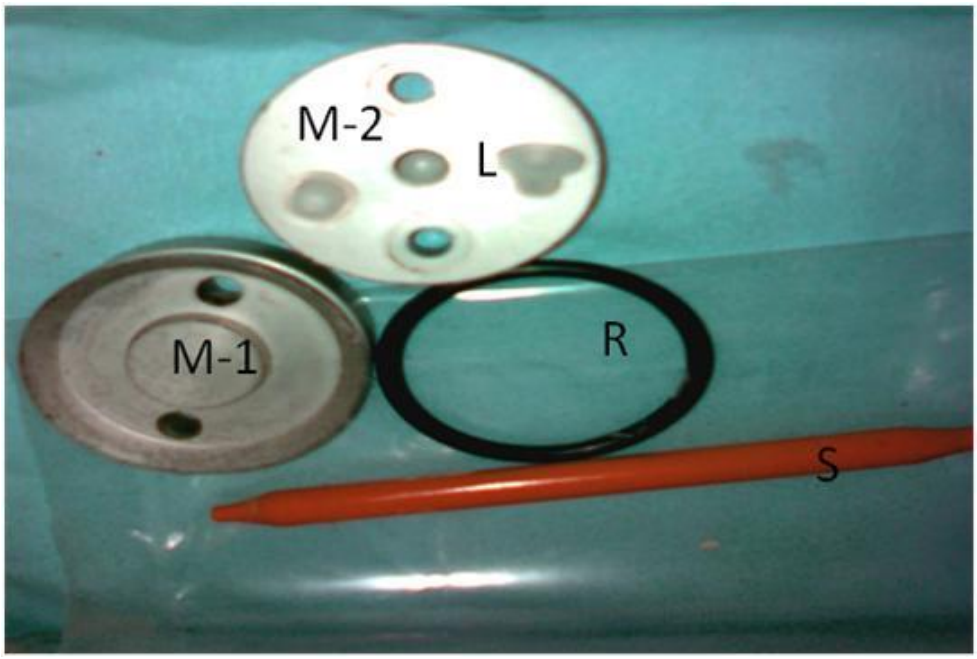


Fig.2.3. The apparatus that makes up the membrane feeder. The letters on the figure illustrate: (M-1 & 2) metal membrane unit front & back side, (L) Plastic lid to close two holes in the metal unit after adding blood, (R) a black rubber ring used to attach the parafilm membrane to metal unit, (S) stick used to release the lids after using them for cleaning.

## 2.6. Wing length measurements

Earlier studies had shown that wing length strongly correlated with body size (Briegel, 1990a, Takken *et al*., 1998, Manoukis *et al*., 2006). When direct measurement of mosquito body size is not viable, e.g. as dehydrated specimens differ from hydrated ones (Jirakanjanakit *et al.*, 2007), body weight could not be used because this varies greatly depending on the amount of food or water provided, or on gravidity (Jirakanjanakit *et al.*, 2007). An isometric relationship has been observed between body mass and body size (wing length) (Briegel, 2003). Here, we corrected for body size by dividing sperm length by wing length; by wing length$^2$ (total body surface) as in testes and accessory glands size; and by wing length$^3$ (body volume) as in proteins estimated, to get a good curve fit.

Adult mosquitoes were killed by freezing and their wings spread on glass microscope slides with drops of PBS saline solution. The wings were measured to the nearest 0.01mm from the alular notch to the distal wing margin, not including the fringe, by using a binocular microscope with an eyepiece graticule (ocular micrometer) (Fig. 2.4). The same microscope magnification was fixed for all samples to minimize the variation between them.

Fig. 2.4. Mosquito wing. The black arrow shows the length measurement from the alular notch to the distal wing margin.

### 2.7. Sex determination of collected pupae

Pupae were collected from the larvae trays by vacuum tube (Weathersby, 1963) and put into pots labelled with the name of the strain, date and sex. A binocular microscope was used to separate the sexes. Less than 20 pupae were picked up by 5ml pipette bulb and put on the petri dish on the microscope stage. The amount of water surrounding the pupae was reduced, to limit pupal movement and a small drops of water were added around the pupae to keep them in a humid area (Barbosa, 1968). After that, a dissecting needle was used to separate the males and females to different sides of the petri dish. Both sexes were distinguished by the shape of the genital segments (Barbosa, 1968). The male has pointed genital lobes, while that of female is more rounded (Benedict, 2007) as shown in Fig. 2.5.

In both sexes, the genital lobe is formed from the 10$^{th}$ abdominal segment, adjacent to the paddles (Clements, 1992). The mosquitoes were picked up separately by 5 ml pipette bulb and put into labelled pots including distilled water. A new petri dish and pipette were used, one for females and the second for males to avoid mixing up the pupae. The work was finished as quickly as possible so as not to expose the pupae to the heat of the microscope light.

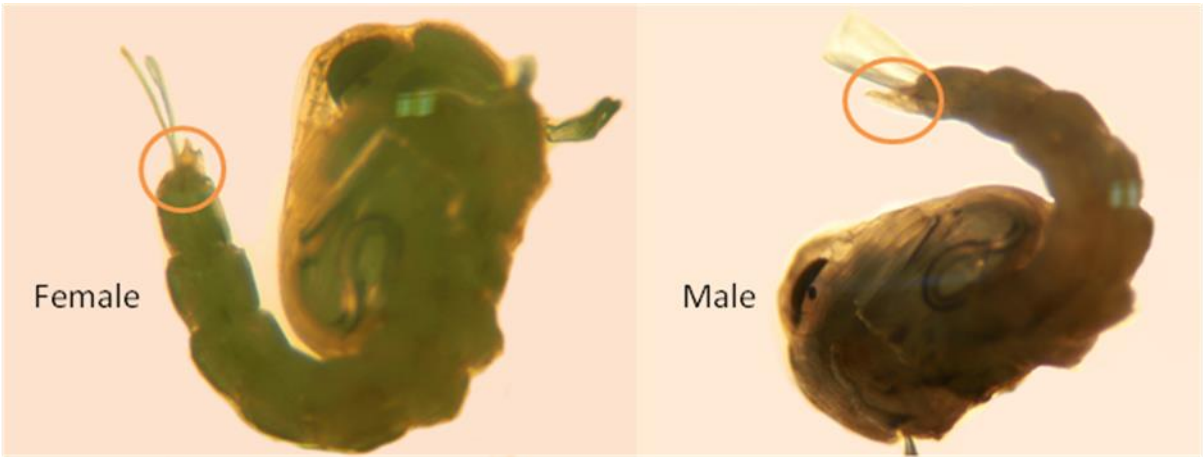


Fig. 2.5. *An. gambiae* s.s. pupae. The female pupa has round genital lobe, while the male has a pointed one.

## 2.8. General methods for statistical analysis

In the current work, data were analyzed using JMP 9 software (Statistical discovery, 2010. SAS Institute Inc., located at SAS Campus Drive, Cary, NC, USA 27513). The statistical processes were as follows:

-Shapiro-Wilk *W* Test (Goodness-of-Fit Test) was used to test data normality.

-Parametric statistic was run using a t-test or ANOVAs, if the data were normally distributed.

-Mean comparisons for ANOVAs test for all pairs was tested by using Tukey-Kramer HSD.

-The critical value of *P* required to reject a null hypothesis (Alpha) was set at 0.05 for all tests.

-If the *P*-value was <0.05 the null hypothesis was rejected in all statistic tests.

-If data were non-normal distributed, a non-parametric test was run by Kruskal-Wallis Tests (3+ groups)

-Nonparametric comparisons for each pair were checked using Wilcoxon tests (2 groups).

-Bars were used to present the predicted differences for parametric tests, while plotboxes were used to display the data of non parametric tests. The median in a plotbox is the 50th percentile, and the median sample value is shown by a vertical line inside the box. The $3^{rd}$ and $1^{st}$ quartiles meanwhile refer to the $25^{th}$ and $75^{th}$ percentiles, as represented by the ends of the box. Emanating from the ends of the box are lines known as whiskers, which continue until they reach the final data point within the range calculated thus:

3rd quartile + 1.5*(interquartile range)

1st quartile - 1.5*(interquartile range)

Lower and upper data points (with the exclusion of outliers) provide the endpoint of the whiskers where the data points fall short of the computed ranges.

# Chapter 3

## Effects of larval growth condition and water availability to adults on reproductive success in *Anopheles gambiae* s.s.

### 3.1. Introduction

A period of maturation known as the teneral phase occurs in mosquitoes after emergence. Of variable duration, this period allows essential development across a range of systems and processes (Briegel, 1990ab). Developments within the teneral phase include; anatomical differentiation, behavioural maturation, development of the flight muscles, the hormonal system, the digestive system, and vitellogenesis (Briegel, 1990ab). This process of maturation relies upon reserves built up in the larval phase (Briegel, 1990ab). Larval food delivery is a main fundamental for adult mosquitoes' reproduction, and that food supply may be affected by several factors such as the quality and quantity of food as well as dietary components. Nayar (1969) counts quantity of food among the factors influencing biological status in

*Ae. taeniokhynohus* mosquitoes. The amount of food at a breeding site has a strong effect on several physiological aspects. For instance, larvae and pupae live for a shorter time where the food supply is limited, and the development period is extended (Reisen, 1975), with pupation occurring later and with a smaller survival rate (Suwanchaichinda and Paskewitz, 1998). A feeding regime of just 7µg/ml/day at up to 30ml water per larva was needed to allow larvae to reach adulthood in *An. gambiae* s.s. (Sutcliffe, 2012). Aboagye-Antwi and Tripet (2010) raised *An. gambiae* s.s. females and males using different quantities of larval food to produce different adult phenotypic qualities. Naksathit and Scott (1998) reared *Ae. aegypti* females at a

range of larval densities and with varied feeding regimes to produce adult mosquitoes of different sizes.

A number of factors can impact on the number of eggs laid by gravid females, such as age and body size of the female as well as the quantity and quality of blood meals and nutrition (Clements, 1992). For example, larger female mosquitoes lived longer and laid more eggs than smaller ones after taking a single blood meal (Takken *et al.*, 1998). A significant correlation exists between female body size and the number of eggs laid, as larger females consume large blood meals and as a result, more mature eggs are laid by them (Briegel, 1990a; El-Akad and Humphreys, 1988; Naksathit and Scott, 1998). Also, large females have a heavier body weight and the ovaries carry a larger number of oocytes (Colless and Chellapah, 1960). Food availability during larval development has been shown to have a clear link with ovarian maturity at the point of adult emergence: adult *An. pharoensis* females who had not established sufficient reserves as larvae to reach the resting stage (ovarian follicles shortly after adult emergence) required more than a single blood meal before oogenesis occurred and mature eggs could be produced (El-Akad and Humphrey, 1990). Small female size and a single blood meal with little blood meant that multiple blood feeds were required for eggs to reach maturity among field –collected *Anopheles* Neotropical species such as Nyssorhynchus (Lounibos *et al.*, 1998). The feeding regime was found to impact upon the number of eggs laid, with females given human blood plus sugar laying fewer eggs than did those fed solely on human blood (Naksathit and Scott, 1998). Among non-blood fed mosquitoes, a positive correlation was suggested between wing length (body size) and the body's protein content (Van Handel and Day, 1989). However, body size does not impact upon egg viability (El-Akad and Humphreys, 1988). With age, a smaller proportion of lipids

were transferred from female body fat into oocytes, leading to reduced fecundity (Briegel, 2003).

In the majority of insect species, development, reproduction and survival are heavily dependent upon warm and moist conditions (Lindsay and and Bayoh, 2004). In the Gambia, *An. gambiae* s.l. were found to increase in terms of adult population when the humidity rose towards the end of the dry season (Jawara *et al.,* 2008). Smaller insects in particular tend to be bound by environmental factors, being unable to tolerate dry conditions. Dehydration is a problem for the smaller insect as a result of its high surface area in relation to its body mass, leading to significant evaporation of water against low storage capacity. This problem has led to three basic physiological solutions: the rate of water loss may be reduced; tolerance of water loss may be increased; or storage capacity may be increased. This last approach may take two forms: water may be stored in bulk, i.e. obtained not from catabolism but from elsewhere; or water storage may be metabolic, where the source of the water molecules is catabolism (Archer *et al.,* 2007).

The most important vectors of malaria in sub-Saharan Africa are *An. arabiensis* Patton and *An. gambiae* s.s. Giles, which are found in different climates. *An. gambiae* s.s. are found in humid areas, while *An. arabiensis* are found in arid regions (Mnzava and Kilama, 1986). Gray and Bradley (2005) demonstrated that *An. arabiensis* displayed more desiccation resistance than *An. gambiae.* At emergence, *An. gambiae* had lower body water content. However, both species displayed similar water loss rates and water content on desiccation. Death occurred at water content levels of 2mg/mg of dry mass in female mosquitoes of both species at emergence, and this became lower with age (Gary and Bradley, 2005). Three parameters are significant in governing resistance to desiccation: initial body water content, rate of water loss, and

water content at death, which occurs when the water content drops below the minimum level critical for survival. Water stored in the haemolymph and tissues forms the initial body water, which is lost via respiration, excretion, and diffusion across the cuticle (Gary and Bradley, 2005). While, through evolution, greater desiccation resistance in *Drosophila melanogaster* may be achieved through reduced rates of water loss or greater storage of bulk water, neither tolerance of dehydration nor metabolic water content may be increased (Archer *et al*., 2007). Temperature and humidity, among other environmental factors, have an impact upon the mosquito metabolism. It is predicted that mosquitoes would favour appropriate microclimatic conditions, given their susceptibility to desiccation (Kessler and Guerin, 2008)(Kessler, 2008). In a study by Huestis *et al.* (2011) conducted in a Sahelian village in Mali during the rainy season, the metabolic rates of *An. gambiae* s.s. and *An. arabiensis* were assessed. The mean metabolic rate of *An. arabiensis* was found to be higher than was measured in M-form *An. gambiae*, after the female gonotrophic status, temperature and flight activity had been accounted for (Huestis *et al.*, 2011). Once body size was also taken into consideration however, the two species were found to have a similar metabolic rate (Huestis *et al*, 2011). It can be concluded from this that mosquitoes are able to reduce their metabolic rate via behaviour and feeding patterns, with implications for survival strategies of aestivating mosquitoes in the dry season. This reduction in metabolic rate can be seen as an evolutionary response to unfavourable environments (Huestis *et al.*, 2011). Aboagye-Antwi and Tripet (2010) found little effect of either larval access to food or water availability on female water, body lipid and glycogen contents as well as wet and dry mass, under normal conditions. In situations where desiccation became a threat however, both these

factors strongly influenced physiological and metabolic parameters as well as survival rates.

So far, desiccation resistance in *An. gambiae* s.s. has not been explained in terms of its physiological mechanisms. In the present study, water availability and larval food availability (which results in variation in phenotypic quality) and their effects on female fecundity and reproductive success of the Mopti chromosomal form of *An. gambiae* s.s. under desiccation stress were experimentally investigated. Marked difference in wing length indicated variation in size or phenotypic quality. Mating cages were set up based upon these groups of contrasting phenotypic qualities. The numbers for eggs laid, eggs laying status, hatching rates, hatching status and number of larvae were recorded.

## 3.2. Materials and methods

### 3.2.1. Mosquito Colony

Samples of the Mopti laboratory strain (MRA-763) of *An. gambiae* s.s. were obtained as live eggs from the Malaria Research and Reference Reagent Resource Centre (MR4) on the 18th of July 2007 and a colony established in the Manson Insectary of the School of Life Sciences, Keele University. This M molecular strain originated from N'Gabacoro Droit, Mali and was colonized from the wild in 2003 by F. Tripet and G. Lanzaro at the University of California Davis. The colony is homozygous for the inversion 2L*a* and polymorphic for the 2R*u* inversion. For a detailed description of the procedures followed for rearing mosquitoes of the requisite phenotypes (good and poor), see section 2.2.2 of Chapter 2. The experimental work in this chapter was done with the researcher's colleague Fred Aboagye-Antwi.

### 3.2.2. Larval food availability and adult phenotypic quality

Mosquito maintenance and generation of good and poor phenotypes for the experiment described here followed that of Aboagye-Antwi and Tripet (2010). Female mosquitoes at 3-5 days old were provided with artificial horse blood, and 2 days post blood feeding, pots were provided as an oviposition site in the cages. Newly emerged first instar larvae were trayed out in groups of 200 per tray as in normal rearing. Thirty-two larval trays were used in total, with half of these utilized for each feeding regime. The feeding continued for between 6 and 10 days, and pupae were collected from each tray. The pupae from each feeding treatment were collected in two pots, good and poor. All pupae appearing after 11days from starting the feeding regimes were brought back to the stock colony. After that, pupae were separated into male and female groups (see section 2.2.7, chapter 2), and each sex group was placed into a uniquely labelled cage.

To minimize the impact of such environmental conditions as temperature, humidity and light under artificial conditions, all of which may have an impact upon larval and adult growth (Nayar, 1968, 1969; Clements, 1992), both cages and trays were moved from their location twice a day. To minimize confounding factors, the cages were labelled using a unique number and symbols, and adults were kept in separate cages to make sure that the adults were virgin. The same food spoon was used to add the larval food during the food courses for all sixteen trays to reduce any variation in the quality of the food added to each larval tray.

### 3.2.3. Determination of sex grouping of pupae

The pupae in each feeding group were separated by sex to ensure all mosquitoes remained virgin (see section 2.2.7, chapter 2). Following sex determination, mosquitoes were placed in cages labelled by sex and phenotypic quality as follows: cage1: ♀ poor, cage2: ♂ poor, cage3: ♀good, and cage4: ♂good. For each of these four categories, two

cages were filled, thus creating replicate cages for each sex-phenotype group. Thirty mosquitoes were randomly picked by removing specimens from various heights within each cage, after shaking the cage, killed by freezing at 1-3 days old, and measurement of wing length was made. These measurements were then subject to analysis to determine variation in body size between the groups from the two feeding regimes.

### 3.2.4. Set-up for mating groups and hydric stress treatments

The first part of the study involved the distribution of female mosquitoes between one and three days old into 568ml pots in groups of 360 (Table 3.1). Mosquitoes were used from each of the larval feeding regimes, and therefore from the 'poor' and 'good' phenotypes. The pots were then allocated to either conditions of hydric stress or optimum water availability, as described in section 2.2.4 of chapter 2, creating four different groups: good phenotypes with constant water availability; good phenotypes with water availability limited to 16 per 24 hours, poor phenotypes with constant water availability; and poor phenotypes with water availability limited to 16 per 24 hours. Sugar cubes *ad-libitum* were always provided. Water availability manipulation was adjusted such that it did not cause significant mortality in stressed females during the seven–day hydric stress period of the experiment (Proportional Hazard likelihood ratio: phenotpic quality: $P>0.6$; hydric stress: $P>0.4$) (Aboagy-Antwi and Tripet, 2010).

Mosquitoes were kept at 65-70% humidity, 26Cº and a 12:12 photoperiod for seven days as in the previously described maintenance conditions (see section 2.2.2, chapter 2). The larval feeding regimen was customized to create two mosquito phenotypic qualities, as mentioned in the previous section (see section 3.2.2.). For each experiment, newly emerged adults at 2-4 days old were kept in mating cages in the following order: 300 ♂good x 300 ♀ good, 300 ♀ good x 300 ♂ poor, 300 ♀ poor x 300 ♂good, 300 ♂ poor x 300 ♀ poor. The pairs in the different cages were kept together for 48 hours for mating

reasons. 60 females from each cage were then divided in two groups and transferred into pots. Of these two pots per group, one was provided with constant water in the form of a moistened cotton bud with distilled water (optimum water conditions), and the other was provided with water for only 16 hours per day (restricted water conditions as a stress condition) (see section 2.2.4, chapter 2) (Fig.3.1).

To eliminate variation, all mosquitoes were supplied with the same type of sugar and laboratory conditions were fixed. A mouth aspirator was used to transfer adult mosquitoes to combination cages to make it easier to check the sex of the adults before transferring them and to count the number of mosquito adults. Confounding effects from the environment were avoided by swapping the positions of each pot daily. The hydric stress regime was continued for seven days.

### 3.2.5. Blood meal provision and reproductive success

On the seventh day of the water availability treatment, all surviving mosquitoes were blood-fed as previously described (Aboagye-Antwi and Tripet, 2010). Two days after blood feeding, gravid females were individually placed into oviposition pots padded with moistened filter paper and cotton wool, and kept for two extra days to allow for oviposition. Following on, the egg batch for each female was counted using a tally counter. The hatching rate of eggs per female was assessed by counting the number of eggs that hatched out each day over a three-day period. Daily recordings of whether females were alive or dead were also taken. At the end of this process, surviving females from each experimental group were frozen to death, and then their wing length was measured.

### 3.2.6. Statistical analysis

All statistical analysis was conducted using the software JMP9 (SAS Institute, Inc). All data were checked for normality test using parametric or non-parametric methods. Because the data of total female fecundity and total reproductive success were found to have deviated from a normal distribution, they were analyzed using Kruskal-Wallis Tests (3+groups) and 1-Way Wilcoxon test (2 groups) to determine the difference between groups. The wing length measurement had not deviated significantly from normality. So,

*t*-Test was used to study the effect of larval feeding regimes on adult quality. Nominal Logistic Regression (Effect Likelihood Ratio (LR) Tests) was used to study the impact of hydric stress treatments on adult mortality, and egg-laying, and egg-hatching. On the other hand, general linear models were used to study the effect of hydric treatments on eggs hatching rate, and number of eggs laid and number of larvae.

Multivariate analysis (general linear models) is conducted using multiple factors and /or covariates. Statistical models were created that included all potential direct sources of variances (main factors) and their interactions in order to test their levels of significance. Non-significant interactions were then eliminated using a stepwise elimination procedure (the least significant one first, then the next, etc….). The final models included all direct effects (significant or not) as well as significant interactions but omitted non-significant interactions. In addition, the significance of replicate effects was tested in all experiments as a direct effect and interactions but is only shown in the results table when and if it was significant and it impacted on the significance of the main factors under study.

Table 3.1. The total number of hydric stress treatments (16h & 24h) by mating cages, the number of replicates per water availability treatment, and the number of mosquitoes in each pot replicate (without dead individuals, see section 3.3.2) in a study of food and water availability and impact upon mating success.

| **Replicate** | **Mating Cage** | **No. ♀ (16h)** | **No. ♀ (24h)** | **Total no. ♀** |
|---|---|---|---|---|
| 1 | Good Female + Good Male A | 23 | 25 | 48 |
| 1 | Good Female + Good Male B | 19 | 29 | 48 |
| 1 | Good Female + Poor Male A | 29 | 23 | 52 |
| 1 | Good Female + Poor Male B | 25 | 18 | 43 |
| 1 | Poor Female + Good Male A | 20 | 21 | 41 |
| 1 | Poor Female + Good Male B | 22 | 21 | 43 |
| 1 | Poor Female + Poor Male A | 22 | 22 | 44 |
| 1 | Poor Female + Poor Male B | 18 | 20 | 38 |
| 2 | Good Female + Good Male A | 22 | 20 | 42 |
| 2 | Good Female + Good Male B | 23 | 17 | 40 |
| 2 | Good Female + Poor Male A | 22 | 27 | 49 |
| 2 | Good Female + Poor Male B | 18 | 23 | 41 |
| 2 | Poor Female + Good Male A | 23 | 13 | 36 |
| 2 | Poor Female + Good Male B | 19 | 19 | 38 |
| 2 | Poor Female + Poor Male A | 21 | 20 | 41 |
| 2 | Poor Female + Poor Male B | 19 | 14 | 33 |
| **Total** | | **345** | **332** | **677** |

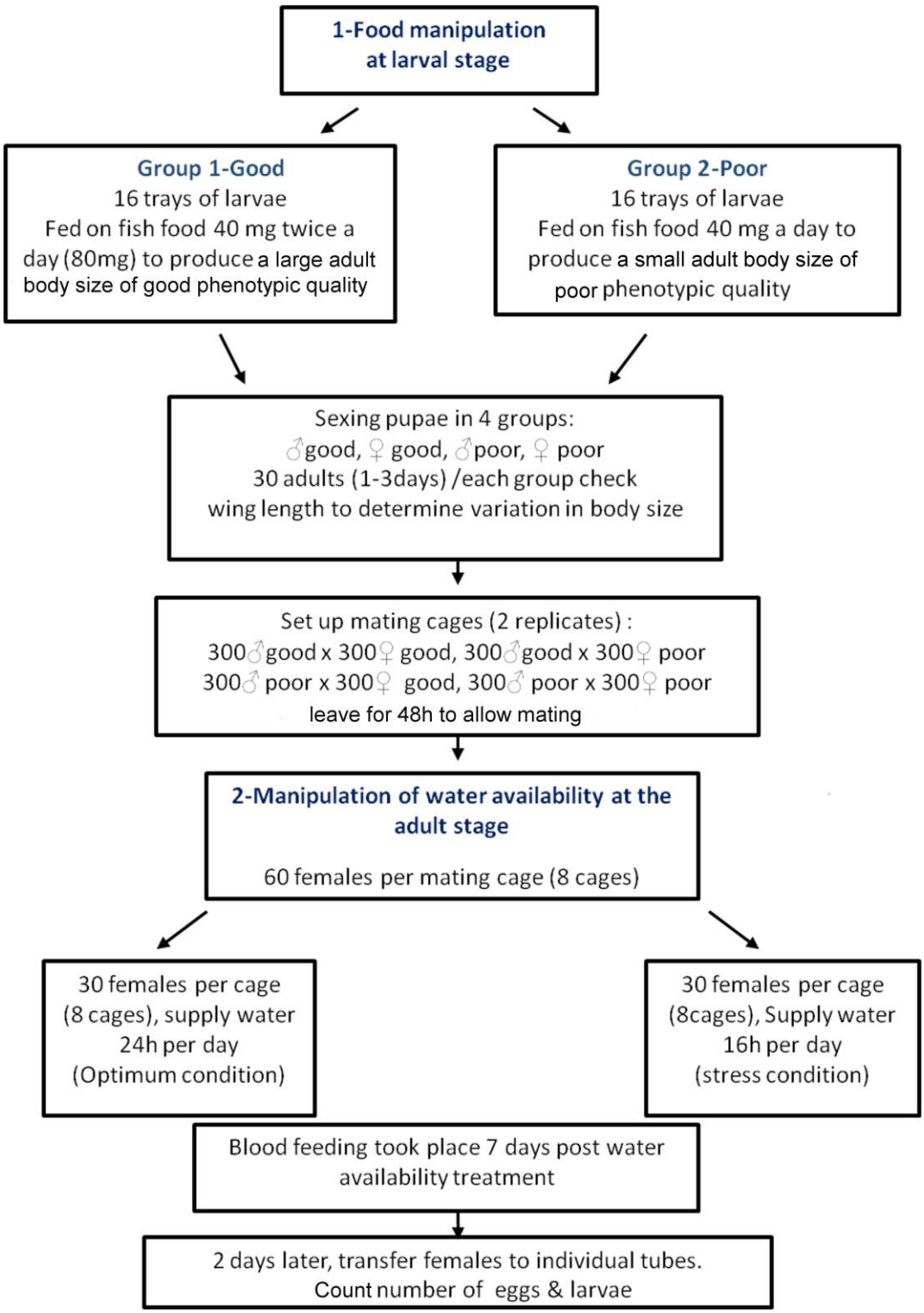


Fig. 3.1. Experimental design for the study of food and water availability and impact upon mating success

## Results

### 3.3.1. Larval nutrition and adult phenotypic quality

Two phenotypes (poor and good quality) of *An. gambiae* s.s. were produced by changing the quantity of food during the larval stages. The mean wing length was 2.89mm for poor, or small body size, females (2.80-2.98CI), compared with 3.27mm for good or large body size females (3.23-3.31CI) (*t*-Test: $n$= 68, $t$= 7.9, $P$<0.001), whereas the mean wing length of the male was 2.83mm (2.79-2.88CI) and 3.18mm (3.14-3.22CI) for poor and good males respectively (*t*-Test: $n$= 67, $t$= 11.5, $P$<0.001). Overall, the two larval feeding regimes resulted in a significant difference in the wing lengths of the two groups (*t*-test: $n$=135, $t$=12.68.70, $P$<0. 001).

### 3.3.2. Effects of hydric stress treatment on mosquito mortality

The total number of *An. gambiae* s.s. mosquito females was 960: however, 283 females died before the water regimes were started and mosquitoes had been transferred into the oviposition pots. The total number of female mosquitoes of the two phenotypes dying after hydric stress treatment was 265 out of 677 (Table 3.2, Fig.3.2). The number of deaths among mosquitoes of good phenotype was 150 for females and 147 for males, whereas for mosquitoes of poor phenotype, the number of deaths was 115 for females and 118 for males (Table 3.2, Fig.3.2).

Nominal logistic regression analysis showed that there was no significant effect from water availability treatment on the survival of female mosquito phenotypes, nor on the males mated with them (Nominal Logistic regression: $n$=677, $\chi^2$=0.42, $P$=0.517) (Table 3.3). In addition, no significant effect of female phenotype on the likelihood of mortality among females ($P$=0.258) was observed. There was, however, a significant effect of male phenotype on the mortality of male mosquitoes ($P$=0.029). Male phenotypes significantly

influence male survival and significantly interacted with female quality ($P$=0.033) (Table 3.3).

The highest percentage mortality rate for both water availability treatments was observed when good females mated with good males, at 50%, while the lowest percentage average, at 30% was when good females mated with poor males (33%) (Fig.3.3, Table3.9).

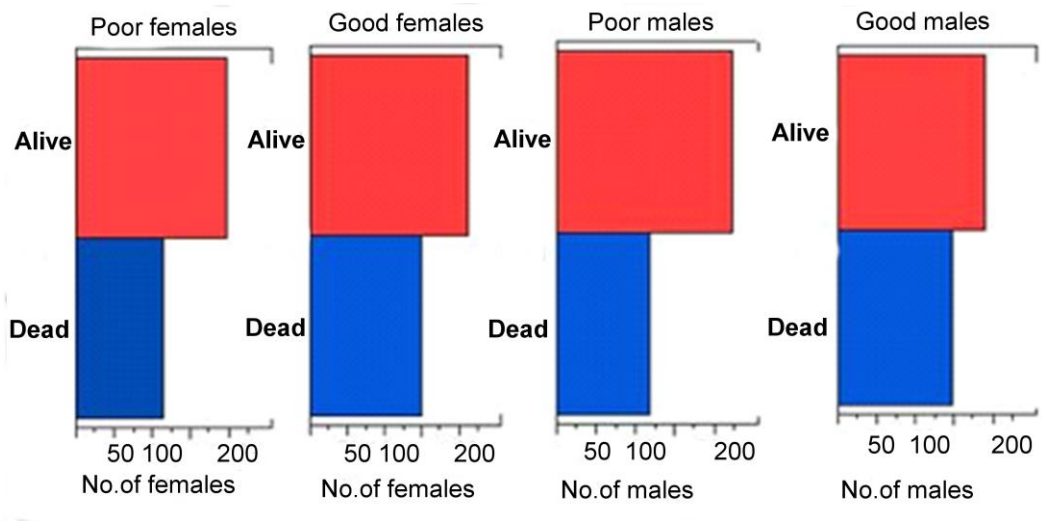


Fig.3.2. Bar plot of mortality of 677 *An. gambiae* s.s. males and females of both phenotypes (good & poor) after 7 days' hydric stress.

Table 3.2. Effect of adult female and male phenotypic quality (good and poor) on mean percentage of mortality in *An. gambiae* s.s. after 7 days of hydric stress treatment. The letters CI are indicative of the confidence interval range

| Phenotype | Sex | Alive adult | Dead adult | % Mortality | | Total |
|---|---|---|---|---|---|---|
| | | | | Alive | Dead | |
| Good | Female | 213 | 150 | 58.68 (0.53-0.63CI) | 41.32 (0.36-0.46CI) | 363 |
| | Male | 189 | 147 | 56.25 (0.51-61CI) | 43.75 (0.38-0.48CI) | 336 |
| Poor | Female | 199 | 115 | 63.38 (0.57-0.68CI) | 36.62 (0.31-0.42CI) | 314 |
| | Male | 223 | 118 | 65.40 (0.60-.70CI) | 34.60 (0.29-.39CI) | 341 |
| | Total | 413 | 265 | 61.00 (0.57-0.64 CI) | 39.14 (0.35-0.42CI) | 677 |

Table 3.3. Nominal Logistic Regression (Effect Likelihood Ratio (LR) Tests) of the effects of female and male phenotypic quality and hydric stress on adult mortality ($n$=677). Interactions are only shown where significant.

| **Source** | **DF** | **LR Chi-Square** | **Probability** |
|---|---|---|---|
| Female Phenotype | 1 | 1.27 | 0.258 |
| Male Phenotype | 1 | 4.75 | 0.029 |
| Male phenotype * Female phenotype | 1 | 4.49 | 0.033 |
| Water availability | 1 | 0.42 | 0.517 |

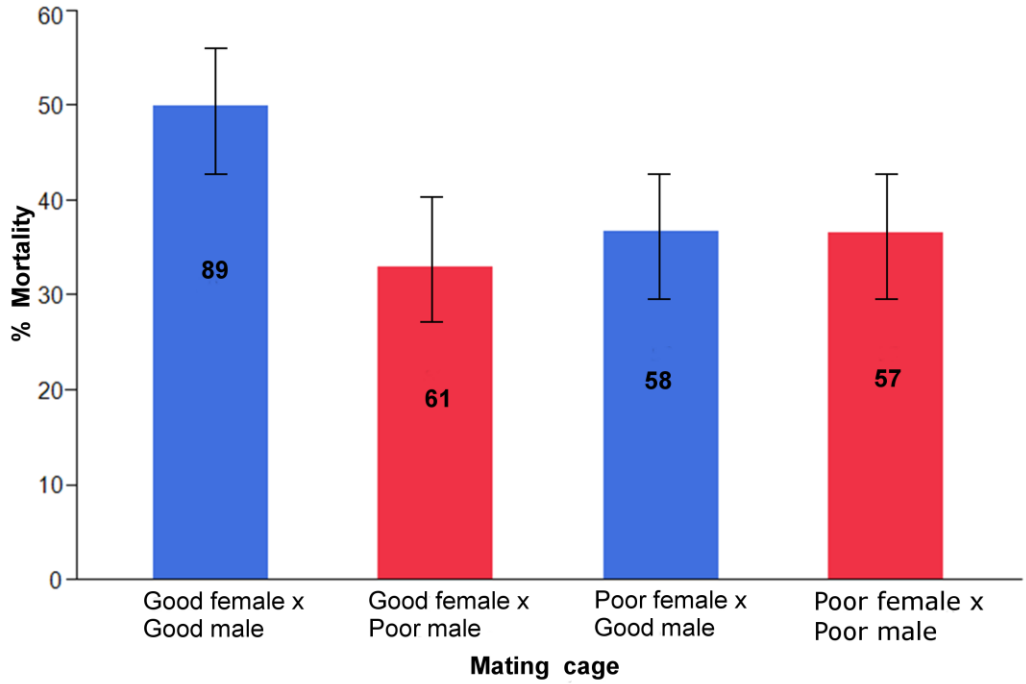


Fig.3.3. Effect of adult female and male phenotypic quality (good and poor) on percentage (±95% CI) of mortality in *An. gambiae* s.s. after 7 days of hydric stress treatment (16h & 24h) across four mating combinations: good female x good male, good female x poor male, poor female x good male, and poor female x poor male. Sample sizes are indicated for dead individuals.

### 3.3.3. Environmental stress factors and their effects on reproductive success

The following reproductive success parameters: egg laying status (ability to lay eggs); number of eggs laid; egg hatching status (whether eggs laid hatched or not); percentage of eggs hatching and number of larvae per mosquito were assessed across two mosquito phenotypes (good and poor).

#### 3.3.3.1. Importance of male and female phenotypes on egg laying, hatching status, and hatching rate.

237 out of 363 good female phenotypes laid eggs, whereas 177 out of 314 poor females laid eggs. Nominal logistic regression analysis showed that there was no

significant effect of water availability or male quality on the likelihood of egg–laying (oviposition), whilst female phenotype had a strong significant effect on egg-laying (Table 3.4).

Table 3.4. Nominal Logistic Regression (Effect Likelihood Ratio (LR) Tests) of the effects of female, male phenotypic quality and water availability on egg-laying (*n*=677).

| Source | No Parameters | DF | LR Chi-Square | Probability |
|---|---|---|---|---|
| Female Phenotype | 1 | 1 | 5.59 | 0.018 |
| Male Phenotype | 1 | 1 | 1.72 | 0.188 |
| Water availability | 1 | 1 | 0.05 | 0.828 |

Nominal Logistic Regression analysis demonstrated that hydric stress and male phenotype did not significantly affect egg hatching status. However, female phenotype did so significantly (Table 3.5).

The percentage hatching rate (number of hatched larvae/ total number of eggs*100) of 414 *An. gambiae* s.s. female mosquitoes was estimated across the four studied groups (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at two water regimes (16h & 24h) (Table 3.9). The mean percentage of eggs hatching at 16h water availability was 31.03 (26.31-35.75CI), and at 24h was 34.81 (29.84-39.77CI) (Table 3.9). The general linear model showed no significant effect of mosquito phenotypes and water availability treatment on hatching rate (Table 3.6). The highest egg-hatching rate at 16h water availability was found in the good female mated with poor male group (Table 3.9, Fig.3.4) and the lowest in the poor female mated with poor male (Table 3.8, Fig. 3.4). At 24h water availability, the highest egg hatching rate was found in poor female x good male (Table 3.9, Fig.3.4), whereas the lowest rate was in the poor female x poor male group (Table 3.9, Fig.3.4).

Table 3.5. Nominal Logistic Regression (Effect Likelihood Ratio (LR) Tests) of the effects of female and male phenotypic quality, and water availability on egg hatching (*n*= 414).

| **Source** | **No Parameters** | **DF** | **LR Chi-Square** | **Probability** |
|---|---|---|---|---|
| Female Phenotype | 1 | 1 | 4.81 | 0.028 |
| Male Phenotype | 1 | 1 | 1.9 | 0.168 |
| Water availability | 1 | 1 | 1.2 | 0.274 |

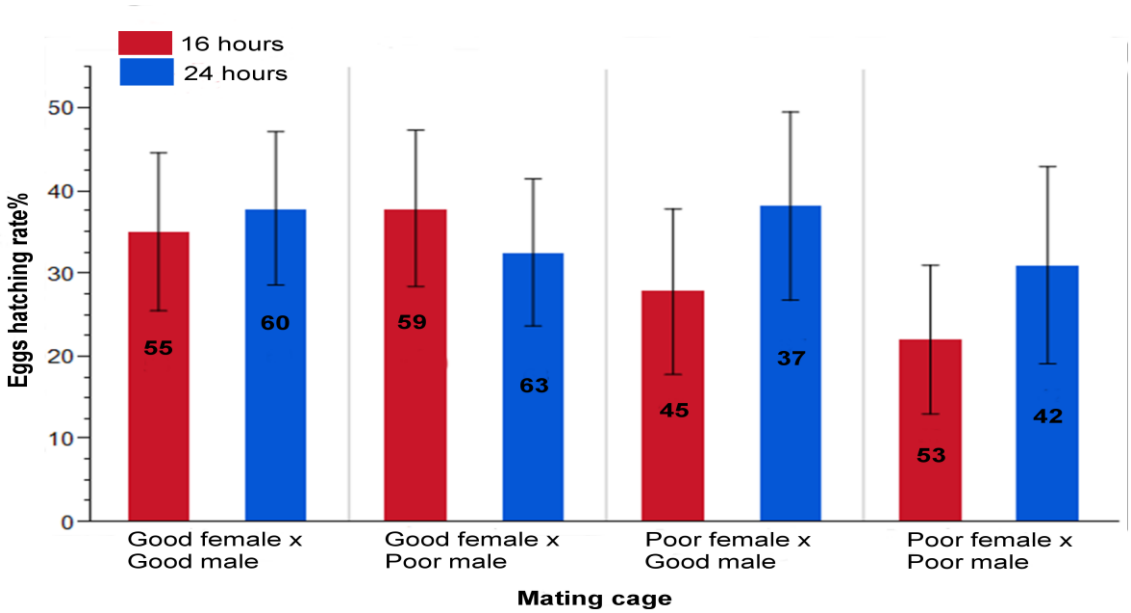


Fig. 3.4. The percentages of laid eggs (± 95% CI) which hatched shown by mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) and water availability (16h (red) or 24h (blue)). Sample sizes are indicated.

Table. 3.6. General Linear Model testing the effect of water availability and male and female phenotypic quality on egg hatching rate (*n* =414).

| Term | Estimate | Std Error | LR Chi-Square | Probability |
|---|---|---|---|---|
| Female Phenotype | 0.10 | 0.05 | 3.64 | 0.056 |
| Male Phenotype | 0.06 | 0.05 | 1.18 | 0.276 |
| Water availability | -0.07 | 0.05 | 1.60 | 0.205 |

### 3.3.3.2. Effect of water availability and mosquito phenotypes on egg numbers

The General Linear Model showed that the female phenotype had a significant effect on the number of eggs but the male phenotype did not (Table 3.7). However, considering water availability in interaction with the male phenotype made a significant effect in the same variable. The effect of male phenotypes depends on water availability (Table 3.7).

The mean number of eggs at 16h water availability was 67.19 (62.29-72.10CI). The mean number of eggs at 24h water availability was 68.02 (62.60-73.44CI). The mean numbers of eggs at 16h & 24h hydric stress across the four mating combinations was assessed as shown in Table 3.9 and Fig. 3.5.

Table. 3.7. General Linear Model testing the effect of water availability and male and female phenotypic quality on the number of eggs (*n*= 414). Interactions are only shown where significant.

| Term | Estimate | Std Error | LR Chi-Square | Probability |
|---|---|---|---|---|
| Female Phenotype | 0.17 | 0.02 | 40.30 | <0.001 |
| Male Phenotype | -0.04 | 0.02 | 3.22 | 0.072 |
| Water availability | 0.017 | 0.025 | 0.43 | 0.507 |
| Water availability*Male Phenotype | 0.07 | 0.02 | 7.52 | 0.006 |

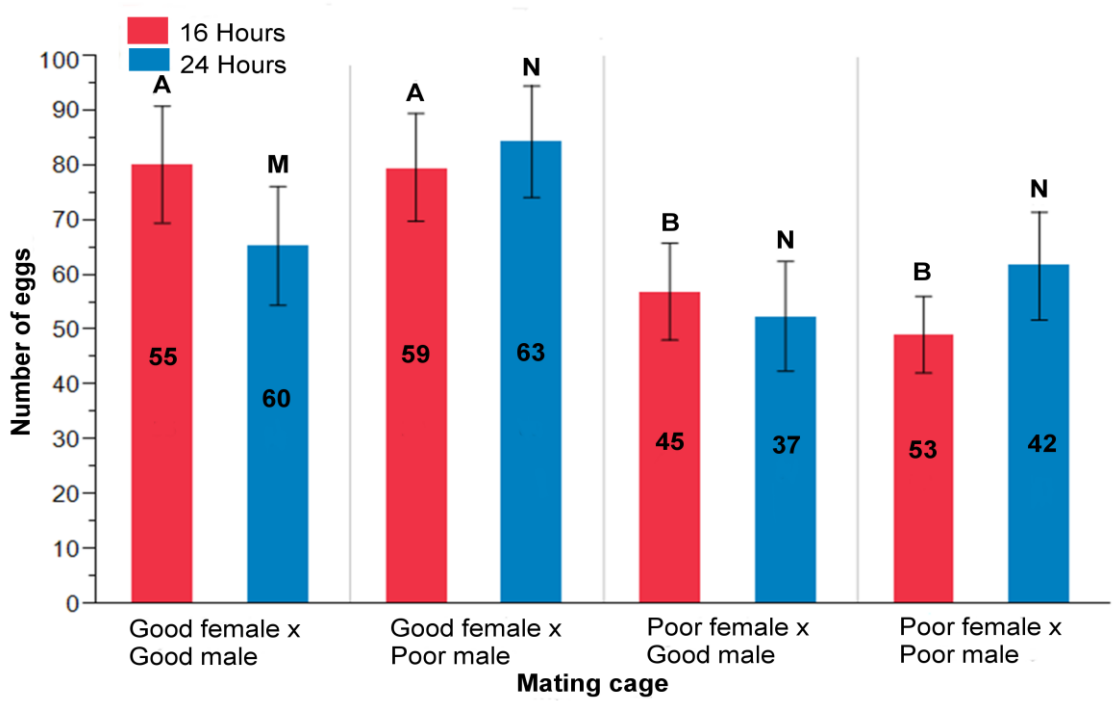


Fig.3.5. Number of eggs laid by productive groups (± 95% CI) shown by mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) and water availability (16h (red) or 24h (blue)). Bars labelled with different letters (A & B for 16h water availability; N & M for 24h) were significantly different (Tukey *P*< 0.05). Sample sizes are indicated

### 3.3.3.3. Impact of water availability and male and female phenotypic quality on the number of larvae

The General Linear Model revealed that there was no significant effect of water regime and male phenotype on the number of larvae, while the quality of the females had a significant influence (Table 3.8). The mean number of larvae at 16h water availability was 44.87 (39.54-50.21CI) (Table 3.9, Fig.3.6). The mean number of larvae at 24h water availability for the same groups was 46.35 (40.33-52.36CI) (Table 3.9, Fig.3.6).

Table 3.8. General Linear Model (Effect Likelihood Ratio (LR)) testing the effect of water availability and male and female phenotypic quality on the number of larvae (*n*=414).

| Term | Estimate | Std Error | t-ratio | Probability |
|---|---|---|---|---|
| Female Phenotype | 8.52 | 2.02 | 4.20 | <0.001 |
| Male Phenotype | 0.66 | 1.97 | 0.34 | 0.735 |
| Water availability | -0.80 | 1.96 | -0.41 | 0.681 |

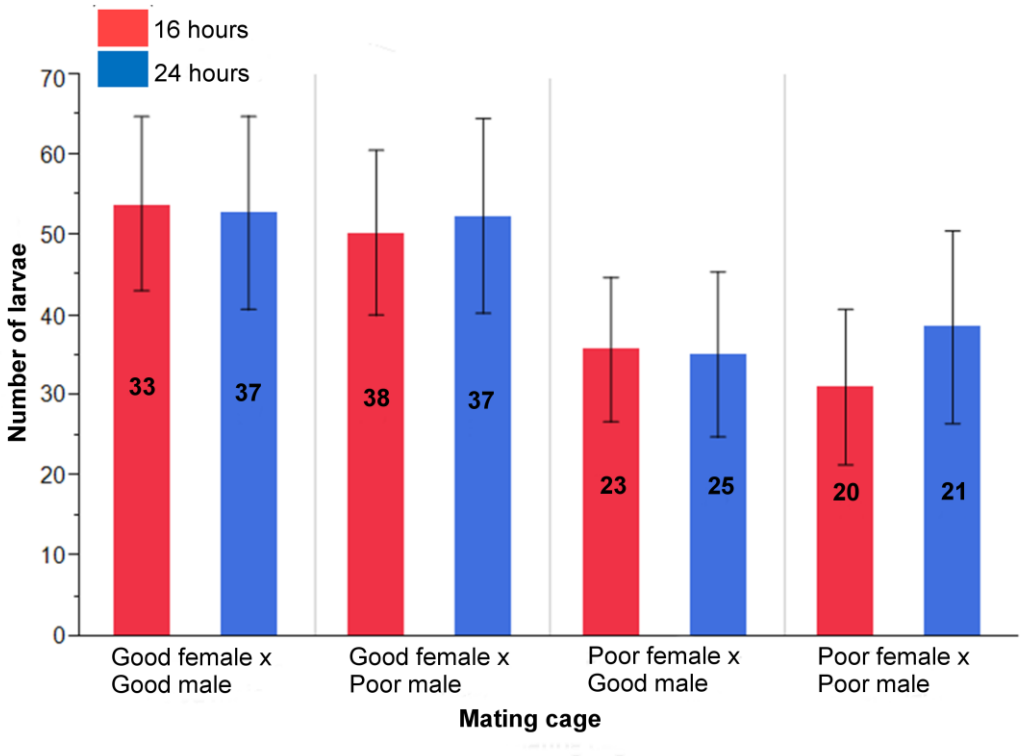


Fig. 3.6. Number of larvae hatched (± 95%CI) shown by mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) and water availability (16h (red) or 24h (blue)). (Females from groups where eggs were not produced/did not hatch are not included). Sample sizes are indicated.

Table 3.9. Effect of mosquito phenotypic quality (good and poor) across four mating combinations and water availability (24h access and 16h access limited) on mean values of parameters of reproductive success in *An.gambiae* s.s. after 7 days hydric stress treatments. Values in brackets are absolute figures out of the total assessed, while those marked with the letters CI are indicative of confidence interval range

| Mating combination | Water availability | Adult mortality % (no. of dead adults/total) | Eggs laying (oviposition)% (no. of eggs laid/total) | Hatching status (No. of hatched eggs / total eggs) | Hatching rate% | No. of eggs laid (mean ± CI) | No. of larvae (mean ± CI) |
|---|---|---|---|---|---|---|---|
| good female x good male | 16h | 49.42 (0.39-0.59CI) (42/87) | 63.22 (0.52-0.72CI) (55/87) | 60.00 (0.46-0.71CI) (33 /55) | 35.03 (25.86-44.21 CI) | 80.05 (71.08-89.02CI) | 53.75 (44.23-63.28CI) |
| | 24h | 50.55 (0.40-0.60CI) (46/91) | 65.93 (0.55-0.74CI) (60/91) | 63.33 (0.50-0.74CI) (38/60) | 37.85 (28.71-46.99 CI) | 65.20 (55.65-74.74 CI) | 52.62 (41.95-63.29CI) |
| good female x poor male | 16h | 28.72 (0.20-0.38CI) (27/94) | 62.77 (0.52-0.71CI) (59/94) | 64.41(0.51-0.75CI) (38/59) | 37.84 (28.99-46.70 CI) | 79.45 (70.80-88.11CI) | 50.10 (41.22-58.98CI) |
| | 24h | 37.36 (0.28-0.47CI) (34/91) | 69.23(0.59-0.77CI) (63/91) | 58.73(0.46-0.70 CI) (37/63) | 32.52 (23.60-41.44 CI) | 84.26 (74.95-93.58 CI) | 52.27 (41.60-62.94 CI) |
| poor female x good male | 16h | 36.90 (0.27-0.47CI) (31/84) | 53.57 (0.42-0.63CI) (45/84) | 51.11(0.37-0.65 CI) (23/45) | 27.83 (17.96-37.97 CI) | 56.82 (46.90-66.73CI) | 35.60 (24.19-47.02 CI) |
| | 24h | 36.49 (0.26-.047CI) (27/74) | 50.00 (0.38-0.61CI) (37/74) | 67.57 (0.51-0.80CI) (25/37) | 38.12 (26.48-49.76 (CI) | 52.32 (40.16-64.48 CI) | 34.96 (21.98-47.94 CI) |
| poor female x poor male | 16h | 37.50 (0.27-0.48CI) (30/80) | 66.25 (0.55-0.75CI) (53/80) | 37.74 (0.25-0.51CI) (20/53) | 22.01 (12.66-31.35 CI) | 49.01 (39.88-58.15 CI) | 30.95 (18.71-43.18CI) |
| | 24h | 35.53 (0.25-0.46CI) (27/76) | 55.26 (0.44-0.65CI) (42/76) | 50.00 (0.35-0.64CI) (21/42) | 30.97 (20.05-41.89 CI) | 61.52 (50.11-72.93 CI) | 38.42 (24.26-52.59 CI) |
| Total | 16h | 37.68 (0.33-0.43CI) (130/345) | 61.45 (0.56-0.66CI) (212/345) | 53.77 (0.47-0.60CI) (114/212) | 31.03 (26.31-35.75 CI) | 67.19 (62.26-72.10 CI) | 44.87 (39.54-50.21 CI) |
| Total | 24h | 40.36 (0.35-0.45CI) (134/332) | 60.84 (0.55-0.65CI) (202/332) | 59.90(0.53-0.66 CI) (121/202) | 34.81 (29.84-39.77 CI) | 68.02 (62.60-73.44 CI) | 46.35 (40.33-52.36 CI) |

### 3.3.4. Impact of water availability and adult phenotypic quality on total fecundity

Total fecundity, i.e. the number of eggs produced by all females, including those which did not lay eggs ($n$= 677), was compared between the mating combinations and for the two water availability regimes. Because the data for each group deviated significantly from a normal distribution (Shapiro-Wilkinson: $P$<0.05 in most cases), non-parametric analyses were used to compare all groups. The median (25-75 quartiles) number of eggs of all 677 females was 27 (0-78), and egg numbers ranged from 0 to 164.

Significant differences in the numbers of eggs were found between the four groups at reduced water availability (Kruskal-Wallis: $n$=345, $df$= 3, $\chi^2$= 12.09, $P$=0.007) and with water always available (Kruskal-Wallis: $n$= 332, $df$= 3, $\chi^2$= 19.43, $P$=0.002) (Fig. 3.7, 3.8). The median (25-75 quartiles) number of eggs laid by 314 small females was 16 (0-60), while the median (25-75 quartiles) number of eggs in 363 large females was 44 (0-96).

Pair-wise comparisons between the four studied groups at reduced water availability showed a significant difference between the number of eggs laid by small females and large females ($P$<0.039 in all cases) (Fig.3.7). The median (25-75 quartiles) number of eggs laid by 164 small females at 16h hydric stress was 24 (0-59.5), whereas the median egg number (25-75 quartiles) on the same water treatment of 181 large females was 50 (0-93).

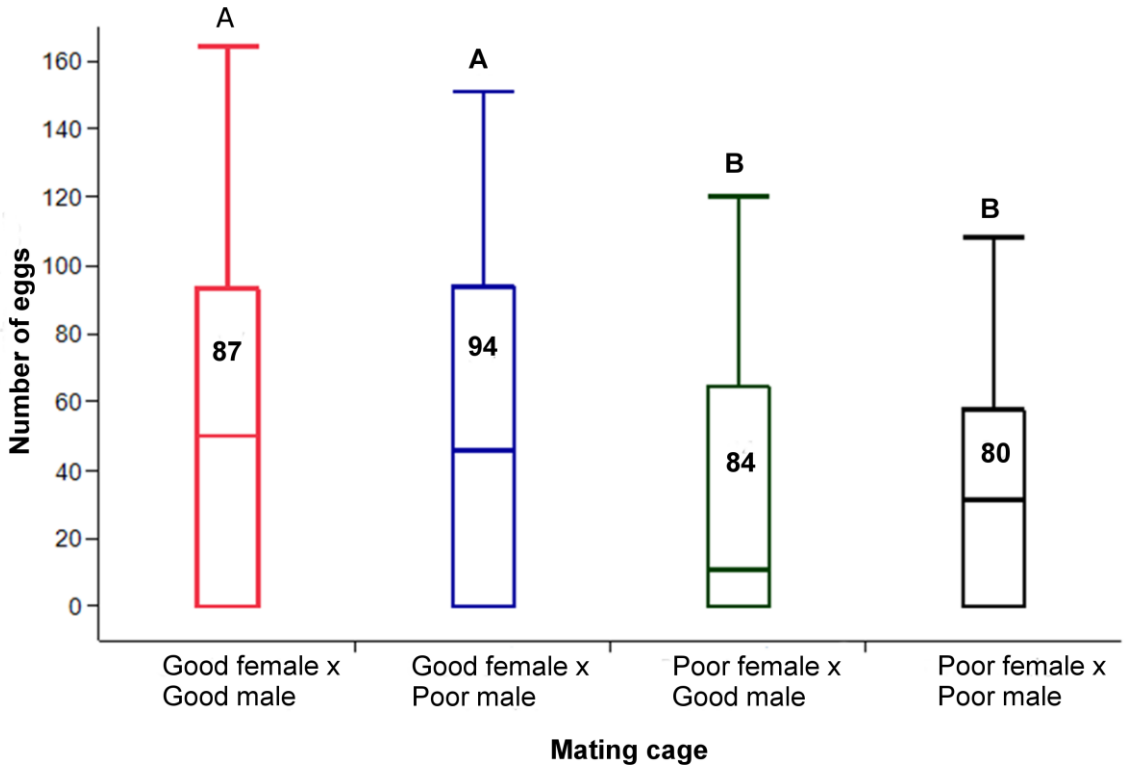


Fig.3.7. Boxplot of the number of eggs of all females (productive or not) in four mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at 16h water availability. The number of eggs followed a non-normal distribution: therefore the bars represent the median. Boxes are interquartile range (75 - 25%). Boxplots labelled with different letters differed significantly (Wilcoxon, $P<0.001$). The whiskers display upper and lower parameter values, excluding outliers. Sample sizes are indicated.

Pair-wise comparisons between the four studied groups at 24h hydric stress showed a significant difference between poor and good females ($P$<0.001 in all cases) (Fig.3.8). The median (25-75 quartiles) number of eggs laid by 150 small females provided with 24h water was 8 (0-61.5), whereas for 182 large females at the same water treatment, it was 42 (0-101).

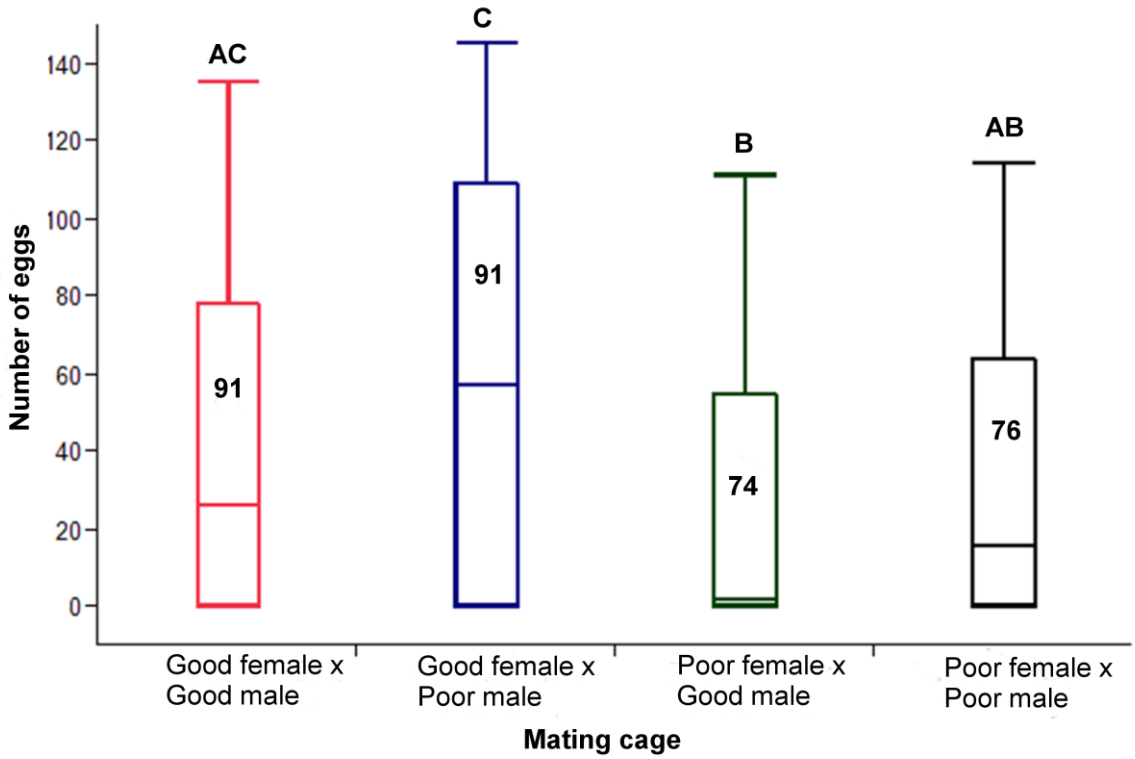


Fig.3.8. Boxplot of the number of eggs of all females (productive or not) in four mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at 24h water availability. The number of eggs followed a non-normal distribution: therefore, the bars represent the median. Boxes are interquartile range (75 - 25%). Boxplots labelled with different letters differed significantly (Wilcoxon, $P$<0.001). The whiskers display the upper and lower parameter values, excluding outliers. Sample sizes are indicated.

### 3.3.5. Impact of water availability and male and female phenotypic quality on total reproductive success

The mosquitoes' total reproductive success was assessed by counting the number of offspring across the four studied groups (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at the two watering regimes (16h & 24h). Because the data for each group deviated significantly from a normal distribution (Shapiro-Wilkinson: $P<0.05$ in most cases), non-parametric analyses were used to compare all groups (Shapiro-Wilkinson: $n= 677$, W= 0.625, $P<0.001$). The median (25-75 quartiles) number of offspring in 677 females for the same mosquito groups was 0 larvae (0-23.5) and larvae numbers ranged from 0 to 126.

Overall, a significant difference in the number of offspring was found across the four groups under the 16h watering regime (Kruskal-Wallis: $n=345$, $df= 3$, $\chi^2= 10.36$, $P=0.015$), but there was no significant difference at 24h water availability ($n= 332$, $df= 3$, $\chi^2= 6.077$, $P=0.107$). The median (25-75 quartiles) number of offspring in 314 small females was 0 (0-4.25), whereas in 363 large females it was 0 (0-39).

Pair-wise comparisons between the four studied groups at 16h hydric stress showed a significant difference in the number of progeny between small and large females across the four studied groups ($P<0.010$ in all cases) (Fig.3.9). The median (25-75 quartiles) number of offspring at 16h hydric stress in 164 small females was 0 (0-2), while in 181 large females at the same hydric stress treatment, it was 0 (0-41.5).

Pair-wise comparisons between the four studied groups at 24h hydric stress showed a significant difference in the number of offspring between poor and good females across the four studied groups ($P<0.040$ in all cases) (Fig.3.10). The median (25-75 quartiles) number of offspring at 24h water availability was 0 (0-6.25) in 150 small females, whereas it was 0 (0-35.5) in 182 large females.

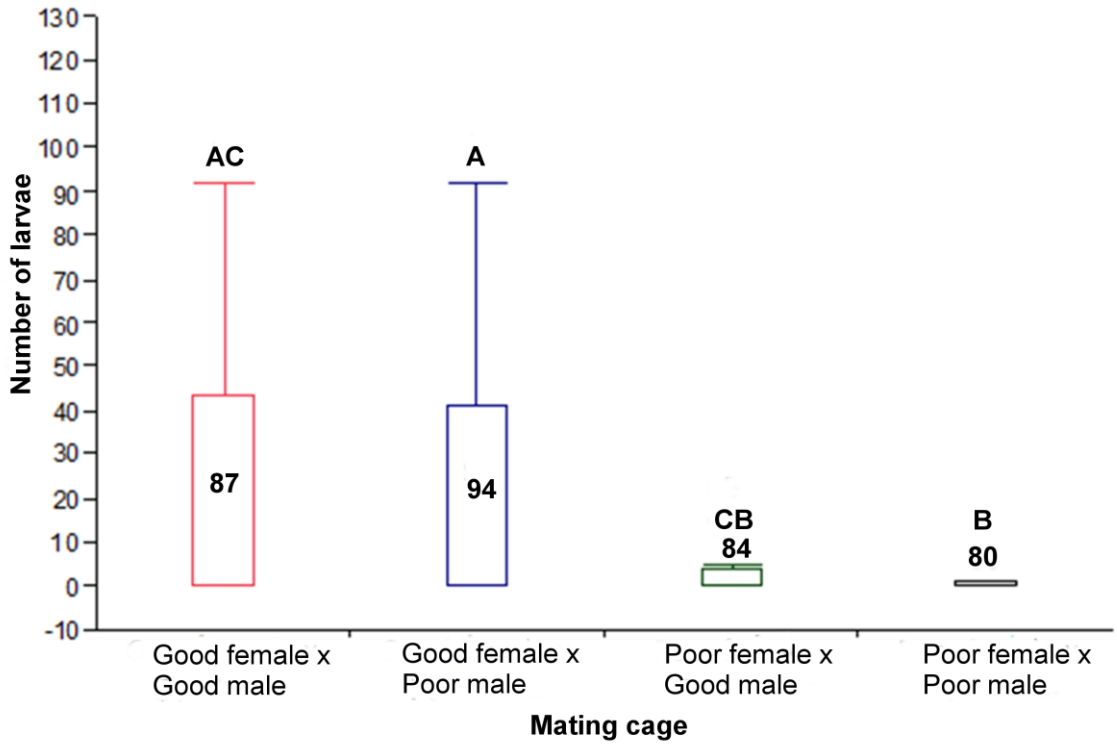


Fig. 3.9. Boxplot of the number of offspring of all females in four mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at 16h water availability. The number of offspring followed a non-normal distribution: therefore, bars represent the median. Boxes are interquartile range (75 - 25%). Boxplots labelled with different letters differed significantly (Wilcoxon, $P<0.001$). The whiskers display upper and lower parameter values, excluding outliers. Sample sizes are indicated.

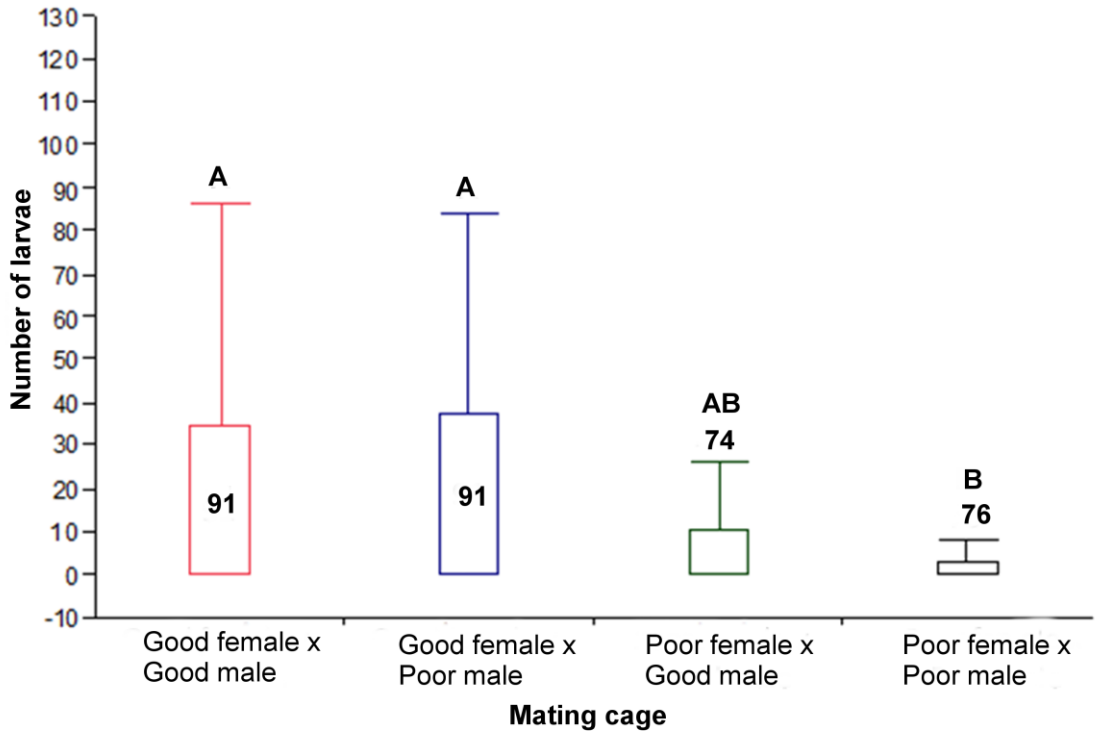


Fig.3.10. Boxplot of the number of offspring of all females in four mating combinations (good male x good female; good female x poor male; poor female x good male and poor male x poor female) at 24h water availability. Number of offspring followed a non-normal distribution: therefore, bars represent the median. Boxes are interquartile range (75 and 25%). Boxplots labelled with different letters differed significantly (Wilcoxon, $P<0.001$). The whiskers display upper and lower parameter values, excluding outliers. Sample sizes are indicat.

### 3.4. Discussion

The impact of larval food and water availability at different levels as an environmental stress on adult mosquitoes was investigated in terms of all features of reproductive success. Larval food availability had an impact on adult size and almost all aspects of reproductive success in females. This indicates a link between reproductive success and female phenotype quality, which in turn, is shown to be connected to environmental conditions. On the other hand, no direct link was observed between reproductive success and the availability of water before the blood meal was taken, it interacted with other factors to affect some of the parameters studied. Mortality prior to oviposition was not affected by any of the factors studied.

The present study revealed a significant difference in body size (based on wing length) related to differing food regimes, with the poor regime producing substantially smaller adults and the good regime creating larger adults across both sexes, the food regime being the determining factor in relation to body size, as reported in other studies (Briegel 1990b; Takken *et al*., 1998; Aboagye-Antwi and Tripet, 2010; Cator *et al.*, 2010). Mosquito larvae accumulate 80-90% of growth and biosynthesis during the fourth and last larval instar, to avoid producing small size adult (Briegel, 2003). Adult mosquitoes with small body size have fewer metabolic reserves. According to Briegel (1990a and 2003), a positive relationship exists between body size and the total amount of protein, glycogen and lipids. The teneral reserves into adulthood, which is a critical feature for adult phenotypic quality, depends on food availability during the larval stage.

The present study demonstrates that there is no statistical effect of water availability regimes and female phenotypes on pre-oviposition mortality. Male quality, however, affected male survival significantly. Maciel-De-Freitas *et al.* (2007) report that food availability during larval stages did not affect either adult male or female survival in *Ae. aegypti* under laboratory conditions, where specimens displayed low levels of mortality irrespective of size. However, in the field, food had a great influence on male survival: larger body size was linked to greater longevity, although the same was not found in females. In fact, females of both phenotypic qualities (small and large) had an overall daily survival rate which was significantly higher than males of both sizes (Maciel-De-Freitas *et al.*, 2007). Moreover, these authors suggest that this may have been a result of the impact of environmental factors such as humidity and temperature on adult survival. Briegel (1990a) demonstrated that the caloric distribution in small *An. gambiae* females was almost 10 times less than that of large females, at approximately, 0.2 cal and 1.95 cal respectively. Meanwhile, caloric content in small females was 30% protein and 43% lipid, whereas in the large females, the content altered to 72% protein and 21% lipid. This increased proportion of lipids may act to counter any difference in the impact of restricted water availability among the two phenotypes by increasing the capacity of smaller mosquitoes to cope with such conditions. Lipid levels in females of poor phenotype may be increased through an increased tendency to take a sugar meal before seeking a blood meal (Briegel *et al.*, 2001; Briegel, 2003). The sugar solution intake by female adult mosquitoes before a blood meal is taken, and the teneral reserve accumulated during larval stages determine female mortality (Briegel, 2003). Females accumulate fat from sugar meals and utilize this as a teneral reserve to allow for greater longevity (Van Handel, 1984). Aboagye-Antwi and Tripet (2010) established that there was no significant variation in glycogen content and dry mass in small and large *An. gambiae* s.s. females.

This was explained by the proposition that small females consumed more sugar meals and drank more water to cope with a shortage of energy reserves. Also, the same authors reported that poor phenotypic quality females stored more total body lipid reserves than larger females.

The pre-oviposition stage of the egg laying process encompasses both behaviours related to reaching a possibly location for oviposition and those associated with site selection, while oviposition encompasses stages related to the physical laying of eggs, including ovulation (Clements, 1999). The present study confirmed the effect of female phenotypic quality on oviposition by gravid females. *An. gambiae* females' selection of an oviposition site depends on a number of environmental factors. For example, the density of larvae and water types and temperature influence site selection (Sumba *et al*, 2008; Impoinvil *et al.*, 2007). The cycle of day and night may, however, exert greater influence over oviposition than do conditions at a particular site (Sumba *et al.*, 2004). Other signals influencing site selection in *An. gambiae* s.s. include volatiles, indicating the presence of particular microbes (Sumba *et al.*, 2004), as well as a larval pheromone and a larval contact deterrent from *An. gambiae* s.s. (Sumba *et al.*, 2008). Egg laying or oviposition by gravid females mostly depends on biotic (presence of larvae in oviposition site) and abiotic (temperature, light, quality of oviposition water) environmental factors as mentioned earlier. The impact of phenotype displayed in the parent, however, remains unexplored. In the current study, all conditions were kept constant apart from water availability and parental phenotype.

The current work confirmed the effect of female phenotypic quality on oviposition by gravid females, where females of good phenotypic quality laid significantly more eggs. One explanation for such a finding is that large females in *An. gambiae* s.s. stored more proteins, lipids and glycogen so had more rapid ovarian maturation compared with smaller

females (Klowden and Chambers, 1988). Another study by Klowden and Russell (2004) found that the amount of blood ingested was directly related to the likelihood of eggs maturing. The blood meal size affected the oocytes' development as increased maturation of oocytes in large females was related to a larger blood meal (Takken *et al*, 1998). The occurrence of oogenesis was limited by size, as it was observed that this only took place in females with a wing length of over 2.8mm in *An. gambiae* s.s. after one blood meal (Fernandes and Briegel, 2005). An additional explanation is that small females have small ovaries with limited ovarioles compared with larger females (Briegel, 1990a)

Results in the present work indicated that there was no significant effect of mosquito phenotypic quality of either sex, nor of limited access to water, on egg hatching rate. This evidence is simply explained by the notion that egg hatching depends on water quality and the temperature of the oviposition site, as time taken for hatching is influenced by the chemical factors present in the water (Yaro *et al.*, 2006a; Sumba *et al.*, 2008; Impoinvil *et al.*, 2007). Hatching may also be influenced by site conditions including flooding, temperature, competition, the amount of moisture in the soil and possibility of any attack upon larvae as they hatch (Koenraadt *et al*., 2003; Yaro *et al*., 2006a; Impoinvil *et al*., 2007). Egg hatching and hatching rates may be further affected by the intrinsic rates of fertilization and semi-sterility due to genetic mutations (Clements, 1992; Lehmann *et al*., 2006). Male and female parental effects may variously impact egg hatching. However, both water and food availability at the larval stage are unexplored in terms of their impact upon egg viability.

Both genetics and environment are held to play a role in the quantity of eggs which female mosquitoes lay (Hogg *et al*., 1996; Lehmann *et al*., 2006). Numbers of ovarian follicles were observed by Arrivillaga and Barrera (2004) to be restricted in high-temperature, water-limited conditions, with an associated decrease in egg production.

According to Rua *et al.* (2005), high temperature led to a reduction in oocyte development time and the gonotrophic cycle in *An. albimanus.* Moreover, large adult females in *An. gambiae* s.s. are more fecund than small females (Briegel, 1990a; Takken *et al*, 1998). A partial explanation for this is found in the greater availability of reserves: good phenotypic quality adults are produced as a result of the teneral reserves created by plentiful food and good conditions during the larval stage (Briegel, 1990a; Briegel *et al*., 2001). An additional reason may be found in the increased blood feeding capacity of larger individuals, such that Takken *et al*. (1998) consider that sufficient blood to complete the first gonotrophic cycle may be taken by large females in a single meal, producing more eggs than their smaller counterparts who must often ingest a further blood meal before the cycle is complete (Takken *et al*., 1998). The current study supports the finding of some previous studies (Briegel, 1990a; Takken *et al*., 1998; and Briegel *et al*., 2001) that egg batch size was primarily dependent upon female phenotype. The numbers of eggs thus seem reliant upon reserves held by the adult female, which acquire even greater importance where moderate environmental stressors are present.

Phenotypic quality in the female was also influential upon the numbers of larvae produced, as may be expected, considering its impact upon other aspects of reproductive success. Thus, adequate food availability for females at the larval stage led to increased numbers of offspring hatching, and this is also supported by Briegel (1990a) and Takken *et al*. (1998). By contrast, when male phenotypic quality was examined as a factor in the numbers of larvae produced, the impact appeared minimal, as supported by Lehmann *et al*. (2006).

When the availability of water is viewed in conjunction with male phenotype in terms of impact upon egg batch size, a complex picture emerges. Where females are provided with ideal water conditions, mating with poor quality phenotype males appears to

produce a considerably greater numbers of eggs. Because male fitness has largely been measured in terms of mating success and the speed with which mating is achieved (Lehmann *et al*., 2006), presumably males of poor phenotypes are more agile in flight and might invest resources in order to optimise the egg number in a single mating (Ng'habi *et al*, 2008). Ng'habi *et al*. (2008) found mating success of male *An. gambiae* s.s. not to be a function of their body size but rather dependent on their size compared to the females. That would mean that poor phenotype males would have an advantage over males of good phenotypic quality if females were predominantly of poor phenotypic quality. However under natural conditions, large males may be more likely to be at an advantage to mate with virgin females, considering the fact that well fed larvae have shorter development time and are the first to emerge as adults (Briegel, 2003). Lehmann *et al*. (2006) consider the greater opportunities for mating afforded to the faster emerging males to be the primary point of advantage for males of good phenotypic quality, rather than any physical differences. Irrespective of this view, good male phenotypic quality was linked to greater reproductive success for the female, and for good quality female phenotypes especially. The expectation is that testes will be smaller with smaller males, and that fewer and less viable sperm will be produced. It is important to consider which features, in fact, characterise high quality sperm in mosquitoes, and here the picture is unclear. Voordouw *et al*. (2008) found that shorter sperm correlated with greater reproductive success, in direct contrast to Helinski and Knols (2009), who found that this was correlated with a lower success rate. Moreover sperm length was not linked by Voordouw *et al*. (2008) to body size nor were viable sperm found to play a significant role in either oviposition or egg batch size (Thailayil *et al*., 2011). It appears that under limited water availability, other factors override any impact of male phenotypic quality, possibly due to females making different strategic choices under the influence of stress.

## Conclusion

The following reproductive success parameters: egg laying status (ability to lay eggs); number of eggs laid; egg hatching status (whether eggs laid hatched or not); the percentage of eggs hatching; and the number of larvae per mosquito were assessed across two mosquito phenotypes (good & poor) at two watering regimes. Good phenotypic quality in the female had a significant effect on almost all parameters of reproductive success, while male phenotypic quality was less important to overall reproductive success. The current study demonstrates that there is no statistical effect of water availability regimes on almost all studied reproductive success parameters. However, when the availability of water is viewed in conjunction with male phenotype in terms of impact upon egg batch size, a complex picture emerges. The present work has established the interaction of environmental factors of stress with mosquitoes' phenotypic quality to influence reproductive success in malaria vector *Anopheles gambiae* s.s. Mopti strain.

# Chapter 4

# Impact of colonization and genetic modification on mating behaviour and reproductive success in *An. gambiae* Sensu Stricto: Test of inbreeding depression hypothesis

## 4.1. Introduction

*An.gambiae* is a vector of malaria that is responsible for more than one million deaths around the world, the majority of which take place in sub-Saharan Africa ((WHO), 2010). Conventional methods minimize contact with mosquitoes by using treated mosquito nets, anti-malaria drugs or by using insecticides (WHO, 2010). These methods protect 10% of the population at risk of malaria in sub-Saharan Africa (WHO, 2010). However, mosquitoes develop resistance against insecticides. In addition, insecticides often have harmful side effects for the environment (Knipling *et al.*, 1968; Crampton *et al.*, 1994). Use of traditional methods has been very successful in controlling malaria, however the potential for emerging resistance is large. Therefore, scientists need to devise new strategies to manage malarial disease with less harm to environment. Since 1962, sterile insect techniques (SITs) have been successfully used in a range of species: these techniques are based upon the concept of altering a population at the genetic level in order to control it (Knipling *et al*., 1968). This general approach includes techniques based upon; cytoplasmic incompatibility, meiotic drive distorted sex ratio, lethal factors, hybrid infertility and mosquito sterilization, whether by means of radiation or chemicals (Knipling *et al*., 1968). Each of these strategies has as its objective the prevention of reproduction within a mosquito population, for example by the release of sterile males into the field (Knipling *et al*., 1968).

SIT techniques include two methods. Firstly, a sufficient number of sterile males are raised under laboratory conditions and released in the field. Secondly, chemosterilant

agents are spread over a natural population, which induces sterility in wild individuals (Knipling *et al*., 1968). Sterile Insect Techniques (SITs) have been implemented both in the laboratory and in the field for the purposes of insect controls (Benedict and Robinson, 2003). For SIT to be successful, the method depends on the ability of males to mate with wild females, which as a result, stop producing offspring. This relies on males transferring sterile sperm and accessory gland secretions to wild females to induce usual post-copulation behaviour in the female, which in *An. gambiae* implies that the female will not mate further, as in this species females only mate once (Catteruccia *et al.*, 2009). The ability of males to mate and transfer sperm to wild females in mating swarms (Charlwood and Jones, 1980) is critical for the success of such approaches. It is thus very important to the further understanding of reproductive ecology and physiology in Anophelines. SIT has various well-known technical problems which have previously contributed to the termination of mosquito releases in the field: such problems include low male fitness, below desired levels of sterile insects due to the absence of sexing strains or hold-ups in production, and migration of the sterile insects from their release regions. Thus, these problems demand attention if SIT is to be improved in terms of its success (Benedict and Robinson, 2003).

Mosquito control methods based on the use of transgenesis to produce sterile insects should consider the effects of this approach on male reproductive fitness, which should ideally be comparable to that of wild males. Therefore, understanding the genetic and physiological bases of reproduction in *Anopheles* mosquitoes should form a fundamental point of future scientific research (Catteruccia *et al*., 2009). Transgenic mosquitoes may lose fitness as a result of gene insertion and expression, and due to inbreeding impact. Catteruccia *et al.* (2003) investigated factors influencing fitness under laboratory conditions with four lines of transgenic *Anopheles stephensi*, a vector species of human

malaria. They found a direct negative effect of the inserted of exogenous gene in at least 3 out of 4 lines, and in addition report a noticeable impact of inbreeding involved in the creation of transgenic homozygotes. Also, the frequency of the transgenic allele in the population declined sharply over time, dying out in between 4 and 16 generations (Catteruccia *et al*., 2003).

Established laboratory colonies are essential for scientific research, but maintaining insects under laboratory colonization long-term leads to significant problems. For example, keeping insects in a small population as in a colony leads to loss of genetic variation, or to individuals becoming homogeneous (Craig 1964). When insects are transferred from the field and reared under laboratory conditions, extreme selection and rapid change in the gene pool is caused (Craig 1964). Also, colonization for several years causes changes in gene frequency (Craig 1964). Inbreeding may be used to achieve uniformity of genes: in mosquitoes this can be achieved through single pair sibling mating continued successively, which by the $20^{th}$ generation should have achieved homozygosity in 99 % of gene pairs (Craig 1964). There is evidence to suggest that environmental buffering is decreased by inbreeding, leading to significant differences in lifespan, length of development and response to toxic substances within groups of identical genotype (Craig 1964). Additionally, homozygosity as a result of inbreeding has an impact on individual differences in gene expression (Fowler and Whitlock, 2002). Competitiveness among males is also impacted by genetic drift, population bottlenecks and selection (Benedict *et al.*, 2009).

Considering the positive impact of hybridism for many traits, one useful approach for the laboratory mosquito colony would be the use of crossbreeding (Benedict *et al.*, 2009). In fact, Menge *et al*. (2005) achieved increased adult size in *An. gambiae* s.s. through this method (Menge *et al.*, 2005). Crossing two inbred strains of mosquito

provides individuals which are not only better than the parents and display consistent development time and responses, but are also frequently fitter than randomly bred individuals (Craig 1964).

In *Ae. aegypti*, crossing of inbred strains produced heterosis such that larvae developed more rapidly, pupation and emergence times became similar for all individuals, greater numbers of mosquitoes survived to adulthood, and adult lifespan and egg production was greater (Craig 1964). Fitness traits important to the ability of genetically modified mosquitoes to breed with wild populations were found to be enhanced by crossbreeding in a study using the Mbita and Ifakara strains of *An. gambiae* s.s, from Western Kenya and Tanzania respectively. After 20 generations, fecundity and body size were markedly increased in comparison with the parent strains (Menge *et al.*, 2005).

Sperm cells produced in the male testes play a significant role in male reproductive success in *Drosophila*. A study of sperm length in *D. subobscura* by Bressac and Hauschteck-Jungen (1996) showed that males have two types of sperm length, long and short. They found that spermatazoa transferred to females during copulation were of both types, with total numbers of approximately twenty thousand, of which 34% were long and 66% short. After 30h post mating, 900 sperm were found in the female spermatheca and receptacle. The proportion of sperm was different in the two organs: long sperm in the spermatheca made up 80% of the total, while in the receptacle this percentage was 89%. However, the proportion of short and long sperm was not constant in the spermatheca (Bressac and Hauschteck-Jungen, 1996). Large variations are observed in sperm length in male *An. gambiae* (Klowden and Chambers, 2004). There are suggestions that variation in sperm length in *An. gambiae* could affect female oviposition behavior (Voordouw *et al.*, 2008). In *An.gambiae* s.s., while Voordouw *et al.* (2008) found that short sperm length was positively correlated with reproductive success, Klowden and Chamber (2004) concluded

that the opposite was the case, based on comparisons of sperm length in the female and the testes. Similar controversy surrounds the effect of sperm length in *Drosophila obscura.*

Research across a range of species provides evidence for a link between the size of testes and accessory glands and reproductive success, including in the stalk-eyed fly (Baker *et al.*, 2003); in *Drosophila* (Pitnick and Markow, 1994b); in the butterfly (Gage, 1994) and in mammals (Gage and Freckleton, 2003; Say and Pontier, 2006). Little is known about male accessory gland size and testes size in mosquitoes. Mahmood and Reisen (1982) studied morphological change in *An.stephensi* males' reproductive system with aging and mating. The findings show a marked decrease in testes length and width with age, with little difference between right and left testes at any age. Additionally, the amount of secretory substance within the male accessory glands also declined with multiple mating, although it was renewed after copulation, as was the number of spermatozoa found within the sperm reservoir (Mahmood and Reisen, 1982). The secretions of the male accessory glands, when transferred to the female along with sperm, are important to several processes, namely sperm storage, egg-laying, and prevention of further mating behaviour (Lung and Wolfner, 2001 , Ram and Wolfner, 2007). This change in behaviour is stimulated via chemical signals to the female's brain (Chapman, 2009). Additionally, yellow gelatinous rods in the seminal fluid go on to form a vaginal mating plug (Giglioli and Mason, 1966). This is proposed by Lung and Wolfner (2001) to be linked both to sperm retention and the cessation of mating activities. Roger *et al.* (2009) (Rogers *et al.*, 2009)described the components of the mating plug in *An.gambiae* in further details.

This study investigates the impact of colonization on potential correlates of reproductive success such as sperm length, testes and accessory gland size in laboratory Mopti strains of *Anopheles gambiae* s.s. of various ages and refreshed strains (refreshed by

crossing with the first generation offspring of field-collected females and males in 2008 and 2009). The impact of genetic modification on the same variables in KIL (the original non-modified strain), in EE (KIL genetically-modified into a docking strain) and in Vida (EE loaded with an antimicrobial peptide) will also be studied. Further, an inbreeding depression hypothesis is tested, as it was hypothesized that the decrease in sperm size in relation to colonization resulted from inbreeding effects (accumulation of detrimental alleles in the homozygous state) rather than from selection for laboratory conditions.

## 4.2. Materials and methods.

### 4.2.1. Mosquito strains

Transgenic and non transgenic mosquitoes are used to assess the impact of colonization and genetic modification on males' reproductive success in *An. gambiae* s.s. For the transgenic lines, EE was KIL genetically modified into a docking strain, and E-Vida was EE loaded with an antimicrobial peptide. Transgenic strains were created from single G1 positive adults by backcrossing to wild type (KIL strain) (Meredith *et al.*, 2011). The Mopti strain was initially established from the village of N'Gabakoro Droit, Mali in 2003 (12°14`16`N, 7°50`20W), by Dr. F. Tripet and Prof. G. Lanzaro (University of California Davis). In July 2007, MRA-763 strain eggs were sourced from the Malaria Research and Reference Reagent Resource Center (MR4), and were reared at the University of Keele (Patrick Manson insectaries). This strain was refreshed by crossing with the first generation of offspring of field-collected females and males in 2008 and 2009. KIL and Kisumu were old strains reared for more than 35 years under laboratory conditions. Gravid field females from the village of N'Gabakoro Droit, Mali were captured and transferred to oviposition tubes. Females laid eggs which after 48h were placed on wet filter paper and covered in a polythene wrap. Within 12-18h, filter papers which contained

field eggs were carried to Keele university insectary. Each egg batch was put into a small tray filled with approximately 200 ml of distilled water. Fish food larvae were added, with the amount depending on the size of the batch. Six larvae individuals from late larval stage three or four were used for DNA extraction. The larvae extractions were identified by Scott/Fanello PCR/RFLP (by the researcher's colleague, Doug Paton). Mopti/M-forms were kept in the insectary, while *An. arabiensis*, and Savannah/S-form offspring were unused. Reciprocal crosses were made with Mopti 200X: Female Mopti 200 X/Male Field and Female Field/Male Mopti 200X (X = 2008 or 2009). Crossing cages were fed a blood meal and eggs laid by females trayed out as a new single strain.

### 4.2.2. Set up of mating cages of different strains to assess the impact of colonization and genetic modification on male reproductive traits

24h post mating, the first blood feeding took place. After this, oviposition pots were added to each mating cage. Eggs were laid by gravid females after two days of blood feeding. After hatching out, 200 first instar larvae were located in 6-8 trays. Larvae go through four stages and a pupae stage within 8-10 days after hatching (see section 2.2.2, Chapter 2). Males were chosen and put into a pot, which was kept in a new cage. Each cage had a sugar solution vial and wet cotton bud on the top of the cage. The following day, the pot which contained the pupae was checked. The remaining pupae were transferred to a new cage, while the male adults were left in the cage. The last steps were repeated each day until sufficient numbers of adults were obtained. This means that there were several cages of the same strain, to ensure all adults in one cage were of the same age. Seven days post emergence as adults, males were picked up using a mouth aspirator/pooter and put into a pot which was covered with net. Male adults were killed by chilling in the freezer for 5min or by placing the pots on ice. Then, samples were divided

into groups: 40 males for imaging of accessory glands and testes surface as fresh samples, because frozen samples are not suitable for dissection (Mahmood and Reisen, 1982); and another 20 males for imaging of sperm length. Samples for sperm length were stored at below -4C° for later preparation of sperm slides. 40 males from each strain were dissected under a dissection microscope (6.3magnification) and images were captured using a digital camera (Olympus, E520) and measurements made with the ImageJ software program.

### 4.2.3. Capturing images of testes and accessory glands in different *Anopheles gambiae* strains

Pupae were picked and separated into females and males (see section 2.2.7, Chapter 2). The virgin male at 7 days old was picked up from the pot with fine forceps: it was held by the legs or head but not by the wings. PBS drops were added to the clean microscopic slide and the male was fixed to the slide using a fine needle on the upper body to hold it in position. A second needle was used to separate the last three segments from the body. The three segments were pulled out, revealing the testes and accessory glands (Mahmood and Reisen, 1982). If the last segments could not be pulled out or the testes remained inside the abdomen, an attempt was made to carefully remove the cuticle to release the organs. Photographs were taken using a digital camera (Olympus, E520) at a magnification of 6.3 under dissection microscope, focusing and repositioning several times for a clear view of testes and accessory glands. The numbers of photos were recorded, followed by the name of the strain and the sample number. Clean tissue was used to clean the slide and the needles were washed with distilled water and dried. After this, the next male was put on the slide. The whole preceding process was repeated for each sample, after which each male was put into the microcentrifuge tube (1.5ml) and labelled with the name of the

sample, sample number and date. Afterwards, all the tubes were kept in a labelled box or bag in the freezer at below -4C° to use for wing length measurements.

### 4.2.4. Measurement of testes and accessory gland length and size

Testes and accessory gland images were captured using a digital camera (Olympus, E520). All images were transferred to a computer and saved. The images were measured with ImageJ software (1.41o Wayne Rasband, National Institutes of Health, USA). The length of organs was measured from the top to the last narrow place in the testes and from the top to the end of the accessory glands (Mahmood and Reisen, 1982).The length measurements of both organs were done, but because it was difficult to determine the last narrow place in organ or stop end point in each organ. Therefore, the data could not use due to huge variation between samples were observed, while for surface, the whole area surrounding the organ was measured (Fig.4.1). All scale data on the images were removed before starting the measurements. Graticule scale measurement photographs were captured at the relevant magnifications: at 6.3x on the dissecting range for testes and male accessory glands. Straight line selection was used to draw a line for photo scale, while Polygon Selection was used to trace the outline of the testes and accessory gland surface (Fig.4.1). Software measured the photographs in pixels. Measurements in pixels were then converted into mm by dividing the pixels output by the number of pixels in 1mm of scale photo. All measurements were repeated twice and an average was taken for each sample.

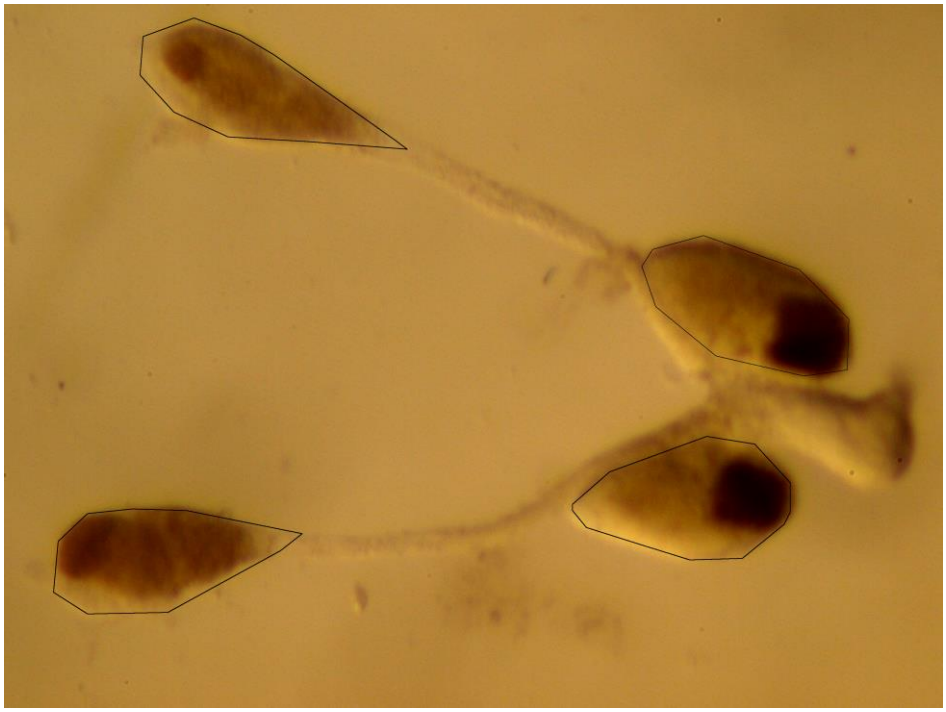

Fig.4.1. Male reproductive system of *An.gambiae* s.s. displaying the different parts measured by Polygon Selection in Image J to trace the outline of the testes and accessory glands surface (black line)

### 4.2.5. Set- up of mating cages of different combinations to test inbreeding depression hypothesis.

Different strains of *An.gambiae* s.s. mosquitoes were used for setting up a number of mating cages to test inbreeding depression hypothesis. Two days post blood feeding (see section2.2.5, Chapter 2), mature female mosquitoes of KIL, Mopti 2008 ref.2009, Mopti 2008 and Kisumu strains laid eggs into white polystyrene pots lined with filter paper and half filled with distilled water. After hatching out, 200 first instar larvae were located in 8 trays for each strain (33 x 23 x 5 cm) including one litre distilled water. Larvae go through

four stages and a pupae stage within 8-10 days after hatching (see section 2.2.2, Chapter 2). First instar larvae were supplied with one drop per tray for a day, of Liquifry (Interpet Ltd., Dorking, UK), then 40 mg to 80 mg of ground flakes baby fish food (Tetra werk, Melle, Germany) until they pupated. Pupae were continually removed from larvae trays for sexing and transferred to two adult mosquito cages, a male cage and a female cage, into pots filled with distilled water where they matured into adults. Each cage had a sugar solution vial and wet cotton bud on the top of the cage. Next day the pot containing the pupae was checked. The remaining pupae were kept in the same cage and a new set of pupae were added every day. Two to six day-old mosquitoes were used to set up the new combinations by crossing equal numbers of males and females (100 individuals in each cage) to set up the following mating combinations: Male KIL x Female Kisumu, Male Kisumu x Female Kisumu and Male KIL x Female KIL. 24h post mating, the first blood feeding occurred. After this, oviposition pots were added to the mating cages. After hatching out, 200 first instar larvae were placed in 6-8 trays. Larvae go through four stages and a pupae stage within 8-10 days after hatching. Male pupae were chosen and transferred to emergence cages. Seven days post emergence as adults, males were picked up using a mouth aspirator/pooter and placed in a pot which was covered with net. 20 males were stored in the freezer at below -4C°. Five to seven male adults per day were dissected under a dissection microscope, and then the slides were examined under a Nomarski microscope (Zeiss standard microscope) and images were captured using a digital camera (Deltapix Infinity X).

### 4.2.6. Preparation of sperm slides in different *Anopheles gambiae* s.s. strains

Sperm slides were prepared to measure sperm cells in different experimental groups. Male mosquitoes of *An.gambiae* s.s. at seven days old were killed by freezing them for five minutes, and they were then dissected under a dissection microscope. One drop of PBS saline solution was added to the glass slide, and then a male was picked up by means of fine forceps and placed on the dorsal side. Two fine needles were then used: one was fixed on the first abdominal segment and the second pulled the last segment. The testes were then isolated and transferred to another saline drop on the same slide, or more saline solution was added to clean the area surrounding the testes. The testes were opened by fixing one needle vertically on the top of testes and with the other needle dispersing/breaking it down, and sperm were separated (Voordouw *et al.*, 2008). Sperm were collected in the middle of the slide by using fine needles to prevent sperm escaping. The sperm slide was covered with a slide cover (0.13-0.17mm thick). After that, DPX was used to seal the slide border completely to prevent the sperm escaping. Twenty slides (5-7 slides per day) were prepared and 20 sperm cells were counted for each of the twenty slides: in total, 300-400 sperm cells were counted for each group. The slides were examined under a Nomarski microscope (at 16x magnification as well as condenser II) which was connected to a digital camera (Lumenera s Infinity X) and a computer in order to capture sperm photos immediately after preparation and thus prevent dry out the slides.

Twenty sperm cells per males/group under standard rearing conditions were chosen to ensure that the mean sperm size per male was a very accurate measurement with a little variance as possible. Preliminary test using the K-means option in JMP and the standard deviation of the first set of sperm cells per male measured showed that a sample size of 20 should allow to detect medium to large biological effects. An example of a power analysis

on the sperm length will be shown in the result section 4.3.1 confirming the high power of sperm length comparisons.

### 4.2.7. Measurement of male sperm length of different cage combinations

Sperm cells were examined under a Nomarski microscope and photographs were taken using a digital camera (Lumenera s Infinity X) which was connected to a Toshiba computer. Photographs were measured by using ImageJ software (1.41o Wayne Rasband, National Institutes of Health, USA). All scale data on the images were removed before starting the measurements. Straight line selection was used to draw a line for photo scale, while segmented lines were used to trace the outline of the sperm cell which started from the sperm head and progressed to the end of the tail. Software measured the photos in pixels. Measurements in pixels were converted into mm by dividing the pixels' output by the number of pixels in 1mm of scale photo. All measurements were repeated twice and the average was taken for each sample.

### 4.2.8. Statistical analysis

All data was checked for deviations from normality and heterogeneity of variances. Based on these tests and visual inspection of the data distribution, it was either analysed by ANOVA and by Tukey`s test to compare group pairs in colonized, genetic modify strains, Field Mopti strain and Super males group. Linear regression was used to analyze the relationship between sperm length, testes and accessory gland size, and wing length. All statistical analysis was carried out using the software JMP9 (SAS Institute, Inc).

## 4.3. Results

### 4.3.1. Effect of colonization on sperm length

The mean sperm length of the 2645 *An. gambiae* s.s. sperm cells across seven mosquito strains at different colonization ages, Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009 (see methods 4.2.1), Mopti 2003, Mopti 2003 refreshed 2008 (see methods 4.2.1), KIL and Kisumu was 0.202mm (0.198-0.205CI). The range of sperm length was 0.014-1.031mm across the seven experimental groups (Fig. 4.2A). Sperm measurements from same mosquitoes are not independent points; hence the statistical unit used for most subsequent analyses was the mean sperm length per mosquito. Consequently, the mean sperm length in the 134 *An. gambiae* s.s. males studied was 0.201mm (0.189-0.214CI). The range of mean sperm length of the same mosquitoes was 0.051-0.531mm (Fig. 4.2B). Across all groups, mean sperm length significantly deviated from a normal distribution (Shapiro-Wilkinson test: $n$= 134, W= 0.956, $P$ =0.003).

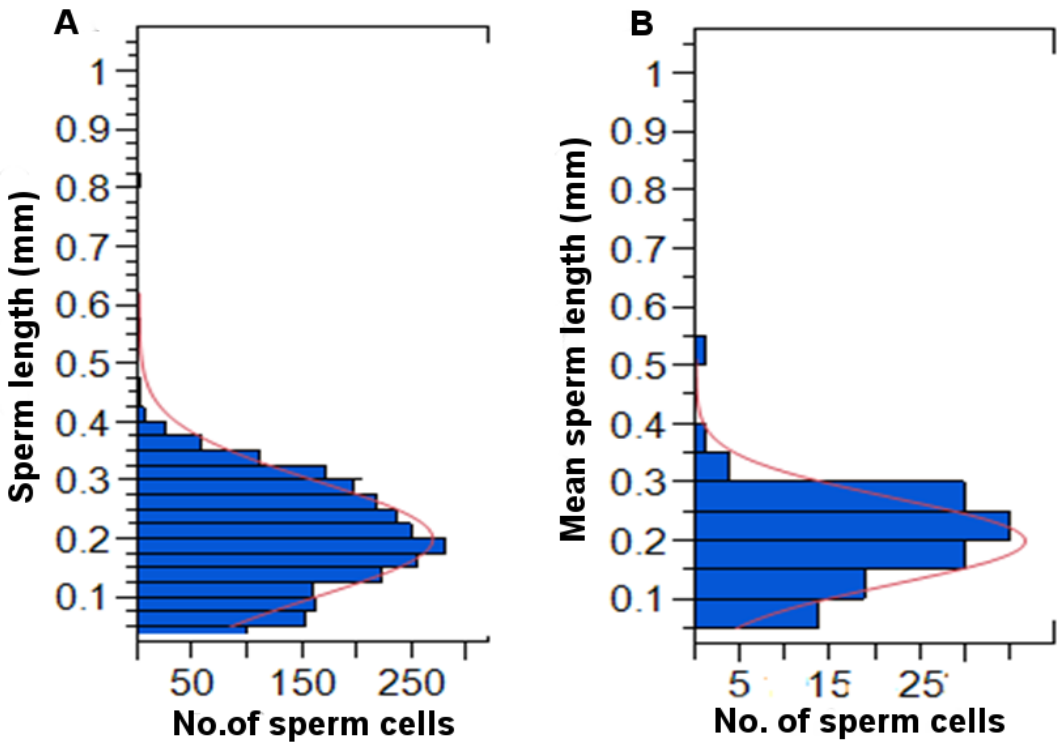


Fig.4.2. (A) Frequency distribution of sperm length (mm) in males (all experimental groups combined: Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL and Kisumu), all sperm measured included, *n*= 2645. The pink line represents a normal distribution based upon the mean and standard deviation in the actual data. (B) Frequency distribution of mean sperm length for each individual, *n*= 134.

The distribution of sperm length and mean sperm length per individual were also studied within experimental groups. Comparison of sperm length distributions overall and averaged by individual for each of the experimental groups (Fig. 4.3.AB) show that sperm length significantly deviated from a normal distribution in all experimental groups. (Shapiro-Wilkinson: $P<0.001$ in all cases) (Fig.4.3A). Mean sperm length did not deviate from normality in the majority of the groups (Shapiro-Wilkinson: $P>0.060$ in all cases) (Fig. 4.3B).

Mean sperm length (*n*= 2645) was 0.250mm (0.244-0.257CI) in Field Mopti, 0.1999mm (0.190-0.208CI) in Mopti 2008, 0.278mm (0.0.272-0.283CI) in Mopti 2008 refreshed 2009, 0.170mm (0.154-0.185CI) in Mopti 2003, 0.221mm (0.215-0.227CI) in Mopti 2003 refreshed 2008, 0.190mm (0.184-0.195CI) in KIL; and 0.102mm (0.096-0.107CI) in Kisumu (Fig. 4.3A).

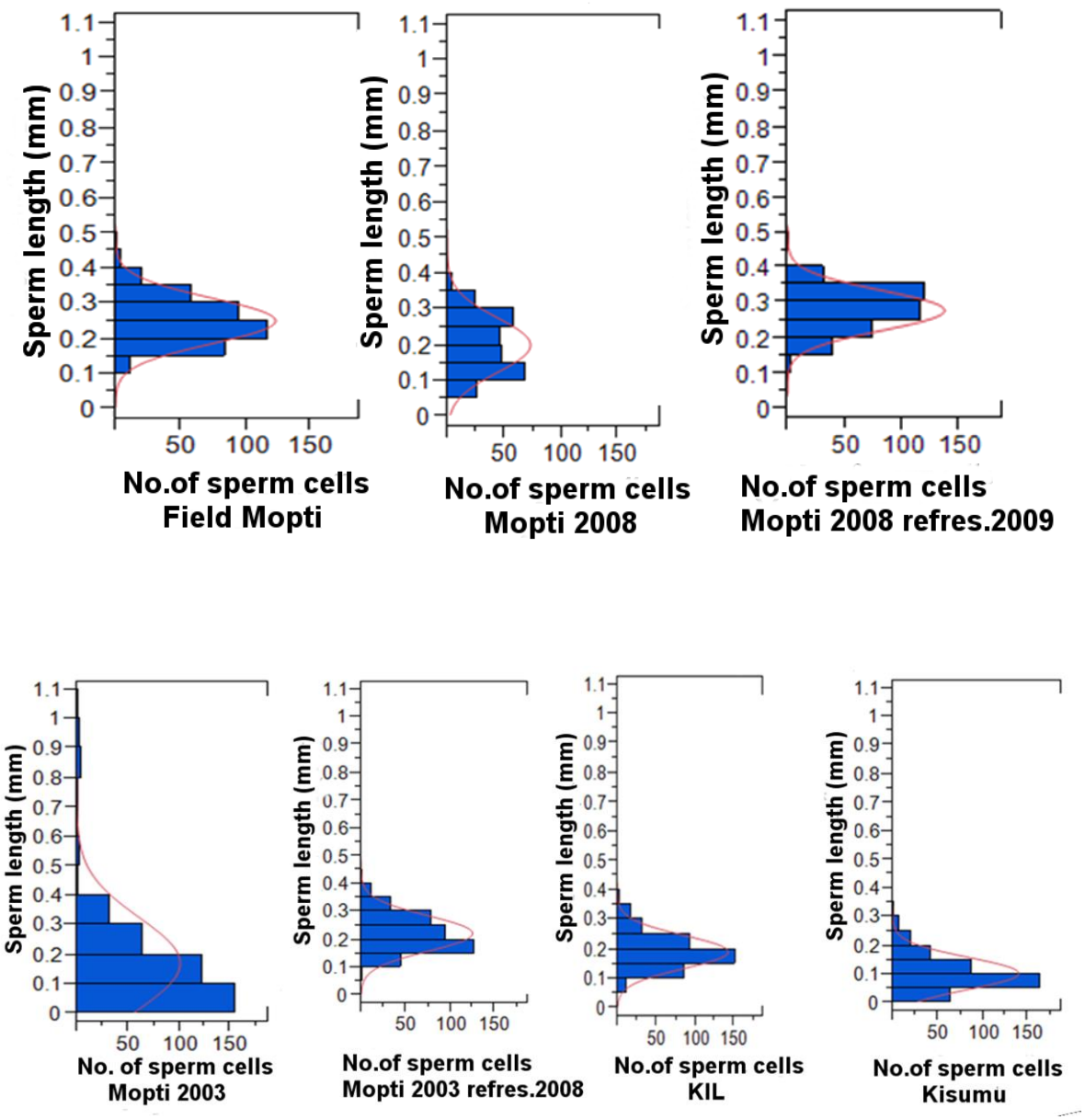


Fig.4.3 (A). Frequency distribution of sperm length (mm) (*n*=2645) for each strain in *An. gambiae* s.s. males in Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL, and Kisumu. The pink line represents a normal distribution based upon the mean and standard deviation in the actual data

Mean sperm length per individual (*n*= 134, 20 sperm per individuals) was 0.250mm (0.235-0.265CI) in Field Mopti, 0.199mm (0.176-0.223CI) in Mopti 2008,0.277mm (0.267-0.287CI) in Mopti 2008 refreshed 2009, 0.168 mm (0.118-0.219CI) in Mopti 2003, 0.221mm (0.206-0.236CI) in Mopti 2003 refreshed 2008, 0.190 mm (0.178-0.201CI) in KIL, and 0.102mm (0.089-0.115CI) in Kisumu (Fig .4.3B). So, Kisumu seemed to have a shortest sperm length, while Mopti 2008 refreshed 2009 had a longest sperm length (Fig.4.3B).

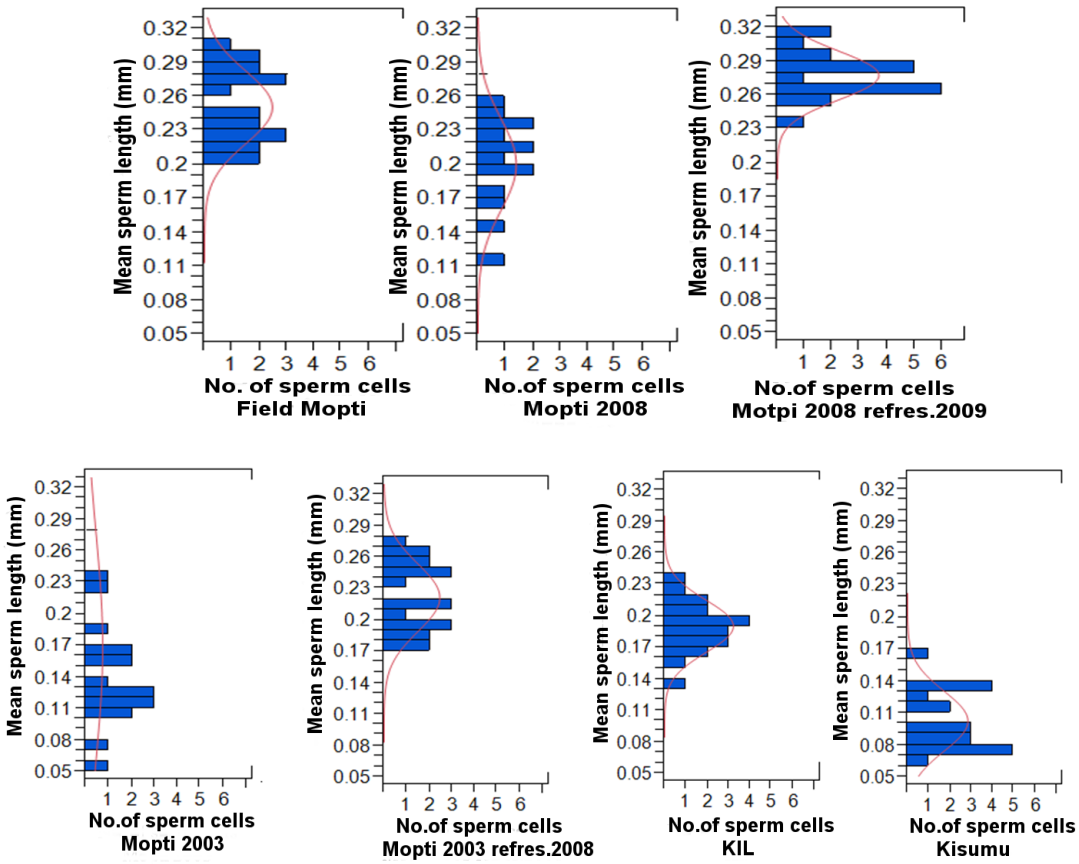


Fig.4.3 (B). Frequency distribution of mean sperm length (mm) of 20 sperm cells per male in 134 *An. gambiae* s.s. males in Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL, and Kisumu. The pink line represents a normal distribution based upon the mean and standard deviation in the actual data

Body size (measured as wing length) was measured and recorded for males of each of the seven groups. The average wing length of the 133 males of Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL, and Kisumu was 2.78mm (2.74-2.81CI). Overall, male wing lengths showed significant difference between groups (ANOVA: $F_{6,133}$= 17.94; $r^2$= 0.460; $P$<0.001) (Fig.4.4). Pair-wise comparisons of wing length between the seven groups showed that there was a significant difference between KIL and all other pairs ($P$<0.001 in all cases) (Fig.4.4).

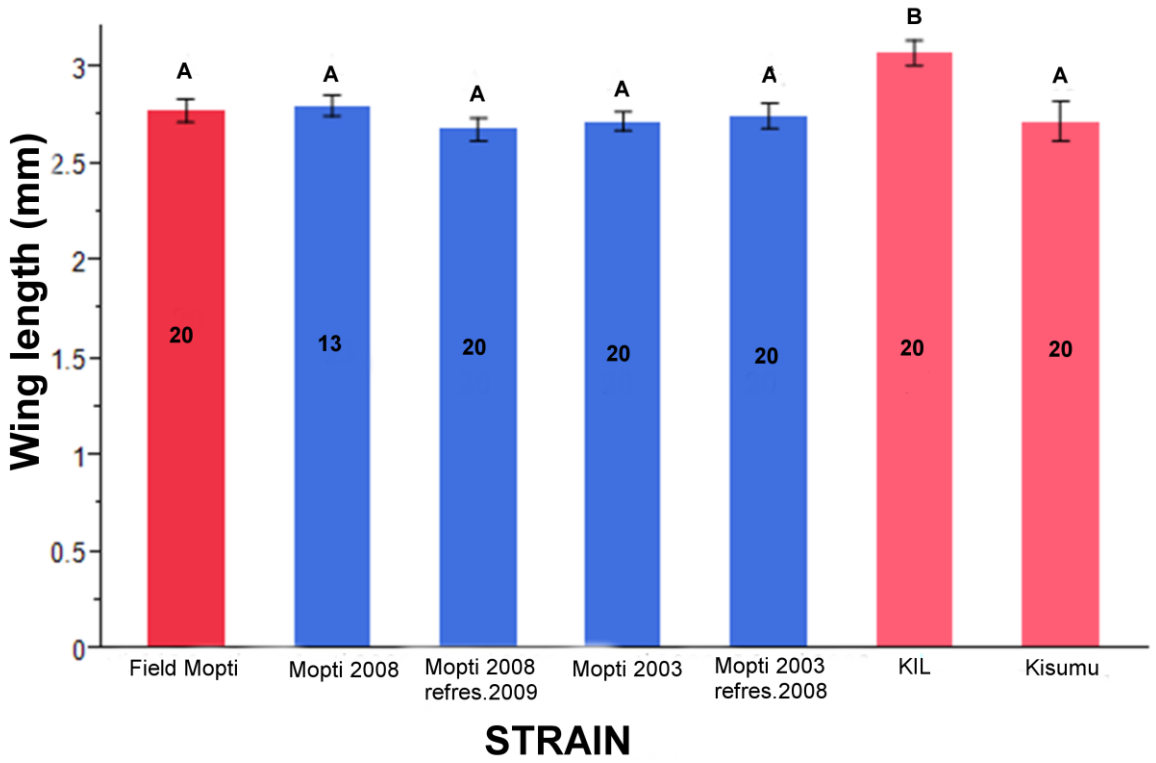


Fig.4.4. Wing length (mm) (±95% CI) in *An. gambiae* s.s. males in Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL, and Kisumu. Bars labelled with different letters differed significantly. Sample sizes are indicated.

The relationship between body size and sperm length was studied. Overall no relationship was observed between mean sperm length and male body size amongst all

combined groups (Linear regression: $n$= 133, $t$= *2.83*; $r^2$= 0.003; $P$=0.479) (Fig. 4.5). The relationship between body size (wing length) and sperm length was studied in each group separately. It was found that there was no relationship between body size and mean sperm length per mosquito in any of the groups (Linear regression: $P$>0.061 in all cases)

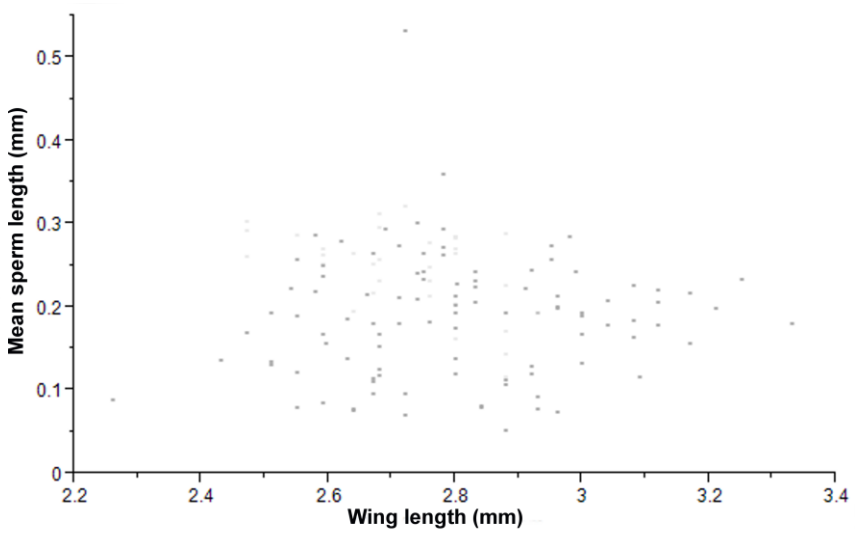


Fig.4.5. Linear relationship between body size (wing length) and mean sperm length (All experimental groups: Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, Mopti 2003 refreshed 2008, KIL and Kisumu; $n$= 133).

Because of significant size differences in body size (wing length) between groups, sperm length was corrected by body size in subsequent analyses. There was a significant difference between all combined experimental groups (ANOVA: $F_{6,133}$ = 26.45, $r^2$= 0.557, $P$<0.001). Pair-wise comparisons between groups showed a significant difference between Field Mopti, newer as well as refreshed strains and old colonized strains ($P$<0.001 in all cases) (Fig 4.6). Sperm length is greatly reduced in old colonized strains compared to the progeny of field-collected females or newer and/or refreshed strains (Fig.4.6). The power analysis for the ANOVA with seven groups and 133 sperm/ wing length measurements

was calculated as alpha was set at 0.05 and sperm length/wing length means of 0.09, 0.061, 0.038, 0.080, 0.062, 0.070, and 0.103 gave a power of 1.00, sigma was 0.018 and delta was 0.020. This confirmed that a sample size 20 sperm/male looked acceptable to detect biological effects.

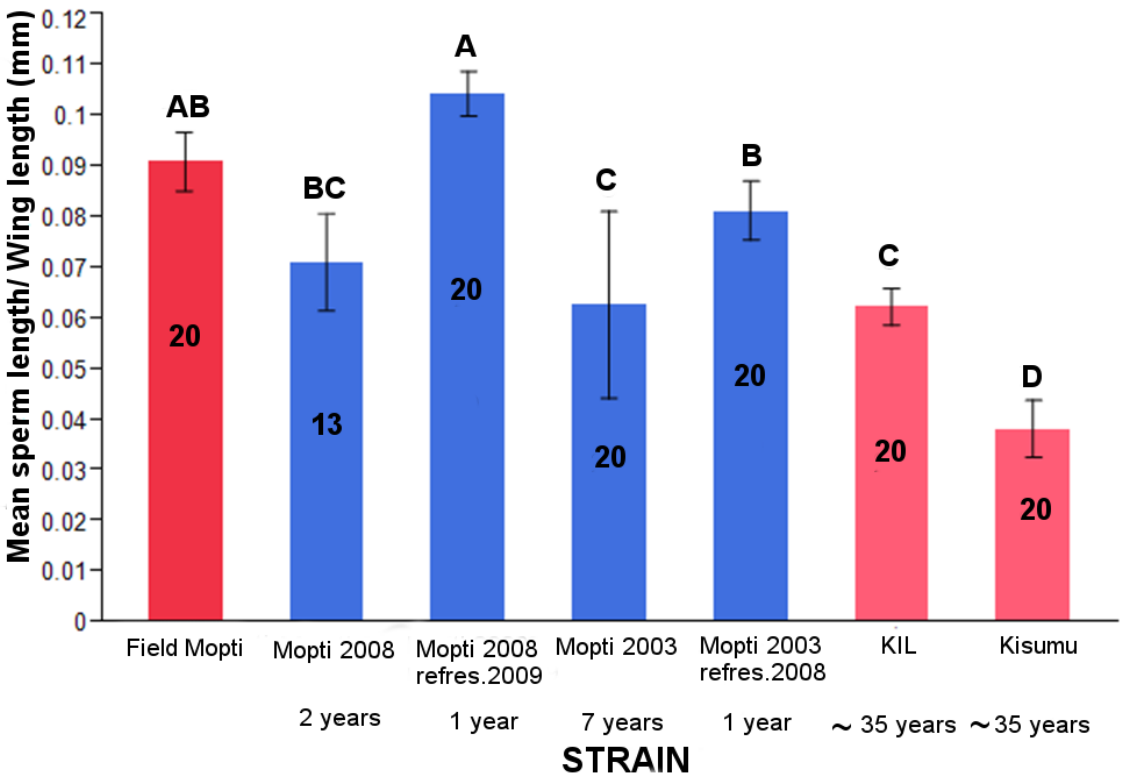


Fig.4.6. Mean sperm length (20 sperm cells were measured in 20 individuals/population) corrected for wing length mm (±95% CI) in male progeny of field-collected Mopti (dark red), in Mopti strains of various age and refreshed or not (blue bars), and in very old strains (light red). Bars labeled with different letters were significantly different (Tukey: $P< 0.05$).

### 4.3.2. Effect of genetic modification on sperm length

To study the effects of genetic modifications on sperm quality, we compared sperm size in Field Mopti, KIL (the original non-modified strain), in EE (KIL genetically-

modified into a docking strain) and in Vida (EE loaded with an antimicrobial peptide). The mean sperm length of the 1578 *An. gambiae* s.s. sperm cells across four mosquito strains was 0.185mm (0.181-0.189CI). The range of sperm length was 0.007 to 0.462mm across the four experimental groups (Fig. 4.7.A). Sperm measurements from same mosquitoes are not independent points; hence the statistical unit used for most subsequent analyses was the mean sperm length per mosquito. Consequently, the mean sperm length in the 80 *An. gambiae* was 0.185mm (0.172-0.179CI). The range of mean sperm length was 0.054-0.301mm (Fig.4.7B). Across all groups, mean sperm length did not significantly deviated from a normal distribution (Shapiro-Wilkinson test: $n$= 80, W= 0.981, $P$=0.316).

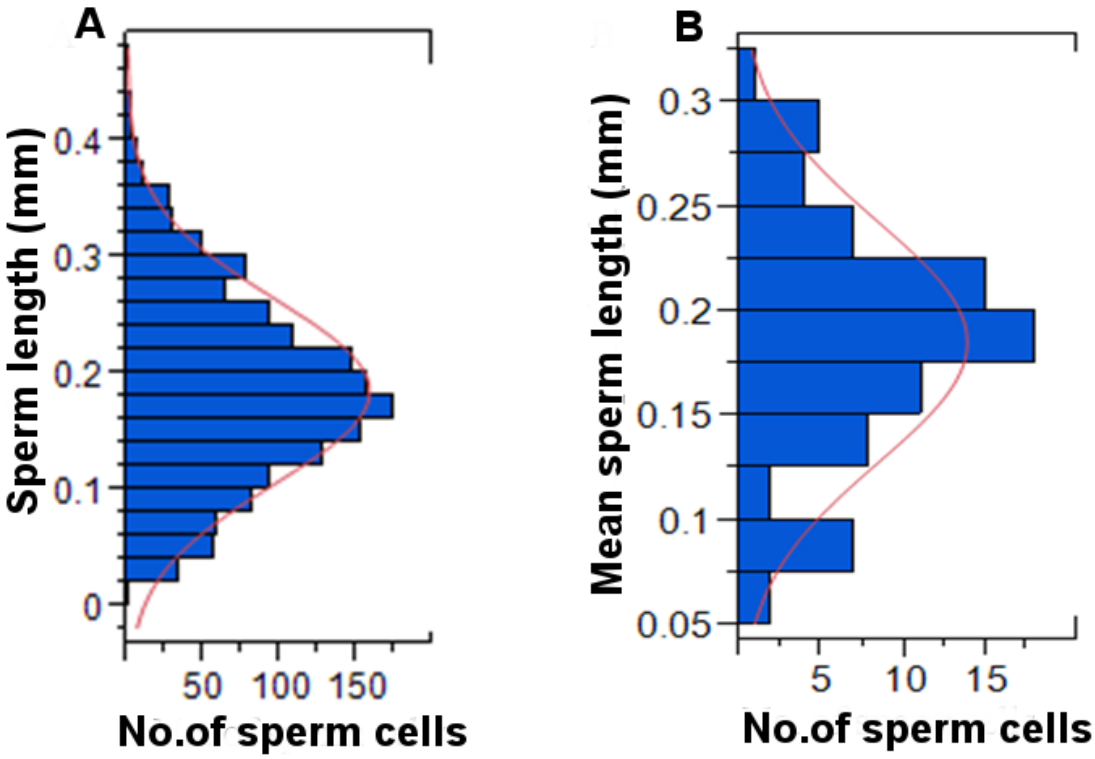


Fig.4.7 (A). Frequency distribution of sperm length (mm) in *An. gambiae* s.s. males in genetically modify strains (EE and Vida) and non transgenic strains (KIL and Field Mopti) (all experimental groups combined), all sperm measured included, $n$= 1578. The pink line represents a normal distribution based upon the mean and standard deviation in the actual data. (B) Frequency distribution of mean sperm length for each individual, $n$= 80.

The distributions of sperm length and mean sperm length per individual were also studied within experimental groups. Comparison of sperm length distributions overall and averaged by individual for each of the experimental groups (Fig. 4.8.AB) show that sperm length significantly deviated from a normal distribution in all experimental groups (Shapiro-Wilkinson: $P<0.001$ in all cases) (Fig.4.8A). Mean sperm length did not deviate from normality in the majority of groups (Shapiro-Wilkinson: $P>0.060$ in all cases) (Fig.4.7B). Mean sperm length ($n$=1578) was 0.250mm (0.244-0.257CI) in Field Mopti, 0.190mm (0.184-0.195CI) in KIL, 0.174mm (0.169-0.180CI) in EE, and 0.124mm (0.116-0.133CI) in Vida (Fig.4.8A).

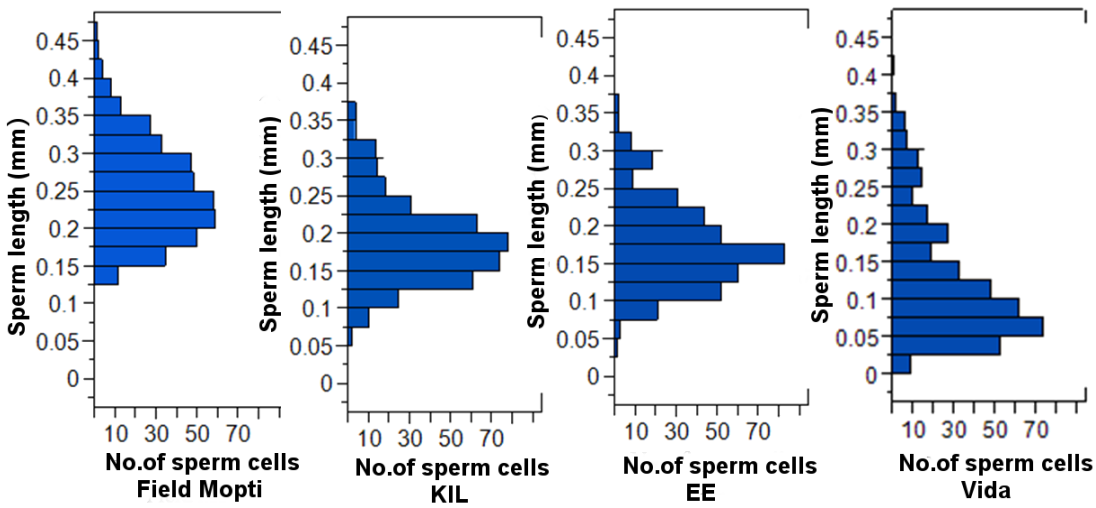


Fig.4.8 (A). Frequency distribution of sperm length (mm) ($n$=1578) for each strain in *An. gambiae* s.s. males in Field Mopti, KIL and transgenic strains, EE and Vida.

Mean sperm length per individual ($n$= 80, 20 sperm per individuals) was 0.250mm (0.235-0.265CI) in Field Mopti, 0.190mm (0.178-0.201CI) in KIL, 175mm (0.164-0.186CI) in EE, and 0.124mm (0.098-0.150CI) in Vida (Fig.4.7B). It seemed that Field Mopti had a longest sperm length, while Vida had a smallest sperm length (Fig.4.8B).

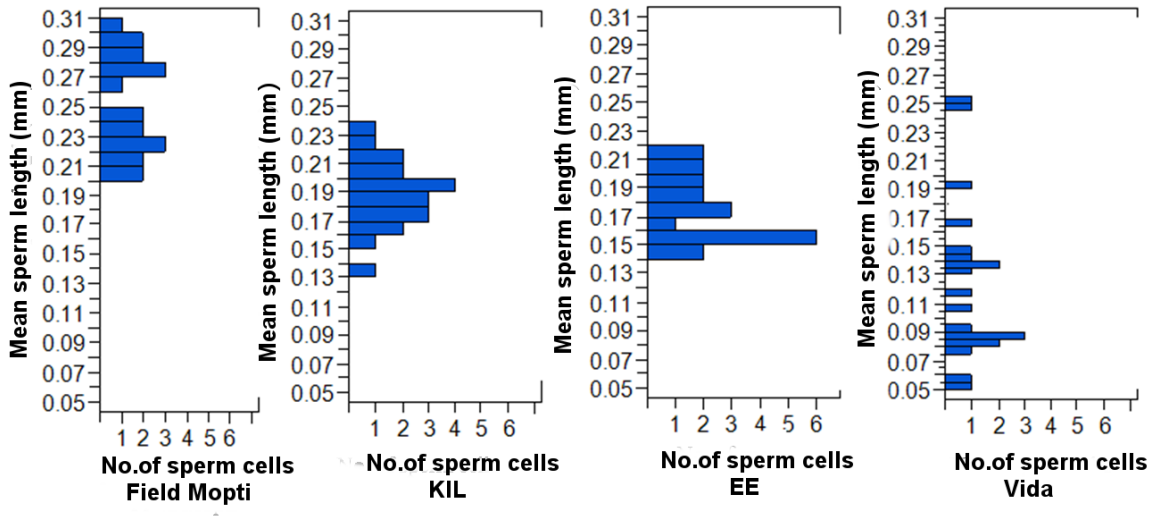


Fig.4.8 (B). Frequency distribution of mean sperm length (mm) for each individual (*n*=80) in *An. gambiae* s.s. males in Field Mopti, KIL and transgenic strains, EE and Vida.

Body size (measured as wing length) was measured and recorded for males of each of the four groups. The average wing length of the 80 males of Field Mopti, KIL, EE and Vida was 2.94mm (2.89-2.97 CI). Male wing lengths showed a significant difference between groups (ANOVA: $F_{3,80}$= 19.28; $r^2$= 0.432; $P$<0.001) (Fig. 4.9). Pair-wise comparisons of wing length between the four groups showed that a significant difference between Field Mopti and KIL, and transgenic strains ($P$<0.001 in all cases), and between KIL and Vida ($P$=0.002) (Fig.4.9).

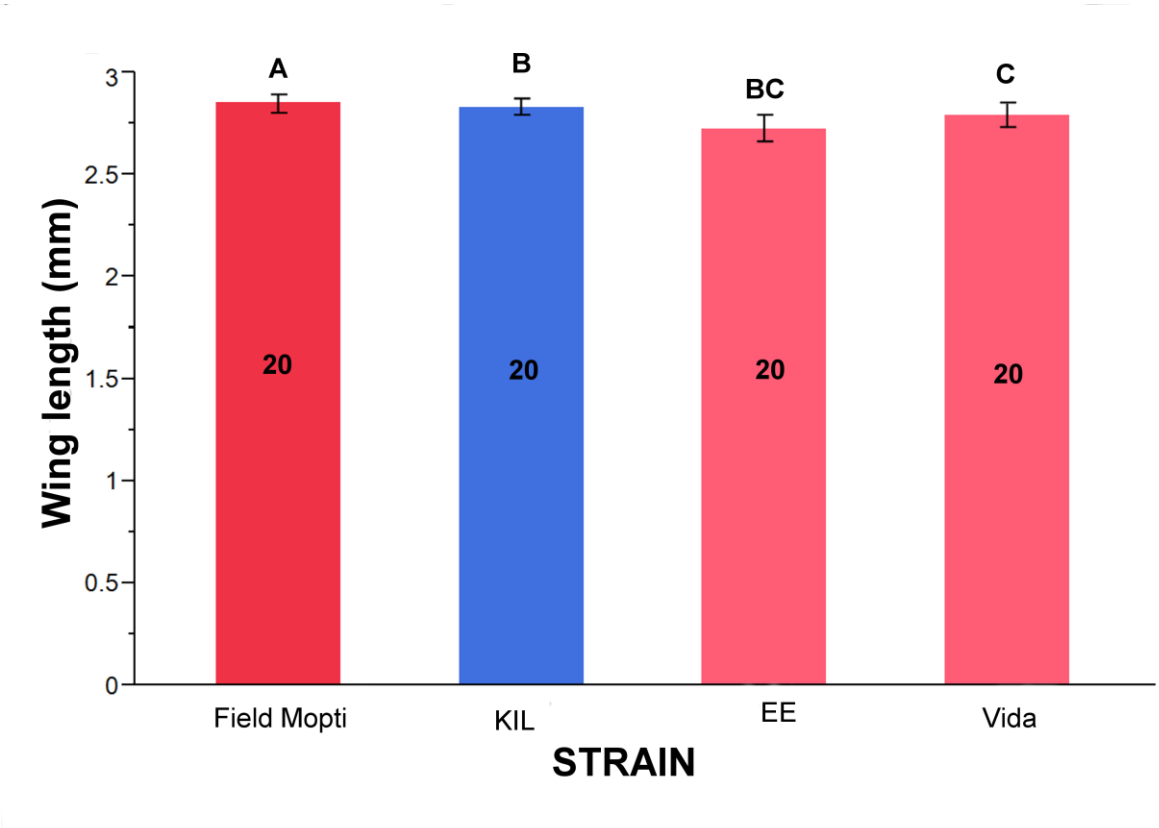


Fig.4.9.Wing length (mm) (±95% CI) in Field Mopti, KIL (non transgenic strains), EE and Vida (transgenic strains). Bars labelled with different letters differed significantly. Sample sizes are indicated.

The relationship between body size and sperm length was studied. Overall no relationship was observed between mean sperm length and male body size amongst all combined groups (Fig. 4.10) (Linear regression: $n$= 80, $t$= 3.62; $r^2$= 0.046; $P$=0.054). The relationship between body size (wing length) and sperm length was studied in each group separately. It was found that there was no relationship between body size and mean sperm length per mosquito in any of the groups (Linear regression: $P$>0.347 in all cases).

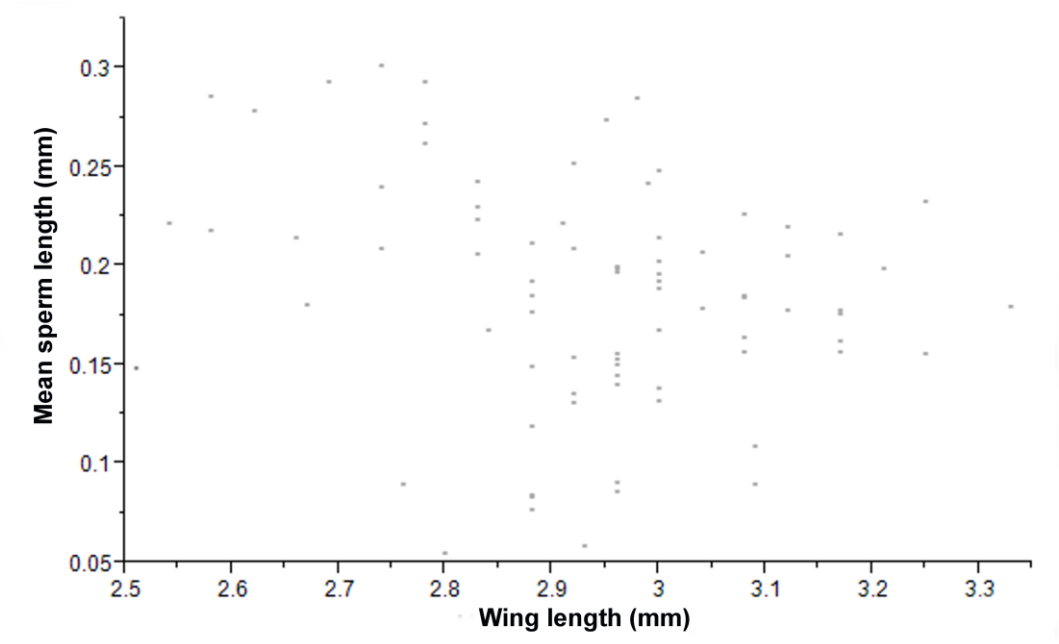


Fig.4.10. Linear relationship between body size (wing length) and mean sperm length (All experimental groups: Field Mopti, KIL, EE, and Vida; *n*= 80).

Because of significant size differences in body size (wing length) between groups, sperm length was corrected by body size in subsequent analyses. There was a significant difference between all combined experimental groups (ANOVA: $F_{3,80}$ = 44.49, $r^2$= 0.661, *P*<0.001). Pair-wise comparisons between groups showed a significant difference between Field Mopti and all non transgenic and transgenic groups (*P*<0.001 in all cases) (Fig.4.11). The transgenic strain EE did not differ significantly from the original KIL strain but sperm size was reduced in the Vida strain (Fig.4.11).

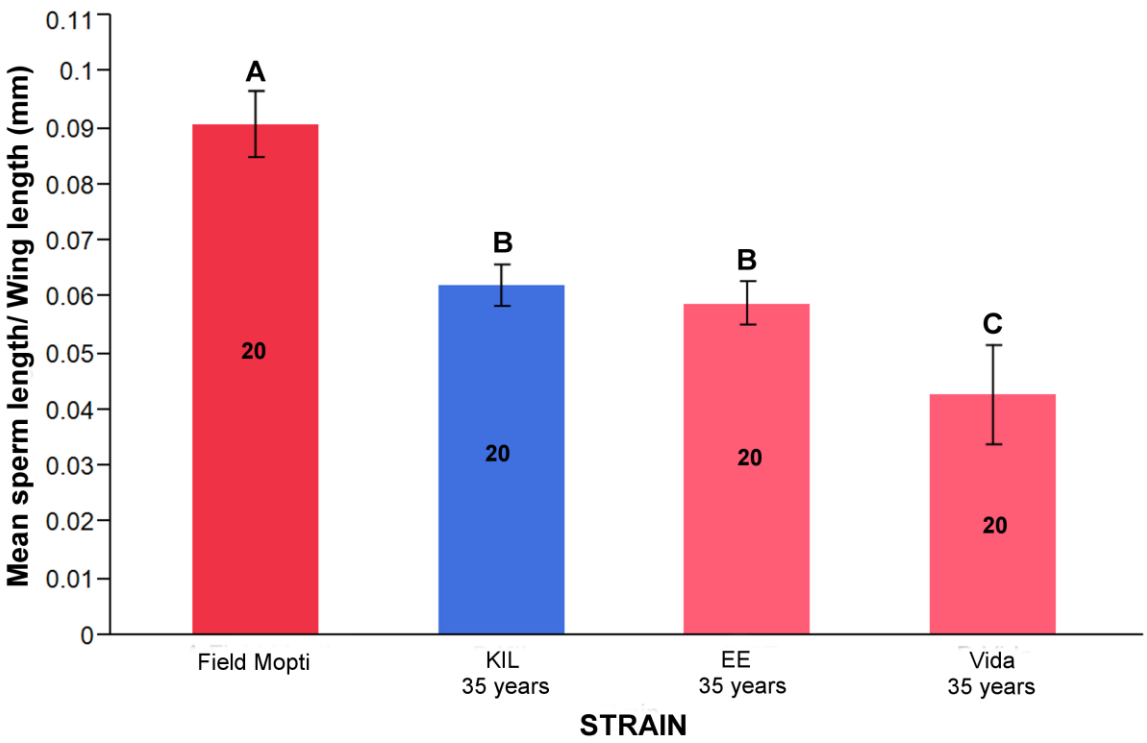


Fig.4.11. Mean sperm length (20 sperm cells were measured in 20 individuals/ population) corrected for wing length mm (±95% CI) in transgenic (EE and Vida) as well as non- transgenic strains (Field Mopti and KIL). Sample sizes are indicated. Graph labeled with different letters (A, B and C) were significantly different (Tukey $P<0.05$).

### 4.3.3. Test of Inbreeding Depression Hypothesis

We hypothesized that the decrease in sperm size in relation to colonization resulted from inbreeding effects (accumulation of detrimental alleles in homozygous state) rather than from selection for laboratory conditions. To test this hypothesis we created crosses between two old strains with reduced sperm length (Male KIL x Female Kisumu). The mean sperm length of the 1525 *An. gambiae* sperm cells across four experimental groups, Field Mopti, Male KIL x Female Kisumu, KIL, and Kisumu was 0.207mm (0.202-0.212CI). The range of sperm length was 0.019-0.525mm across the four experimental groups (Fig. 4.12.A). Sperm measurements from same mosquitoes are not independent

points; hence the statistical unit used for most subsequent analyses was the mean sperm length per mosquito. Consequently, the mean sperm length in the 77 *An. gambiae* s.s. males studied was 0.206mm (0.187-0.225CI). The range of mean sperm length of the same mosquitoes was 0.069-0.434mm (Fig. 4.12B). Across all groups, mean sperm length did not significantly deviate from a normal distribution (Shapiro-Wilkinson test: *n*= 77, W= 0.968, *P*=0.0523).

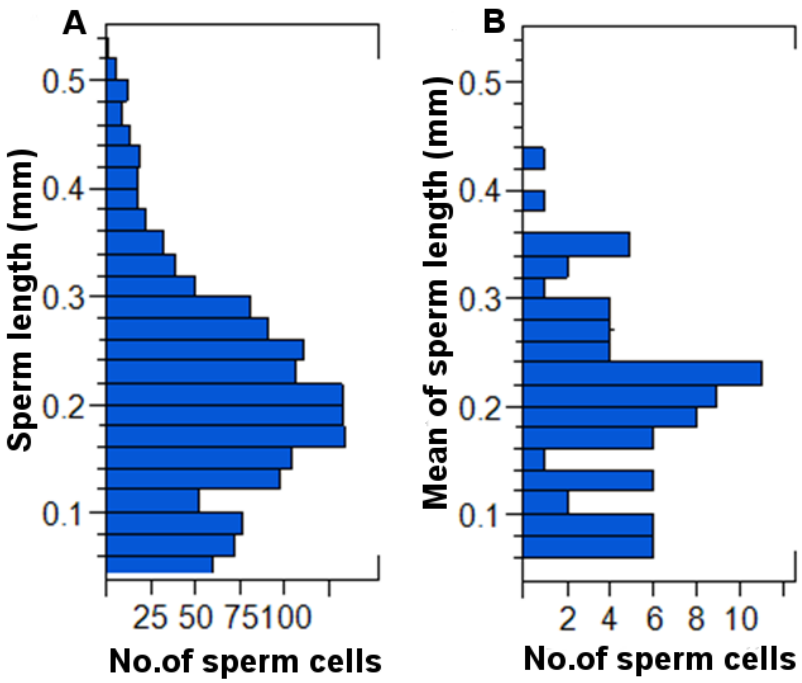


Fig.4.12. (A) Frequency distribution of sperm length (mm) in males (all experimental groups combined: Field Mopti, Male KIL x Female Kisumu, KIL, and Kisumu), all sperm measured included, *n*= 1525. (B) Frequency distribution of mean sperm length for each individual, *n*= 77

The distributions of sperm length and mean sperm length per individual were also studied within experimental groups. Comparison of sperm length distributions overall and averaged by individual for each of the experimental groups (Fig. 4.13.AB) show that

sperm length significantly deviated from a normal distribution in all experimental groups. (Shapiro-Wilkinson: $P<0.001$ in all cases) (Fig.4.13A). Mean sperm length did not deviate from normality in a majority of groups (Shapiro-Wilkinson: $P> 0.054$ in all cases) (Fig. 4.13B).

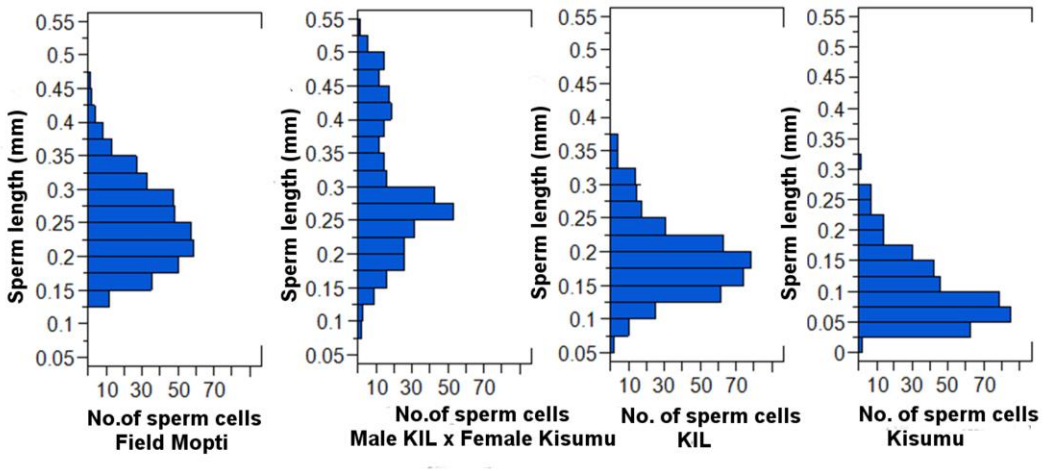


Fig.4.13 (A). Frequency distribution of sperm length (mm) of 1525 sperm cells in *An. gambiae* s.s. males in Field Mopti, Male KIL x Female Kisumu, KIL, and Kisumu

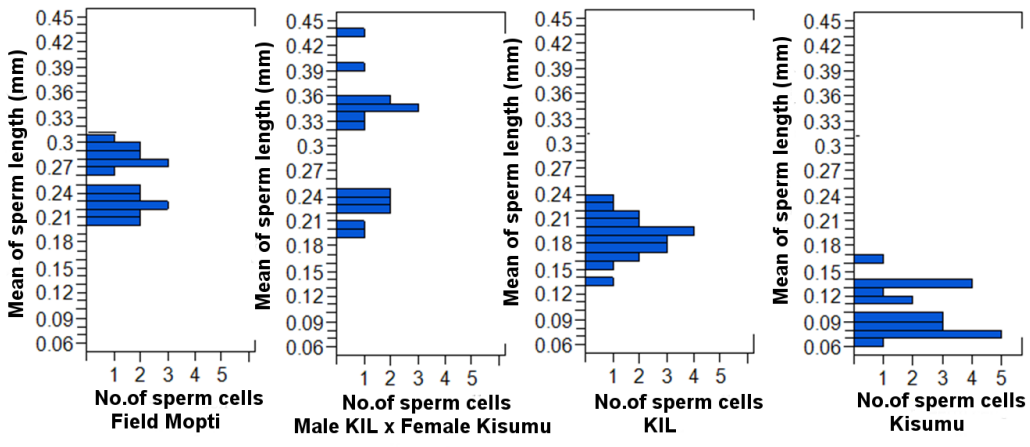


Fig.4.13 (B). Frequency distribution of mean sperm length (mm) of 20 sperm cells per male in 77 males in Field Mopti, Male KIL x Female Kisumu, KIL, and Kisumu.

Body size (measured as wing length) was measured and recorded for males of each of the four groups. The average wing length of the 77 males of Field Mopti, Male KIL x female Kisumu, KIL, and Kisumu was 2.90mm (2.854-2.958CI). Male wing lengths showed a significant difference between groups (ANOVA: $F_{3,77}$= 34.81; $r^2$= 0.588; $P$<0.001) (Fig. 4.14). Pair-wise comparisons of wing length between the four groups showed that there was a significant difference between Male KIL x Female Kisumu and Kisumu, and Field Mopti ($P$<0.001 in both cases), between KIL and Kisumu, as well as Field Mopti ($P$<0.001 in both cases) (Fig.4.14).

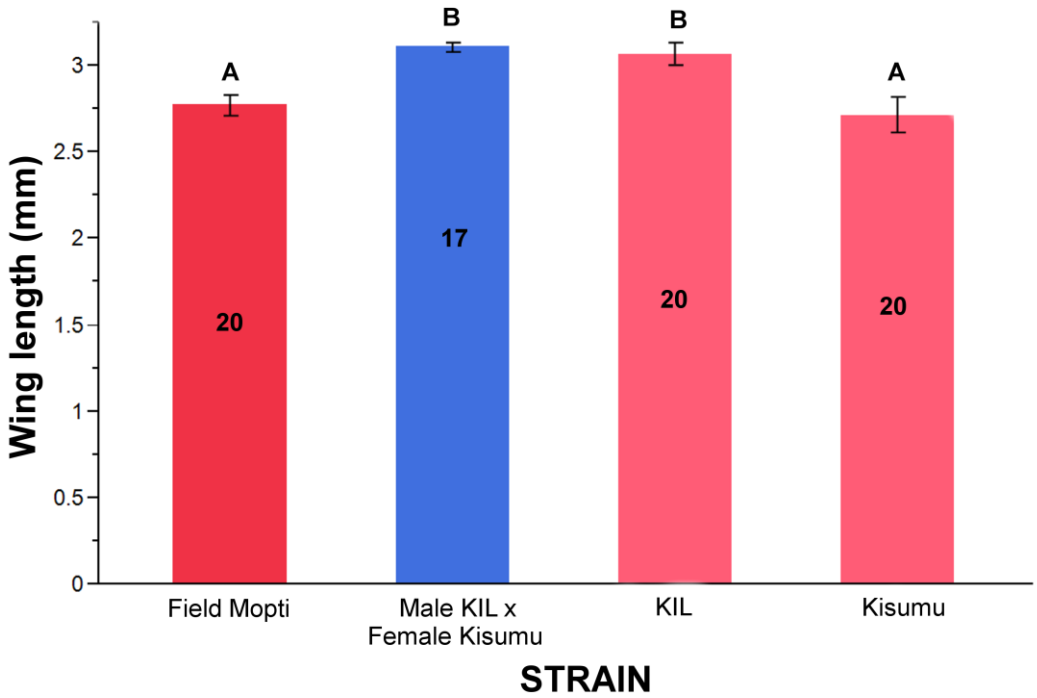


Fig.4.14. Wing length (mm) (±95% CI) in Field Mopti, Male KIL x Female Kisumu, KIL and Kisumu, Bars labelled with different letters differed significantly. Sample sizes are indicated

The relationship between body size and sperm length was studied. A positive overall relationship was observed between mean sperm length and male body size amongst all

combined groups (Fig. 4.15) (Linear regression: $n$= 77, $t$= *-1.47*; $r^2$= 0.124; $P$=0.017). The relationship between body size (wing length) and sperm length was studied in each group separately. It was found that there was no relationship between body size and mean sperm length per mosquito in any of the groups (Linear regression: $P$>0.282 in all cases).

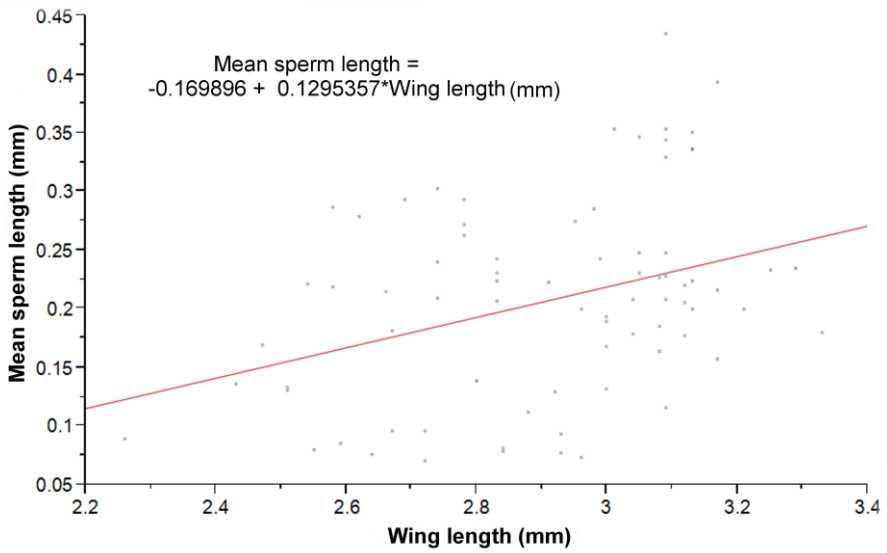


Fig.4.15. Linear relationship between body size (wing length) and mean sperm length (All experimental groups: Field Mopti, Male KIL x Female Kisumu, KIL, and Kisumu; $n$= 77). The linear equation is indicated.

Because of significant size differences in body size (wing length) between groups, sperm length was corrected by body size in subsequent analyses. There was a significant difference between all combined experimental groups (ANOVA: $F_{3,77}$= 63.55, $r^2$= 0.723, $P$<0.001). Pair-wise comparisons between groups showed there was a significant difference between colonized strains and Field Mopti as well as the crossing ($P$<0.001 in all cases) (Fig.4.16). The sperm length of crosses was fully restored as in a Field Mopti (Fig.4.16).

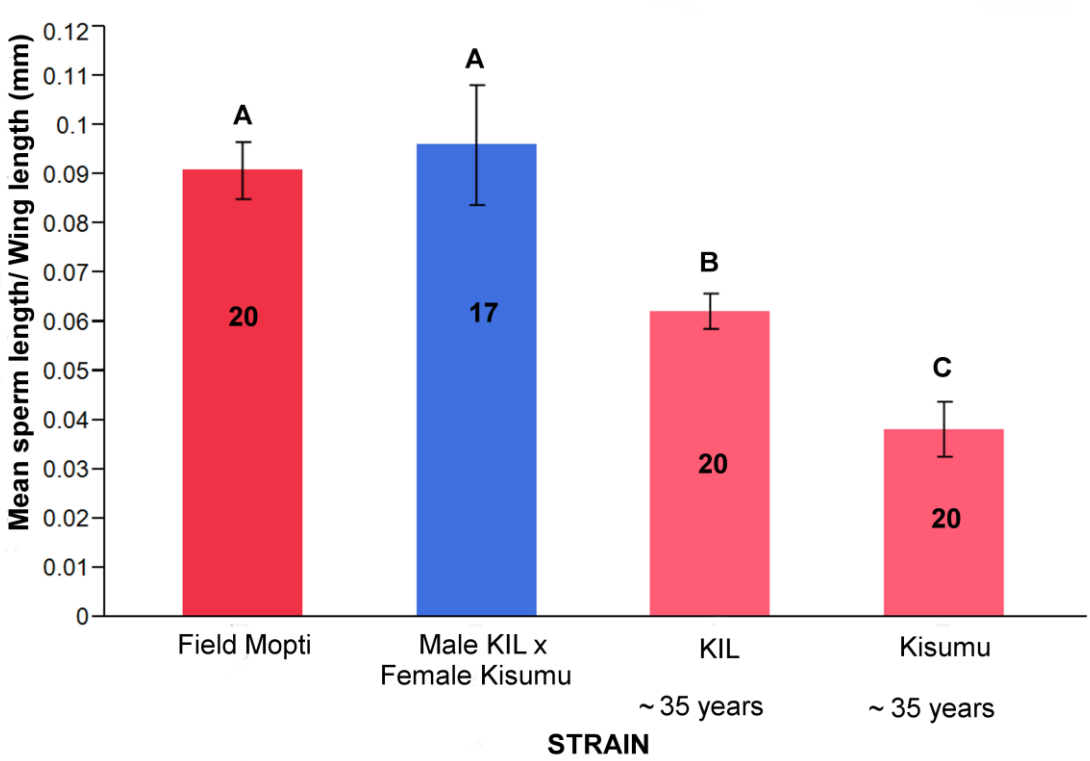


Fig.4.16. Mean sperm length (20 sperm cells were measured in 20 individuals/ population) corrected for wing length mm (±95% CI) in non- transgenic strains (Field Mopti, Kisumu and KIL) as well as cross ♂ KIL x ♀Kisumu. Sample sizes are indicated and bars labeled with different letters (A, B and C) were significantly different (Tukey $P<0.05$).

### 4.3.4. Effect of colonization on testes and accessory glands size

The mean testes size (surface of image) of 236 males across six experimental groups, Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu was 0.035mm (0.034-0.036CI) (Fig.4.17A), While the mean of male accessory glands (MAGs) size (surface of image) of 238 *An.gambiae* glands for the same mosquitoes was 0.028mm (0.028-0.029CI) (Fig.4.17B). The range of MAG size was 0.015- 0.045 $mm^2$ , while testes size range was 0.003- 0.061$mm^2$ (Fig.4.17AB).

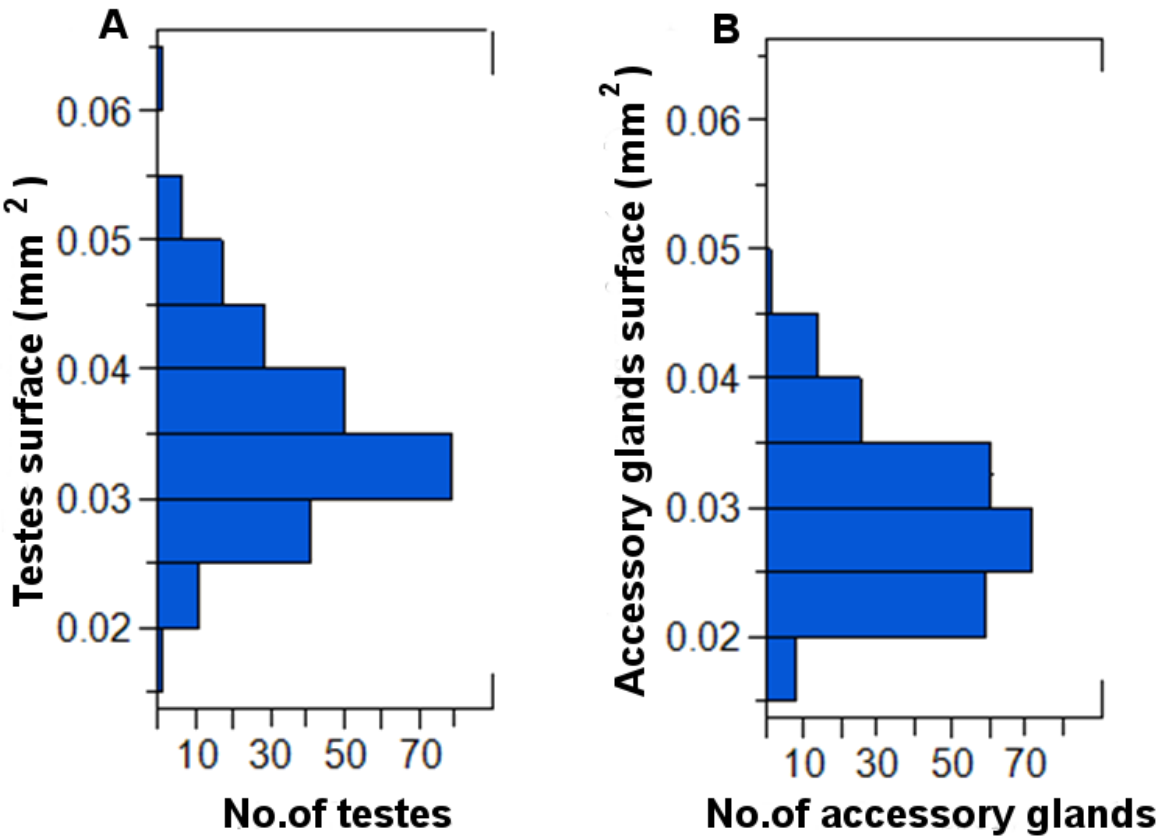


Fig.4.17 (A). Frequency distribution of testes size ($mm^2$) (surface of testes image) (*n*= 236) and (B) accessory gland size ($mm^2$) (surface of accessory glands image) (*n*= 238) in *An. gambiae* s.s. males (all experimental groups combined).

The highest mean of testes size (surface of image) across the six groups was 0.040$mm^2$(0.037-0.044CI) in Kisumu, while the lowest mean of testes size was 0.031 (0.030-0.033CI) in Field Mopti and 0.031 (0.029-0.032CI) in Mopti 2008 (Table 4.1). However, Field Mopti had a largest accessory glands size across all six studied groups (mean=0.036$mm^2$ (0.034-0.37CI)), whereas Mopti 2008 had a smallest accessory glands size amongst all studied groups (mean=0.024$mm^2$ (0.023-0.025CI)) (Table 4.1).

Table 4.1. Mean of male testes size $mm^2$ (surface of image) and accessory glands size $mm^2$ of *An. gambiae* s.s. across six studied groups: Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu. The letters CI are indicative of the confidence interval range and SD letters are revealing the standard division.

| Mosquito line | Testes size ($mm^2$) (surface of image) Mean± SD | Sample size | Accessory glands ($mm^2$) (Surface of image) Mean± SD | Sample size |
|---|---|---|---|---|
| Field Mopti | 0.031±0.003 (0.030-0.033CI) | 39 | 0.036±0.005 (0.034-0.037CI) | 42 |
| Mopti 2008 | 0.031±0.004 (0.029-0.032CI) | 40 | 0.024±0.004 (0.023-0.025CI) | 39 |
| Mopti 2008 refreshed 2009 | 0.033±0.004 (0.031-0.34CI) | 39 | 0.025±0.003 (0.024-0.027CI) | 39 |
| Mopti 2003 | 0.033±0.005 (0.031-0.035CI) | 37 | 0.028±0.004 (0.026-0.029CI) | 39 |
| KIL | 0.039±0.006 (0.037-0.041CI) | 40 | 0.029±0.004 (0.027-0.030CI) | 39 |
| Kisumu | 0.040±0.010 (0.037-0.044CI) | 41 | 0.029±0.005 (0.027-0.031CI) | 40 |

Body size (measured as wing length) was measured and recorded for males of each of the six groups. The average wing length of 245 males of Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu was 2.782mm (2.759-2.805CI). Overall, There was a significant difference in body size (wing length) between the six groups (ANOVA: $F_{5,245}$= 30.89, $r^2$= 0.392, $P$<0.001). Pair-wise comparisons showed a significant difference between Kisumu and Mopti 2008, and Mopti 2008 refreshed 2009, and Mopti 2003, and Field Mopti, as well as KIL ($P$<0.001 in all cases), between Field Mopti and Mopti 2008, and Mopti 2008 refreshed 2009, and Mopti 2003 ($P$<0.001 in all cases), between KIL and Mopti 2003 ($P$<0.001). It seemed that Kisumu had a largest body size (mean=2.970 (2.926-3.014CI) (Fig. 4.18). Because of that difference, testes size and AC size were corrected for effects of body size by dividing them by wing length$^2$.

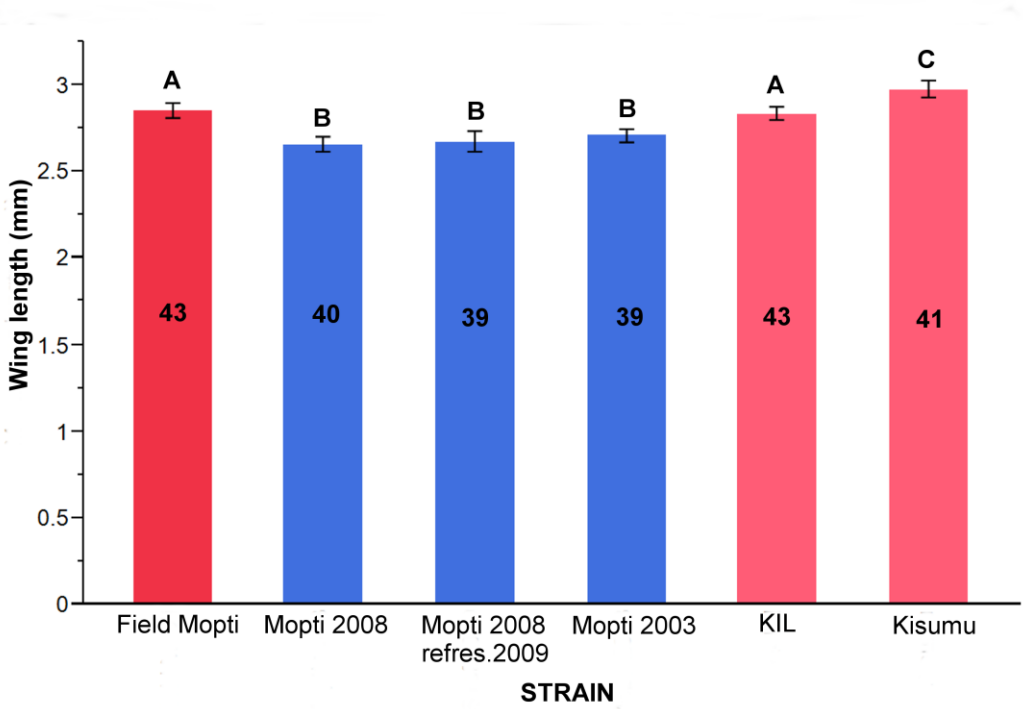


Fig.4.18. Wing length (mm) (±95% CI) in Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu. Bars labelled with different letters differed significantly. Sample sizes are indicated.

The relationships between body size and sexual organs size were studied to assess whether large males have large sexual organs or not. A positive overall relationship was observed between mean testes size and accessory glands size and male body size amongst all combined groups (Linear regression: Testes, $n$=236, $t$=-0.44, $r2$=0.116, $P$<0.001; Accessory glands, $n$=238, $t$=-3.88, $r^2$=0.278, $P$<0.001) (Fig.4.19AB).

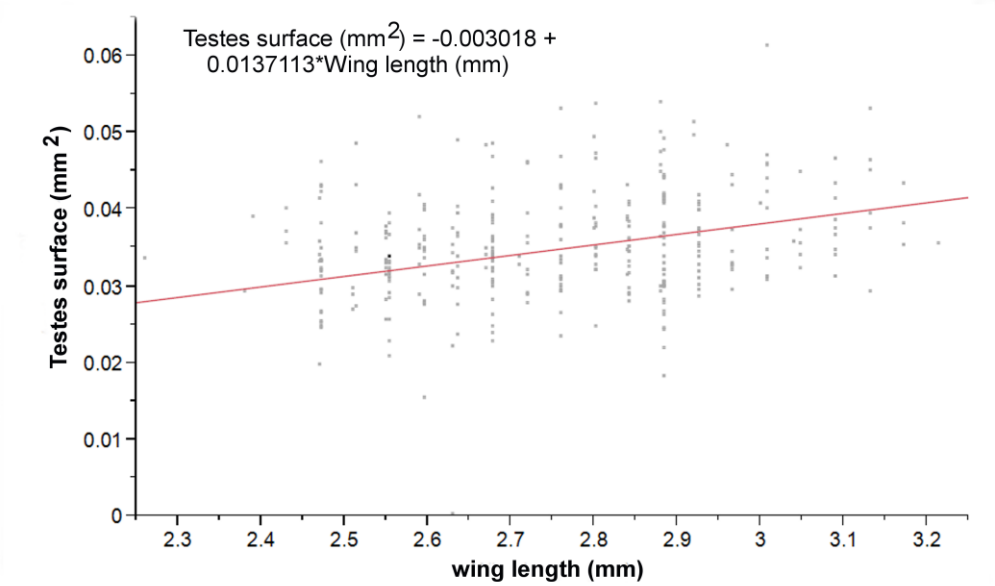


Fig.4.19 (A). Linear relationship between body size (wing length ($mm^2$)) and male testes size ($mm^2$) (surface of AC image) in all combined experimental groups (Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu, *n*=236).

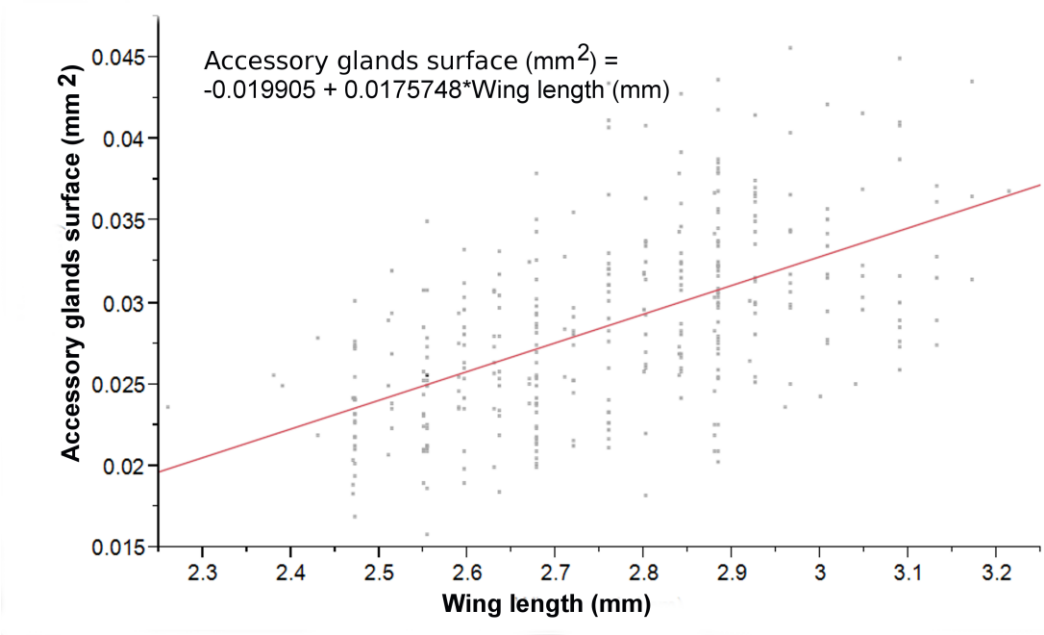


Fig.4.19 (B). Linear relationship between body size (wing length ($mm^2$)) and male accessory glands size ($mm^2$) (surface of AC image) in all combined experimental groups (Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL, and Kisumu, *n*=238).

The relationship between body size (wing length) and testes size and accessory glands size were studied in each group separately. It was found that there was no relationship between body size (wing length) and testes size in any of the experimental groups (Linear regression: $P>0.277$ in all cases). However, a very strong relationship between body size and accessory gland size was found in five groups (Linear regression: Field Mopti $n= 42$, $t= -2.7$, $r^2= 0.433$, $P<0.001$, Mopti 2008 $n=39$, $t=-1.89$, $r^2=0.297$, $P=0.003$, Mopti 2008 refreshed 2009 $n=39$, $t=-0.52$, $r^2=0.268$, $P=0.007$, Mopti 2003 $n= 39$, $t= -1.8$, $r^2= 0.101$, $P=0.047$, and Kisumu $n= 40$, $t= -1.01$, $r^2= 0.178$, $P=0.006$) (Fig. 4.20).

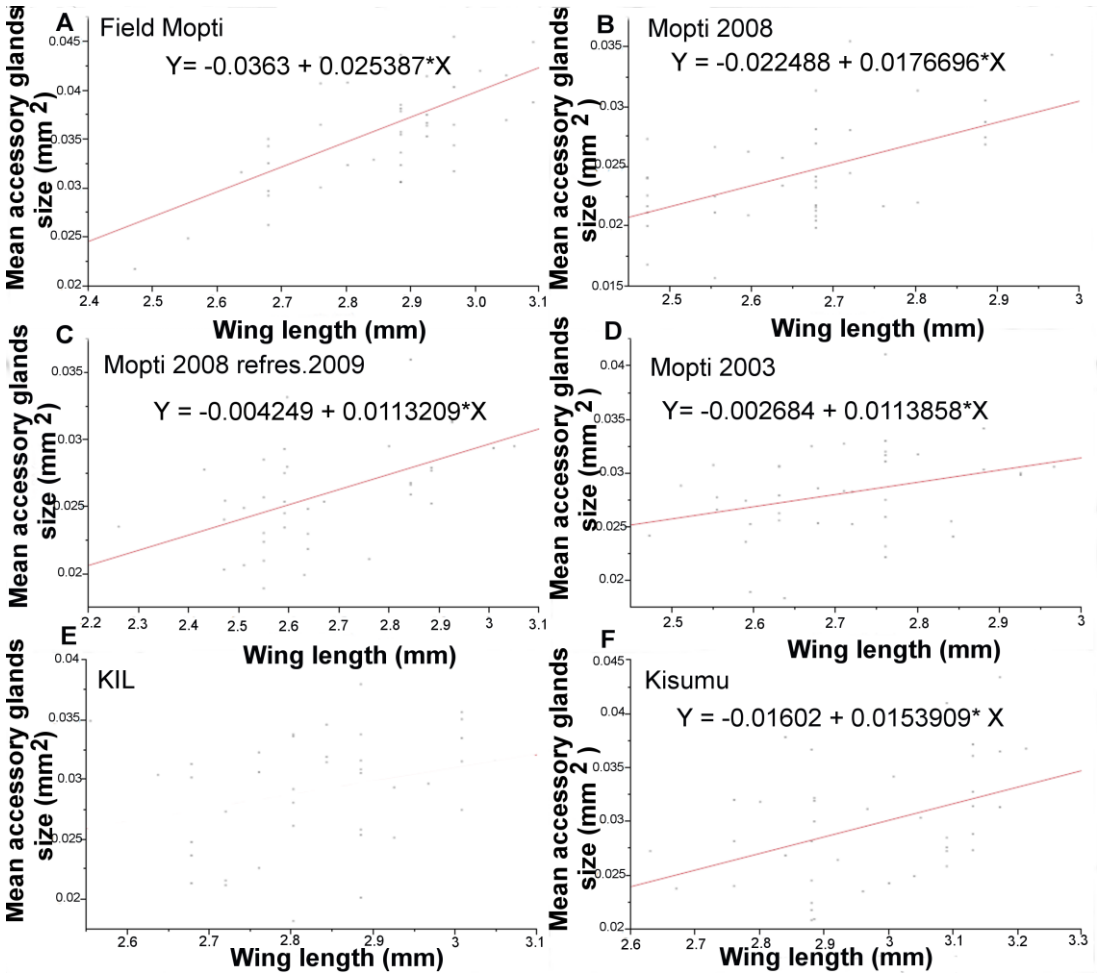


Fig.4.20. Linear relationship between body size (wing length ($mm^2$)) and male accessory gland size ($mm^2$) (surface of AC image) in six experimental groups: (A) Field Mopti, (B) Mopti 2008, (C) Mopti 2008 refreshed 2009, (D) Mopti 2003, (E) KIL, (F) Kisumu. Linear equations are indicated in significant relationship groups

Testes size and accessory glands size were corrected for the effects of body size by dividing them by wing length$^2$. The two variables were then checked for normality once more. Testes size and AC size distribution overall showed that the two variables did not deviate from normality in all six studied groups (Shapiro-Wilkinson: $P$>0.165 in all cases). Testes size (surface of testes image) showed a significant difference overall amongst the six studied groups (ANOVA: $F_{5,236}$= 4.910, $r^2$= 0.096, $P$=0.003) (Fig. 4.21). Pair-wise

comparisons showed that Field Mopti significantly difference from other groups ($P$<0.001 in all cases) (Fig.4.21). In contrast, accessory gland size (surface of MAG image) demonstrated a significant difference overall across the six studied groups (ANOVA: $F_{5,238}$ = 18.863, $r^2$= 0.289, $P$<0.001) (Fig. 4.21). Pair-wise comparisons confirmed Field Mopti significantly differed from other studied groups ($P$<0.001 in all cases). In all colonized strains, accessory glands were significantly smaller than in the progeny from field captured females. In contrast, testes size were larger in colonized strains than in the progeny of field individuals (Fig.4.21)

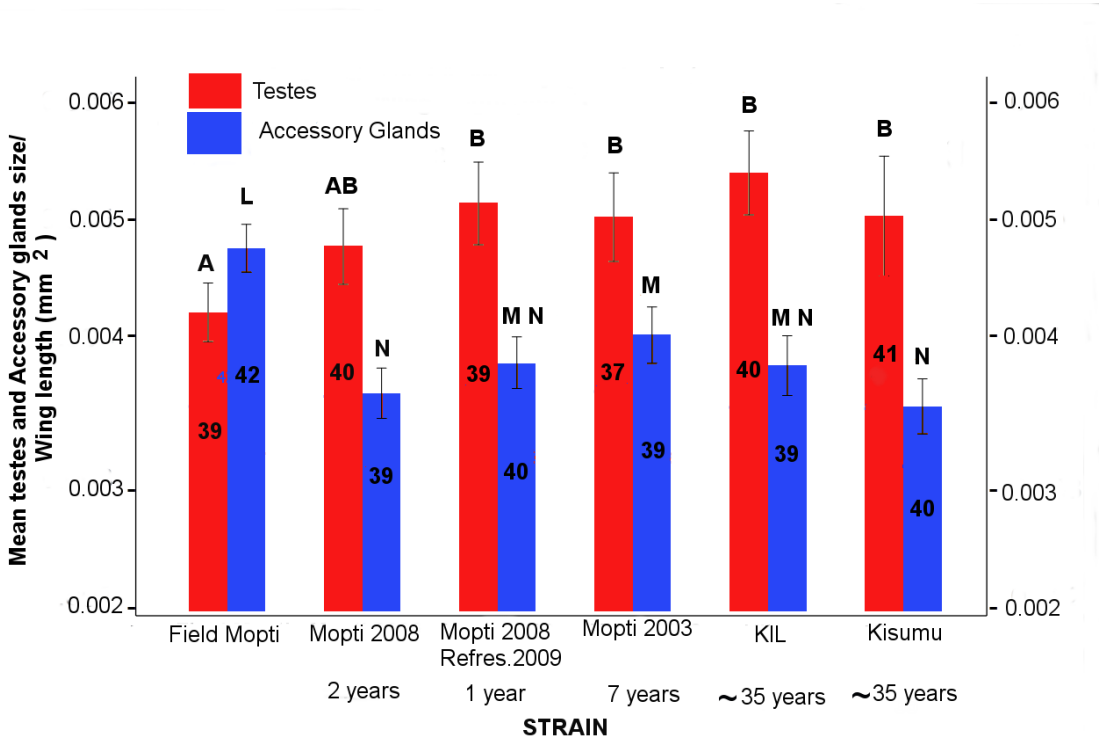


Fig.4.21. Mean testes (red) and accessory glands (blue) size (mm) corrected with wing length (mm$^2$) (±95%CI) in non-transgenic (Field Mopti, Mopti 2008, Mopti 2008 refreshed 2009, Mopti 2003, KIL and Kisumu). Bars labeled with different letters (A, B and C for testes; L, M and N for accessory glands) were significantly different (Tukey $P$< 0.05).

### 4.3.5. Effect of colonization and genetic modification on accessory glands and testes size

The mean testes size (surface of image) of 156 males across four experimental groups, Field Mopti, KIL, EE and Vida was 0.035mm$^2$ (0.034-0.036CI) (Fig.4.22A), While the mean of male accessory glands (MAGs) size (surface of image) of 157 *An.gambiae* s.s. glands for the same mosquitoes was 0.030mm$^2$ (0.029-0.031CI) (Fig.4.22B). The range of MAG size was 0.014- 0.045 mm$^2$, while testes size range was 0.021- 0.053mm$^2$ (Fig.4.22AB).

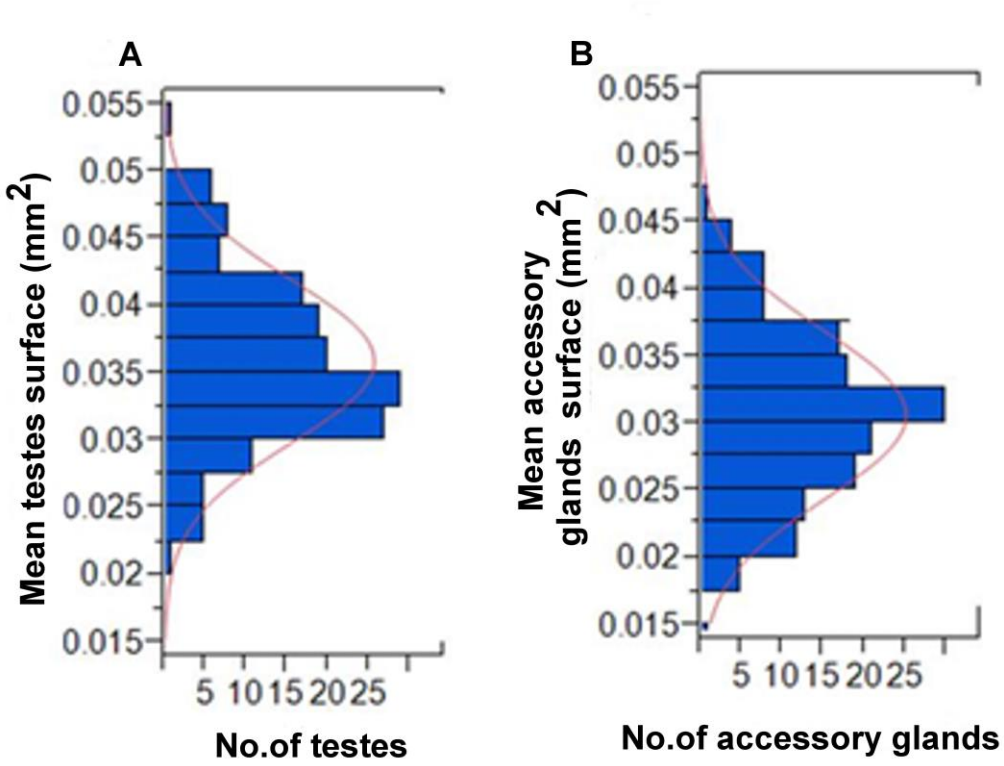


Fig.4.22 (A). Frequency distribution of testes size (mm$^2$) (surface of testes image) (*n*= 156) and (B) accessory gland size (mm$^2$) (surface of accessory glands image) (*n*= 157) in *An. gambiae* s.s. males (all experimental groups combined).

The highest mean of testes size (surface of image) across the four studied groups was 0.039mm$^2$(0.037-0.041CI) in KIL, while the lowest mean of testes size was 0.031 (0.030-0.033CI) in Field Mopti. However, Field Mopti had a largest accessory glands size across all four studied groups (mean=0.036mm$^2$ (0.034-0.37CI)), whereas Vida had a smallest accessory glands size amongst all studied groups (mean=0.027mm$^2$ (0.026-0.029CI)) (Table 4.2).

Table 4.2. Mean of male testes size mm$^2$ (surface of image) and accessory glands size mm$^2$ of *An. gambiae* across four studied groups: non-transgenic strains (Field Mopti, KIL), transgenic strains (EE, and Vida). The letters CI are indicative of the confidence interval range and SD letters are revealing the standard division

| Mosquito line | Testes size (mm$^2$) (surface of image) Mean± SD | Sample size | Accessory glands size (mm$^2$) (surface of image) Mean± SD | Sample size |
|---|---|---|---|---|
| Field Mopti | 0.031±0.003 (0.030-0.033CI) | 39 | 0.036±0.005 (0.034-0.037CI) | 42 |
| KIL | 0.039±0.006 (0.037-0.041CI) | 40 | 0.029±0.004 (0.027-0.030CI) | 39 |
| EE | 0.036 ±0.005 (0.034-0.038CI) | 35 | 0.029±0.006 (0.026-0.031CI) | 37 |
| Vida | 0.035±0.005 (0.034-0.037CI) | 42 | 0.027±0.004 (0.026-0.029CI) | 39 |

Body size (measured as wing length) was measured and recorded for males of each of the four groups. The average wing length of 165 males of Field Mopti, KIL, EE, and Vida was 2.799mm (2.773-2.824CI). Overall, There was a significant difference in body size (wing length) between the four groups (ANOVA: $F_{3,165}$= 4.66, $r^2$= 0.079, $P$=0.003). Pair-wise comparisons showed a significant difference between EE and KIL as well as Field Mopti ($P$<0.004 in all cases). It seemed that Field Mopti and KIL had a largest body size (Field Mopti, mean =2.847(2.799-2.896CI), KIL, mean = 2.830 (2.781-2.878CI)). Because of that difference, testes size and AC size were corrected for effects of body size by dividing them by wing length$^2$

The relationships between body size and sexual organs size were studied to assess whether large males have large sexual organs or not. Overall, there was no relationship between mean testes size and male body size amongst all combined groups (Linear regression: $n$=155, $t$=4.15, $r^2$=0.005, $P$=0.780) (Fig.4.23A). A positive overall relationship was observed between mean accessory glands size and male body size amongst all combined groups (Linear regression: $n$=156, $t$=-4.15, $r^2$=0.320, $P$<0.001) (Fig.4.23B).

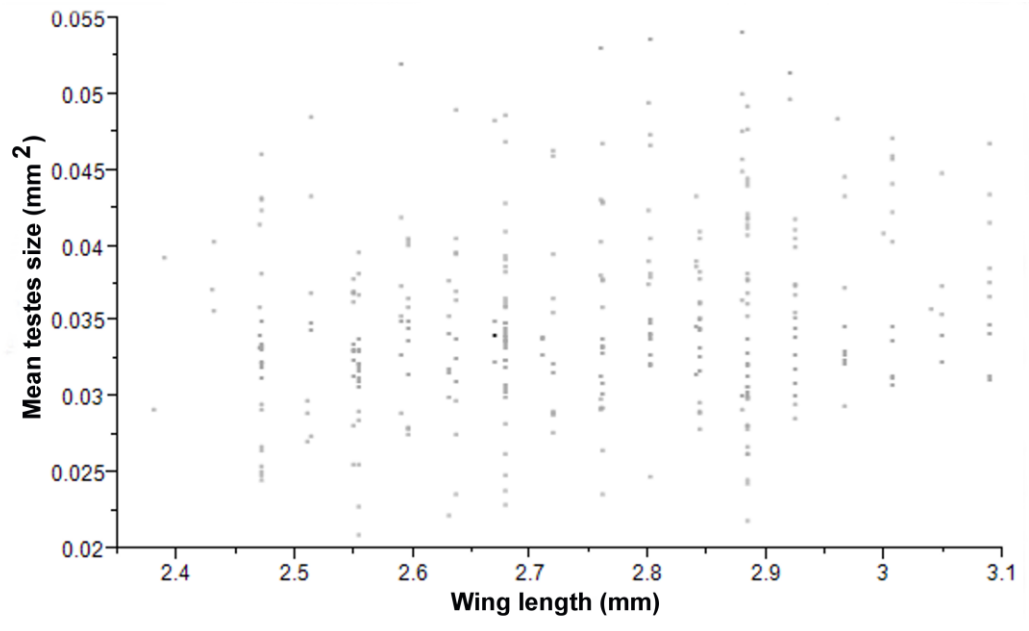


Fig.4.23 (A). Linear relationship between body size (wing length ($mm^2$)) and male testes size ($mm^2$) (surface of testes image) in all combined experimental groups (Field Mopti, KIL, EE, and Vida, *n*=155).

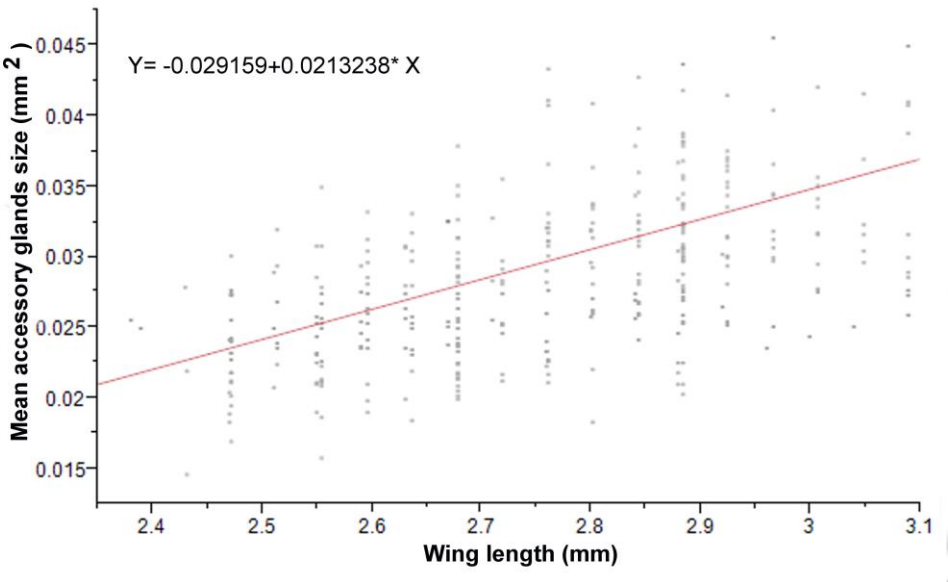


Fig.4.23 (B). Linear relationship between body size (wing length ($mm^2$)) and male accessory glands size ($mm^2$) (surface of AC image) in all combined experimental groups (Field Mopti, KIL, EE, and Vida, *n*=156).

The relationship between body size (wing length) and testes size and accessory glands size were studied in each group separately. It was found that there was no relationship between body size (wing length) and testes size in any of the experimental groups (Linear regression: $P>0.438$ in all cases). However, a very strong relationship between body size and accessory gland size was found in three groups (Linear regression: Field Mopti $n= 42$, $t= -2.7$, $r^2= 0.433$, $P<0.001$, EE $n=37$, $t=-2.75$, $r^2=0.439$, $P<0.001$, Vida $n=38$, $t=-2.32$, $r^2=0.483$, $P<0.001$). But there was no relationship between accessory glands size and male body size in KIL (Linear regression: $n= 39$, $t= -1.5$, $r^2= 0.078$, $P=0.084$).

Testes size and accessory glands size were corrected for the effects of body size by dividing them by wing length$^2$. The two variables were then checked for normality once more. Testes size and AC size distribution overall showed that the two variables did not deviate from normality in all four studied groups (Shapiro-Wilkinson: $P>0.199$ in all cases).

Testes size (surface of testes image) showed a significant difference overall amongst the four studied groups (ANOVA: $F_{3,155}= 10.22$, $r^2= 0.168$, $P<0.001$) (Fig.4.24). Pair-wise comparisons showed that Field Mopti significantly differed from other groups ($P<0.001$ in all cases) (Fig.4.24). On the other hand, accessory gland size (surface of MAG image) demonstrated a significant difference overall across the four studied groups (ANOVA: $F_{3,156}= 20.39$, $r^2= 0.287$, $P<0.001$) (Fig.4.24). Pair-wise comparisons confirmed Field Mopti significantly differed from other studied groups ($P<0.001$ in all cases). Also, there was a significant difference between EE and Vida ($P=0.046$).

In Field Mopti, testes size was significantly smaller than in KIL and transgenic stains, EE, and Vida. In contrast, accessory glands size were larger in the progeny of field

individuals than in non transgenic strain, KIL and transgenic groups, EE and Vida (Fig.4.24)

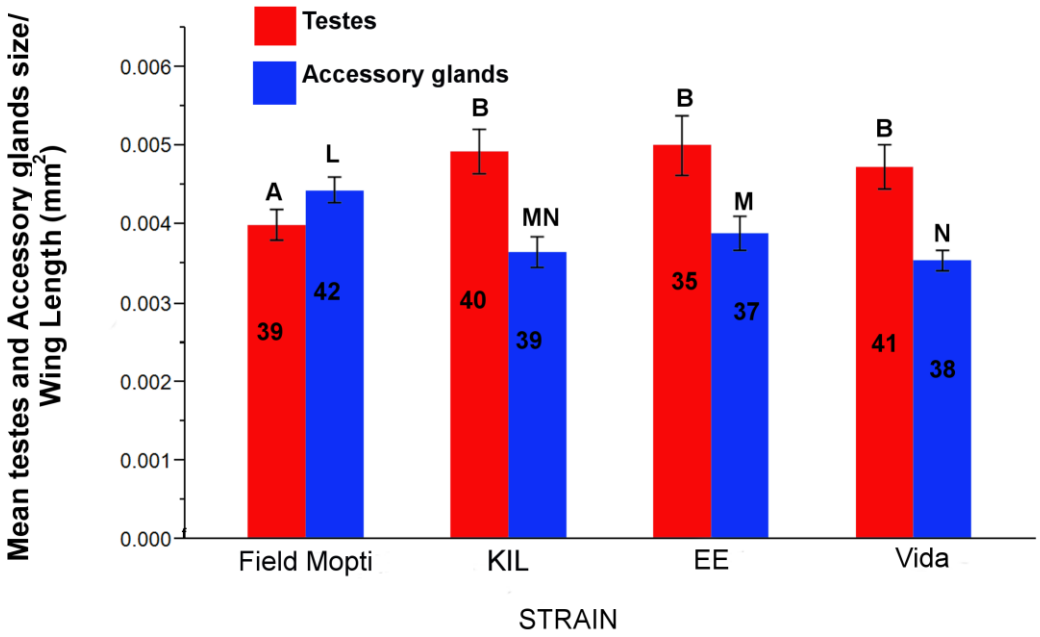


Fig.4.24. Mean testes (red) and accessory glands (blue) size (mm) corrected with wing length ($mm^2$) (±95%CI) in non-transgenic (Field Mopti, KIL) and transgenic (EE, Vida). Bars labeled with different letters (A, B and for testes; L, M and N for accessory glands) were significantly different (Tukey $P< 0.05$).

## 4.4. Discussion

The comparative impacts of both sperm quantity and quality have formed the subject for much recent research into sperm competition. In the fruit fly, sperm size has been observed to differ widely between species and is thus considered to be important in the evolution of those species (Joly *et al*., 2004). Heteromorphism is a known phenomenon in several *Drosophila* species, which means that sperm cells are produced at different levels of length in the same male (Bressac and Hauschteck-Jungen, 1996). Males in *Drosophila* species produce two types of sperm size, long and short sperm cells (Joly *et al*., 1989)

(Pasini, 2010) but in *An. gambiae* s.s. mosquitoes there is great variation in sperm length (Klowden and Chamber, 2004; Voordouw *et al.*, 2008). In the present study, a large variation in sperm length was found across seven groups of colonized strains. The sperm variation between groups which reported here may have a fitness consequence on male mosquitoes whether, is a positive or negative effect is, at present, unclear and further studies are needed. The mean sperm size was 0.201mm (0.189-0.214CI) with a range of mean sperm length between 0.051 and 0.531mm. Unfortunately, sperm size in *An.gambiae* s.s. is poorly documented. The first study, by Klowden and Chamber (2004), reported that mean sperm length in *An. gambiae* s.s. males was 280μm, but in fact this varies widely, range between 26 and 500 micrometers. Another study by Voordouw *et al.* (2008) recorded that the mean sperm length (mean ±SE) was 197±7.2μm with a range of between 100 and 250μm. In *Drosophila* species however there is much documentation concerning sperm quality. For example, (Pitnick *et al*., 1995) investigated sperm length variation amongst 42 species within the genus *Drosophila* and observed a large variation between species with a range of between 0.32 and 58.3mm. An additional study by Joly *et al.* (1989), which measured sperm size in 27 *Drosophila* species, found a mean length which ranged from 0.113mm in *D. obscura* to approaching 20mm in *D. littoralis*.

Sperm size variation may be due to different selective pressures correlated to both biological and physiological limitations such as fertilization and sperm competition (Joly *et al.*, 2004). It is possible that sperm length impacts upon reproductive success as a result of interaction with the female reproductive organs, or alternatively through competition where multiple mating occurs before the storage of sperm is achieved (Joly *et al.*, 2004). Further researchers have posited that the size and shape of the sperm tail, rather than being tailored to the most effective form for its journey to the fertilisation site, is rather a feature of compatibility which acts as a mechanism for species specific discrimination (Joly *et al.*,

2004). However, another possibility in the case of *Drosophila* is that the differences noted are a result of phenotypic plasticity (Joly *et al.*, 2004): that is, physiology, morphology and behaviour are affected by alterations in environment (Price *et al*., 2003). This has positive results for fitness (Price *et al.*, 2003). Changes in environmental conditions introduce selection pressures under which variation from the features of preceding generations may present benefits (Price *et al.*, 2003). The production of long sperm necessitates a longer development period to achieve sexual maturity (Pitnick and Markow, 1994b, Pitnick *et al*., 1995), possibly because of the need for larger testes (Pitnick, 1996), and also places restrictions upon the numbers of sperm produced (Pitnick, 1996; Pitnick and Markow, 1994b). In addition, female fitness is also linked to the production of long sperm in sperm-monomorphic *Drosophila* (Pitnick and Markow, 1994a b, Pitnick *et al*., 1995, Pitnick, 1996). A further discussion of sperm length variation will be presented in Chapter 5.

Reproductive activity is the most challenging characteristic of behaviour to transfer successfully to laboratory conditions (Baker, 1964). SIT depends upon success in this area (Fried, 1971). Moreover, the impact of colonization on sperm length is unknown. In the current study, it was observed that sperm length is greatly reduced in old colonized strains compared to the progeny of field-collected females or newer and/or refreshed strains. Variation when comparing colonised and wild mosquitoes occur gradually through the process of colonisation, and are considered using founder, drift and selection models (Benedict *et al*., 2009). In this view, laboratory colonies become homogeneous and less competitive, while increasingly differing from field populations in genetic terms (Benedict *et al.*, 2009). However this does not present a full picture (Norris *et al*., 2001), as certain countering forces must also be taken into account, although the genetic foundation of these is currently not clear (Munstermann, 1994). For example a study of isozyme polymorphism in two

$12^{th}$ generation brother-sister mated strains of *Aedes triseriatus* revealed surprisingly high polymorphism levels in more than one locus, leading the authors to suggest that heterozygosity is strongly selective, occurring here through the balancing effect of recessive lethal (Matthews and Craig, 1987). In other studies with less intensive inbreeding (Knop *et al*., 1987) heterozygosity remained close to or higher than parental levels, supporting this hypothesis. Research in Anophelines and interestingly in *Anopheles gambiae* yield similar results, and in *Anopheles gambiae* s.s., paracentric inversion polymorphism has been shown to display higher than expected levels both in the wild and under laboratory conditions (Brooke *et al*., 2002). Resion (2003) reported that mosquito colonized strains became very inbred within a few generations. In the current work, there was a link between sperm length and inbreeding degree as measured by age of colony. However, to estimate inbreeding degree directly molecular techniques assessing heterozygosity and allelic richness would be needed.

Fitness can be identified as an individual's ability to pass genes to the subsequent generation (Massonnet-Bruneel *et al*, 2013). For mosquitoes, fitness can be measured by survival (larvae/adult), larval growth rate, adult emergence rate and development time, and by reproduction measured by fertility, fecundity and mating competitiveness (Massonnet-Bruneel *et al*, 2013). Rearing insects under laboratory conditions could lead to reduced male mating competitiveness due to the impact of inbreeding depression along with diversity loss as a result of genetic bottlenecking (Ferguson *et al*, 2005). Male mating competitiveness with wild populations can decline and it has frequently been found that over time assortative mating behavior can develop quickly under laboratory conditions; within just a few generations of colonization (Reisen, 2003). For instance, sterile males of both *Culex tarsalis* & C*x. Tritaeniorhynchus,* despite remaining competitive under laboratory conditions, could

not compete effectively for field females due to an assortative mating phenotype induced by laboratory colonization (Reisen, 2003). Also, Benedict & Robinson (2003) reported that before sterile males can be released, it must be known that males would mate with field females because adaptation to mating conditions on the laboratory may have a negative affect on their performance in a field setting (Benedict & Robinson, 2003). The inability of laboratory–reared males to copulate with field-type females was interpreted as the result of assortative mating behavior developed under laboratory conditions (Davidso *et al*, 1970; Reisen, 2003). Also, colonized insects become less choosy with regards to their partner for mating under limited space (e.g. cages) such that colonized insects will mate with both wild and laboratory strains whereas field-derived insects will not mate with colonized insects because for their unnatural mating behavior (Howell and Knols, 2009). In order to produce mosquitoes for the sterile insect technique (SIT), a method must be found to mass-produce competitive yet sterile males which will mate with wild females (Benedict *et al*., 2009).

In the current study**,** sperm length was used to determine the quality of genetically modified *An. gambiae* s.s. males in order to study genetic manipulation costs and colonization costs on male fitness. It was found that transgenic mosquitoes had greatly shorter sperm length than field individuals. The transgenic strain EE did not differ significantly from the original KIL strain, but sperm size was reduced in the Vida strain. The reasons for such a finding could include the impact of bottleneck, inbreeding, genetic manipulation costs and colonization as well as genetic modification together.

The term population bottleneck is used to signify a significant drop in the size of population which is not permanent (Futuyma, 2009). One example of this is where small

numbers of founders establish a new colony, and grows slowly, leading to the founder effect, or random genetic drift (Futuyma, 2009). This is less likely to occur where the population grows quickly, as heterozygosity in this case will not be greatly affected (Futuyma, 2009). (Fowler and Whitlock, 2002) investigated the effect of inbreeding and environmental stress (high temperature and high adult density) on phenotypic variance and genetic variance in 52 lines of *D. melanogaster* created by single-pair population bottlenecks. They found that inbreeding does not increase the developmental effects of stress. In the same way, environmental stress does not increase the level of inbreeding depression. There was no relationship between genetic variance and environmental stress: however, alterations in environmental conditions rendered variance components of inbred strains less predictable, with certain strains showing markedly different sensitivity to environment while most remained similar to control groups (Fowler and Whitlock, 2002). Both phenotypic and genetic variance are made less predictable by changed environmental conditions, while such changes also throw up greater possibilities for divergence in key parameters for evolution (Fowler and Whitlock, 2002). Other explanations exist for the finding of the current study related to sperm length reduction in transgenic lines due to the insertion location in the chromosome, and the cost of gene expression (Catteruccia et al., 2003, Li *et al*., 2008). Catteruccia *et al*. (2003) report that the frequency of the transgenic allele declined harshly over time in *An. stephensi* transgenic mosquito populations. Transgene frequencies were lower than expected levels and continued to decline until they became extinct within four to sixteen generations. This decline and eventual loss of transgenic alleles may be related to expression costs or chromosomal insertion, or may be due to links fixed as homozygous transformed lines were founded with deleterious alleles. The findings may indicate that fitness is negatively affected by the insertion, or the expression, of transposon-engineered genetic cassettes (Catteruccia *et al*, 2003; Li *et al*,

2008). Moreover, the reduction in sperm length in transgenic lines could be an impact of colonization as discussed earlier, as the KIL originals for transgenic mosquitoes have been reared under laboratory conditions for more than thirty years. Therefore, the sperm reduction in genetically modified mosquitoes could be a combination of effects from both colonization and genetic manipulation.

To explain the reduction in sperm length across the colonized strains as mentioned earlier, there are two assumptions: natural selection for laboratory conditions or an inbreeding effect. Natural selection may be described as a response to environment leading to different phenotypes being more or less successful in terms of reproduction (Campbell and Reece, 2002), or as variation in the part played by various entities or groups of entities in the generations which follow (Futuyma, 2009). Differences may be caused by alleles or genotypes and are generally heritable (Futuyma, 2009). Evolution refers to variation between generations in terms of the occurrence of alleles in a group, and comes about as a result of natural selection (Campbell and Reece, 2002). Microevolution includes small-scale change at the level of single genetic loci, through which natural selection works to allow a group to change in response to environmental conditions, by selecting such changes as bring benefit, thus repeating those changes more than others (Campbell and Reece, 2002). Only differences with a genetic basis may be passed to subsequent generations however and thus contribute to evolution (Campbell and Reece, 2002).

Inbreeding is reproduction which takes place between close kin, and may result in a decline in the fitness of progeny termed inbreeding depression (Hedrick and Kalinowski, 2000, Futuyma, 2009). Further, such depression may apply to the majority or all of a population where genetic drift has occurred, fixing deleterious alleles: this may occur where groups are small or population bottlenecks have taken place, and are of particular note to conservationists because of the frequency with which this can occur in highly

endangered species with their small populations (Hedrick and Kalinowski, 2000; Futuyma, 2009). Homozygosity lies behind inbreeding depression, through its effect in partially recessive harmful mutations and in overdominance, or alleles at loci with heterozygote advantage (Charlesworth and Willis, 2009, Futuyma, 2009). While deleterious alleles exist at low levels within populations, overdominant alleles at a locus exist at higher levels due to balancing selection (Charlesworth and Willis, 2009, Futuyma, 2009). The negative impact of inbreeding depression upon offspring's genetic value is significant (Charlesworth and Charlesworth, 1987). In a study by (Hunt and Drummond, 1983) reciprocal crosses of field and colony groups of ticks, reproduction characteristics were comparable between wild females whether mated to colony or wild males, while those of colony females were similar whether mated to wild or colony males.

Here, it was hypothesized that the decrease in sperm size in relation to colonization resulted from inbreeding effects (accumulation of detrimental alleles in homozygous state) rather than from selection for laboratory conditions and this hypothesis was tested by creating crosses between two old strains with reduced sperm length. It was reasoned that if the shortest sperm occurs as a result of natural selection, then sperm length in crossing will be as short as their parents for reasons of inheritance, but if the sperm length in crossing lengthens, this impact of inbreeding as the accumulation of recessive alleles that have deleterious effect would be fewer in the progeny of non-related parents. As predicted, the sperm length of crosses was fully restored. This validates the hypothesis which supports the finding as a result of the impact of colonization (inbreeding depression) on mosquito sperm length rather than natural selection.

In the present study, using a standard microscope, images were captured and it was possible to measure organs' actual size in *An.gambiae* s.s. males. Here, the size of testes and accessory glands were estimated. The mean testes size from all colonized and genetic

strains was 0.035mm$^2$ (0.034-0.036CI), with a range of 0.003-0.053mm$^2$. Meanwhile, the mean accessory gland size was 0.028mm$^2$ with a range of 0.015-0.045mm$^2$. In another study, Mahmood and Reisen (1982) recorded the length and width of testes in *An. gambiae* s.s. (mean ±CI) (327±17 and 121±5 µm respectively) at seven days, and also measured 238±13µm for length of the accessory glands and 127±5µm for width at the same age. The size of insects' organs such as testes and accessory glands may be used to predict the age of males (Mahmood and Reisen, 1982, Huho *et al.*, 2006), and also to study male mating status (Mahmood and Reisen, 1982). Reductions in the volume of the male accessory glands (MAG) volume found in those species which mate more than once indicate that the glands play a role in controlling female mating frequency (Mikheyev, 2004). In *Cyrtodiopsis dalmanni*, rapid accessory gland growth preceded sexual maturity, with mature sperm bundles and motile sperm being present in the testes several days prior to maturity. Further, accessory gland size impacted upon mating frequency for males (Baker *et al*., 2003). This relationship may be due to a restriction placed upon spermatophore numbers or components by gland size, but this remains uncertain (Baker *et al*, 2003). Chapters Five and Six will provide more detail about the role of accessory glands in male reproductive success.

Testes are significant in creating and storing sperm: however, there is evidence that female behaviour after mating, including egg-laying and avoidance of further mating, is not dependent in *An. gambiae* on sperm, but rather is under the influence of male accessory gland secretions (Thailayil *et al*., 2011). (Hatsumi and Wakahama, 1986) observed a positive correlation between testis length and sperm length in *Drosophila nasuta* subgroup. Further, (Pitnick and Markow, 1994b) found positive correlation between re-mating in females and testes volume, as well as to a lower degree with relative dry weight of testes in *D. pachea* and similar species. These *Drosophila* may have developed large testes due to

their necessity for the production of large sperm (Hatsumi and Wakahama, 1986, Pitnick and Markow, 1994a).

The data here showed that accessory glands in colonized and transgenic mosquitoes were significantly smaller than in the progeny from field captured females. In contrast, testes size was larger in colonized strains than in the progeny of field individuals. These patterns are thought to result from adaptations to laboratory conditions. This variety however is problematic to measure, due to the hidden pool of recessive genes in heterozygous groups and the limited number of genes which actually display their capacity to induce change, and many variable traits not being easily observable, as they may be physiological rather than morphological in nature, necessitating highly involved genetic techniques which are limited in their availability (Craig 1964).

Male body size (as measured by wing length) could be a factor with influence on male reproductive success (Ng´hab *et al*., 2008). The data here reported that there was no relationship between body size and sperm length or testes size across all studied groups. This means that large males should not have large testes with the longest sperm, and nor do small males have small testes with the shortest sperm. So, it was not possible to predict the testes size and sperm length from male body size. However, (Pitnick *et al*., 1995) found a significant relationship between sperm length and male body size among 42 *Drosophila* species. The authors justified their finding as relating to delayed male maturity in development of large testes as a cost of producing long sperm. A study by (Ponlawat and Harrington, 2007) aimed to uncover the relationship between male body size of *Ae. aegypti* and number of spermatozoa in their testes. Researchers observed a significant relationship between total spermatozoa numbers and male body size, as large males (2.27mm wing length) produced more sperm cells than did small males (1.85mm wing length) suggesting that greater size was linked to enhanced reproductive fitness. Sperm production in

adulthood may increase male capacity for multiple mating (Ponlawat and Harrington, 2007).

There is a body of evidence to support the importance of adult size for reproductive success in *An. gambiae*, with clear implications for SIT programmes. As an example, larger females appear to be universally preferred for mating even in conditions of high demand for females, indicating that this is related to egg capacity (Okanda *et al*., 2002). Research indicates that medium-sized males found six times greater reproductive success than others in the swarm (Ng'habi *et al.*, 2008). At the same time however, large males had more energy stores and lived for 13% longer when compared with medium sized males (Ng'habi *et al.*, 2008) When the 3 size groups were examined separately it was found that males which mated tended to be of greater size than males who failed to mate (Ng'habi *et al*., 2008). Medium sized males were considered more successful possibly due to a balance of agility and energy storage which favoured them, or because of size parity with females (Ng'habi *et al*., 2008). Another finding in the present data is that a positive relationship was observed between accessory gland size and male body size amongst all studied groups. This correlation indicates that male body size was a major predictor of MAG size. Therefore, it was expected that large males would have more reproductive success than small males, as found in a study by Ponlawat and Harrington (2009) in which reproductive success was high in large males because they delivered more sperm for the number of females than did the smaller males. Also, accessory gland proteins (Acps) play a key role for the male in successful reproduction when transferred to females during copulation (Tram and Wolfner, 1999) regarding which greater detail will be provided in the following chapter.

## Conclusion

The ability of males to mate and transfer sperm to wild females in mating swarms is critical for the success of SIT. It is thus very important to further the understanding of the reproductive ecology and physiology of Anophelines. Here, the impact of colonization and genetic engineering on potential correlates of reproductive success in *An. gambiae* s.s. males was assessed by comparing sperm quality, testes size and accessory gland size in the progeny of wild mosquitoes versus transgenic and non-transgenic mosquito colonies. The results indicate that inbreeding associated with the colonization and/or genetic modification processes strongly affects sperm length. It was hypothesized that the decrease in sperm size in relation to colonization resulted from inbreeding effects (accumulation of detrimental alleles in the homozygous state) rather than from selection for laboratory conditions. The hypothesis was tested by creating crosses between two old strains with reduced sperm length (Male KIL x Female Kisumu). As predicted, the sperm length of crosses was fully restored. In contrast, the offspring of field collected females and recently colonized strains tended to have smaller testes than long established strains, suggesting potential selection on this trait. The accessory glands of the progeny of wild-caught females were significantly larger than all colonized strains. In the current work, it was observed a variation in sperm length between groups but weather it is fitness positive or negative is at present unknown.

# Chapter 5

# Study of sperm quality, sexual organ size and male accessory glands' protein production in hybrid *Anopheles gambiae* s.s. males as well as inbreeding strains

## 5.1. Introduction

Female mosquitoes of the genus *Anopheles* are carriers of *Plasmodium* parasites which cause malarial disease around the world. One indirect strategy to combat malaria is the release of sterile male mosquitoes which compete with wild males and transfer the sterile components (sterile sperm) to females, as a result of which the field population declines (Lofgren et al., 1974). However, this approach is only viable where sterile male mosquitoes successfully compete in terms of mating. The outcome from previous work on SIT of over more than twenty years showed that sterile males, when placed in competition with wild males in the field, were not successful (Benedict and Robinson, 2003). Therefore, it is essential to improve quality in the sterile Anopheline males produced in the laboratory, and this firstly requires a good understanding of the reproductive biology in the male of this species, in order to allow accurate evaluation of male fitness (Benedict and Robinson, 2003).

Mature sperm are generated in the testes (Huho et al., 2006). Earlier studies among *Drosophila* species revealed that the sexual maturation rate in males varies considerably from one species to another, being slower than that of females in 24 species, comparable with females in five species and faster than the female rate in 13 species (Pitnick *et al*., 1995). Great variations are observed in sperm length in males *An. gambiae* (Klowden and Chambers, 2004). Studies show that sperm polymorphism is found in several species. For example, in moths (Lepidoptera) (Morrow and Gage, 2000), beetles (Coleoptera) (Green, 2003), and cockroaches (Pterygota) (Harris *et al.*, 2007). There are suggestions (Voordouw

*et al*., 2008) that variation in sperm length in *An. gambiae* could affect female oviposition behaviour. In mammals, sperm length is positively associated with sperm swimming speed (Gomendio and Roldan, 2008). The male`s competitive ability may be measured either through the average swimming speed or through determining the maximum swimming speed (Gomendio and Roldan, 2008). Male fertility is positively affected where sperm are long, with a resultant high swimming speed (Gomendio and Roldan, 2008). Among mammals however, sperm length is not selected for in sperm competition (Gage and Freckleton, 2003). Numerous studies in vertebrates have contributed greatly to the current understanding of the male reproductive success. For example,Gomendio and Roldan (1991) found in both primates and rodents a positive correlation between sperm length and sperm velocity. Also, the same authors observed that males in polyandrous species have longer sperm than in monoandrous species. Male body size in *Drosophila hydei* was also identified as having implications for reproductive fitness, with smaller body size negatively related to the number of offspring, number of sperm transferred during mating, and the number of sperm bundles developing in the testes at a given time (Pitnick and Markow, 1994b). In addition, smaller males required longer to reach reproductive maturity post-eclosion, and did not have as many mating partners as did their larger counterparts (Pitnick and Markow, 1994b). In addition, a significant relationship was found between male age at maturity and sperm length, and another positive correlation between age at maturity and body mass (Pitnick *et al*., 1995).

Numerous studies establish a positive relationship between body size and sperm length, and body mass and testes as well as accessory glands size (Pitnick and Markow, 1994b, Pitnick, 1996, Gage and Freckleton, 2003, Garamszegi, 2005, Rogers *et al*., 2005a). A significant relationship was found between sperm length and body size (wing length) in *An. gambiae* (Voordouw *et al.*, 2008); in the bumblebee *Bombus terrestris* (Baer *et al*.,

2003); in butterflies (Gage, 1994) and in *Drosophila* (Pitnick *et al*., 1995). Studies have shown that *Drosophila hydei* males which were comparatively small in overall size, while having less length and thickness in the testes than larger males, nonetheless dedicated a greater proportion of their growth to the testes (Pitnick and Markow, 1994b). There is a significant body of data that connects the size of sexual organs to male reproductive success. For example, the newly emerged adult males of a stalk-eyed fly (*Cyrtodiopsis dalmanni)* have small testes and accessory glands which increase gradually to reach sexual maturity. Accessory gland size in this species is an essential factor in sexual maturity and mating frequency (Baker *et al*., 2003). In butterfly species, testes size is a good indicator of male reproductive success, and males have large testes in species with strong sperm competition (Gage, 1994). Large testes size is also found in mammalian species that have multiple mating (Soulsbury, 2010). Two factors, singly or in combination, can affect the quantity of tissue in the testis capable of producing sperm (Lüpold *et al*., 2009). These factors are gross size; and how much of the testis' volume is composed of seminiferous and how much of somatic tissue. A significant amount of research work has shown that a competitive environment is related to larger testes size proportionally to body size, and in fact this measurement is frequently used to assess the degree of sperm competition (Lüpold *et al*., 2009). In contrast however, the relationship between levels of competition and either the balance of tissue types or speed of sperm production within the testes has been less widely researched (Lüpold *et al*., 2009). Lüpold *et al*. (2009) approach this in their study of New World blackbirds (Icteridae), concluding that testes histology is an indirect basis for selection in that species. This finding was based upon the relationship found in the study between this histology and sperm length, and also upon the link seen between sperm competition and the proportion of sperm-producing tissue (Lüpold *et al*., 2009). There is plentiful data to indicate that the incidence of sperm competition is linked with large size

among many taxa. For instance, feral cats, *Felis catus* L., when living in rural areas, have a larger testes size than individuals living in extreme environments (Say and Pontier, 2006). In mammals, there was no relationship between sperm length and testes size (Gage and Freckleton, 2003).

Accessory gland proteins (Acps) play a key role for the male in successful reproduction. After mating however, Acps remain of importance, for example being required in order for the female to successfully store sperm (Tram and Wolfner, 1999). In *D. melanogaster,* Acps also work to reduce the likelihood of the female mating again, as well as to facilitate a greater numbers of eggs to be produced and laid: these effects last for 24 hours after copulation, and for longer only where sperm is stored by the female. Tram and Wolfner (1999) suggest that this limits the effects to situations where they are of benefit to the female: i.e. where sperm are present. However, Wolfner (2002) also finds that the presence of Acps reduces female life expectancy. Further roles of Acps include being the constituents of the mating plug firmed in the female reproductive tract after copulation, and as antibacterial agents, with potential protective benefits for males, females, sperm and eggs (Lung and Wolfner, 2001; Lung *et al.*, 2001). Rogers *et al*. (2009) identified twenty-seven MAGs proteins in *An.gambiae* by using mass spectrometry analysis and RT-PCR methods. One of the most essential proteins in the glands was named Plugin (AGAP009368), found in MAGs and originating in the anterior region of the male accessory glands. It was demonstrated that a transglutaminase enzyme is responsible for the formation of the mating plug, through the connecting together of seminal proteins. Rogers *et al.* (2009) suggest that Plugin forms the primary substrate for TGase in the male accessory glands. Transglutaminase (AGAP009099) is required for the formation of the mating plug (Rogers *et al.*, 2009). Tripet *et al*. (2005), in their study of the effects of cross-mating in *An. gambiae* s.s. between M and S molecular forms, did not find any difference

in the effect of mating with either a male of the same form or an alternate form in preventing further female mating and stimulating egg-production, indicating that the proteins from the male accessory gland secretions involved in these processes do not differ between the two forms, in contrast to previous research on the topic (Tripet *et al*., 2005). Hatching rates and numbers of larvae surviving to adulthood were affected by cross-mating: a finding which the authors suggest may be due to the hybrid form rather than any effect of MAG secretions. Shutt *et al*. (2010) injected MAG secretions into the intra-thoracic region in both *Anopheles stephensi* Liston and molecular forms M and S of *An. gambiae* s.s., as well as crossing substances to be injected between these two forms of *An. gambiae* s.s., and found in all cases that further mating by females did not occur, thus indicating that the substances involved are the same in both forms. The authors further suggest, as a result of these findings that, where remating occasionally occurs in *An.gambiae* s.s., this is not as a result of mating incompatibility between M and S forms.

Several studies have observed the impact of male age on the quantity of male accessory glands proteins. For example, Nagalakshmi *et al*. (2004) used a direct Elisa method to detect DrmSP proteins (as a primary antibody) in the reproductive tissue of male moths (*Helicoverpa armigera*), and discovered that older males (3–7 days old) generated a greater amount of proteins than younger males (1-2 days old). Another study by Muse (1993) observed that in the grasshopper, *Zonocerus variegates* (Orthoptera), the newly emerged males have a lower level of male accessory gland proteins than that seen in older males. Muse (1993) also found that the amount of proteins declined by 17.6% during copulation. Male accessory gland size in the stalk–eyed fly *Cyrtodiopsis dalmanni* was smaller in mated males than virgin individuals, but the glands became larger again during the 48 hours post-copulation (Rogers *et al*., 2005a). Gland size correlated significantly with mating frequency, while there was no positive relationship between testes size or

length and mating status (Rogers *et al.*, 2005). *Aedes aegypti* males were found by Jones (1973) to be limited to copulation with approximately five mates, as although depleted reserves of spermatozoa and accessory gland secretions are rebuilt, most males were found not to seek further copulation,or were unable to ejaculate upon doing so (Jones, 1973). In addition, Ramalingam (1983) found that in males copulating with more than five females within 24 hours, accessory gland volume was diminished, by up to 67% upon full depletion, in line with the volume of secretions lost upon ejaculation. Ramalingam (1983) further states that following full depletion, the original capacity of the accessory glands could not be regained. Further, in bedbugs, seminal fluid was found to be depleted more rapidly than were spermatozoa, and it was a lack of seminal fluid which determined the limits of mating potential (Reinhardt *et al.*, 2011). Similarly, in the *D. melanogaster* male it was observed that copulation was limited to approximately five females, due to depletion of seminal fluid (Linklater *et al*., 2007).

In populations kept in captivity, restrictions in genetic variation become problematic. Such restriction may be assessed by determining the heterozygosity of individuals at one locus. Additionally, the alleles at one locus may be counted (Allendorf, 1986). Allele substitution is mainly attributed either to genetic drift or natural selection. However, genetic drift is also held to bear the major responsibility for variations in DNA sequences among species (Futuyma, 2009). In inbred populations, greater numbers of homozygotes are found. This may, according to the partial dominance hypothesis, lead to deleterious, recessive or partially recessive alleles being increasingly expressed. According to the overdominance hypothesis, inbreeding depression may stem from decreased numbers of heterozygotes and their consequent combination, with potential advantages over the homozygotes (Charlesworth and Charlesworth, 1987, 1999). The results of inbreeding depression are wide-ranging, including reductions in viability, fecundity and mating

success, and an increase in sterility, time taken for development and response to environmental stressors (Lacy, 1997; Roff, 1998; Bijlsma *et al.*, 1999; 2000; Pedersen *et al.*, 2011).

Inbreeding is likely to occur in mosquito colonies because of the colonization process. It was observed in chapter 4 that colonization strongly affects sperm length negatively. In addition, the male offspring of field collected females and recently colonized strains tended to have smaller testes than long established strains, suggesting potential selection on this trait. The accessory glands of the progeny of wild-caught females were significantly larger than all colonized strains. This impact of colonization may cause problems for mosquito releases in the SIT programme. For that reason, one easy solution which does not involve complicated back-crossing schemes, is to create heterozygous males from two inbred strains in order to take advantage of hybrid vigour (see Chapter 4).

*An. gambiae* has been subdivided into five chromosomal forms based on extensive Polytene chromosome studies (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985). These forms are Savanna, Bamako, Mopti, Forest and Bissau and each live in specific environmental zones (Toure *et al.*, 1983; 1998). *An. gambiae* is also subdivided into two molecular forms, M and S (Favia *et al.*, 1997; 2001) based on sequence divergence of the ribosomal DNA region on the X chromosome (Favia *et al.*, 1997: 2001).

So far, the previous literature has shown a lack of knowledge about *Anopheles* male reproductive success. The experimental hypothesis is that there will be a statistically significant difference between the four groups, Field Mopti, KIL, Mopti 2003 and Super males. The null hypothesis is that there will be no statistically significant difference between the four groups. In the current work, sperm length, testes and accessory glands size in four experimental groups were firstly determined, to establish whether or not there was a difference between inbreeding strains, Field individuals and Super males produced

by no relative crossing of M molecular forms, as used in the previous chapter with SM molecular forms. The relationship was then examined between body size and sperm length, and body size and size of sexual organs. After this, the amount of Plugin and Transglutaminase in virgin male accessory glands was investigated, to discover the original quantity of proteins before their transfer to females during copulation. In addition, the relationship between body size and the amount of male accessory gland proteins was examined. The inbreeding hypothesis was also examined to discover whether male quality could be improved under laboratory conditions.

## 5.2. Materials and methods.

### 5.2.1. Set-up of mating cages

Two days post blood feeding, mature female mosquitoes of KIL and Mopti 2003 strains laid eggs into white polystyrene pots lined with filter paper and half filled with distilled water. After hatching out, 200 first instar larvae were located in 8 trays for each strain (33 x 23 x 5 cm) each with one litre of distilled water. Larvae go through four stages and a pupa stage within 7-10 days after hatching. First instar larvae were supplied with one drop per tray per day of Liquifry (Interpet Ltd., Dorking, UK), then 40 to 80mg of ground flakes of baby fish food (TetraWerk, Melle, Germany) until pupation (see section 2.2.2, Chapter 2). Pupae were continually removed from larvae trays for sexing (see section 2.2.7, Chapter 2) and transferred to four adult mosquito cages which housed respectively, Mopti 2003 males, Mopti females, KIL males and KIL females. The pupae were put into pots filled with distilled water where they matured into adults. Adults were transferred to the separate cages using a mouth separator. The number of adults was recorded every day, and therefore the total number of adults was known. Mating cages were set up of 2 to 4-

day old mosquitoes as follows: 200 ♀KIL x 200♂KIL and 200♀Mopti 2003 x 200♂ Mopti 2003. There were two replicates for each mating cage. 24h post mating, the first blood feeding was provided, as mosquitoes need a blood meal to develop eggs (Gerberc, 1970).

To minimize the impact of environmental conditions such as temperature, humidity and light under artificial conditions that may affect larval and adult growth, both cages and trays, kept on shelves, were moved twice a day. To minimize confounding factors, the cages were labelled using a unique number and symbols in a different colour. 70-75% humidity, a temperature of 26-27°C and a 12:12 photoperiod were maintained at all times.

### 5.2.2. Creation of hybrid males

To produce hybrid individuals (Super males) a new combination was created by crossing 200 ♀KIL x 200♂Mopti 2003 as two replicates. 24h post mating, the first blood feeding took place. Thereafter, oviposition pots were added to the mating cages. After hatching out, 200 first instar larvae were moved to 6-8 trays. The larvae went through four stages and a pupal stage within 7-10 days after hatching. Male pupae chosen as Super males were transferred to adult mosquito cages in ♀KIL x ♂Mopti 2003 combinations. At the same time, male pupae of KIL and Mopti 2003 were sexed and transferred to two separate adult cages, and adult numbers were recorded each day.

### 5.2.3. Standard curve preparation and calculation

Standard curve stock solution was prepared by adding 10 lower male reproductive tracts in 880µl of PBS with PI to a microcentrifuge tube (1.5ml) for Plugin protein, and ten final abdominal segments in 440µl PBS with PI for Transglutaminase: these were stored under -80C° if stopping at this stage (Table 5.1). Each standard curve tube was subject to

30 seconds of grinding (Sonicator: Vibra-cell 300W High Intensity Ultrasound Processor, Sonics & Materials Inc, Connecticut, USA) to break down the tissue and tubes were then kept on ice. With each use, the homogenizer probe was cleaned using tissue soaked with absolute ethanol alcohol to prevent contamination between samples. All tubes were put in a Bioruptor sonicating water bath (Bioruptor $^{Tm}$ UCD-200, diagenode) for 10 minutes at high speed to break the tissues up further, as final abdominal segments surrounded with a hard cuticle were being used. The tubes were kept at -80$^{\circ}$C in a freezer (Thermo Scientific) for 10min, before being placed in a cold centrifuge (Thermo Fisher Scientific, Heraeus, Fresco21 centrifuge, Germany) for 15 minutes at 4$^{\circ}$C at 13.000 rpm.

Eight dilutions were prepared by adding 110µl of PBS plus PI to each microcentrifuge tube (1.5ml). 110 µl was taken from the stock tube and transferred to the first tube vortex, and 110 µl from the 1st tube was transferred to the 2$^{nd}$ tube, and so on until the 8$^{th}$ tube. Each time the tube was vortexed well and put on ice. Eight standard curves were prepared to make sure that at the end of the process there would be six dilutions, with a margin for error in case of mistake and to cover the range of sample absorbance. Two independent standard curves were prepared for each plate and loaded into two separate plates. Six 2-fold dilutions of standard curves were used to calculate the standard curve formula. Elisa work was carried out essentially as described in Wigby *et al.*, 2009. To minimize the variation between samples used to prepare standard curves, all males were at 4 days old and from the same generation or same egg batch. The standard curves were prepared freshly a few minutes before use.

Table 5.1. Summarized method of standard curve, amount of buffer used and number of male accessory glands in both proteins.

| Protein | Number of males | Volume of PBS with PI (Standard curve) | MAGS equivalent in a whole volume (Standard curve) | MAGS equivalent in 110µl |
|---|---|---|---|---|
| Plugin | 10 males | 880 µl | 20 | 2.5 |
| Transglutaminase. | 10 males | 440 µl | 20 | 5 |

The two independent standard curves for each plate were created by plotting the absorbance curves for each curve (x axis) against the dilution curve for each plate (Y axis). A best fit curve was drawn through the points in the graph using Polynomial Fit degree- 2 via JMP software. If both curves were good (by checking whether the R Square value =0.9 and the absorbance curves are within the linear section of the standard curve dilution), then the average was taken to create a formula for dilution curves. If one curve was better than other, then the standard curve was used to calculate a formula of dilution curves. To determine the male accessory gland protein in each sample, the obtained standard curve formula was multiplied by 4 for Transglutaminase and 8 for Plugin, as a sample dilution of 1/4 was used for Transglutaminase and 1/8 for Plugin (Table 5.2).

### 5.2.4. Measurement of protein quantity in male accessory glands via Elisa

In the current work, two primary antibodies were used to assess the quantity of each protein in the male accessory glands. Affinity–purified polyclonal antibodies against Transglutaminase (AGAP009099) and Plugin (AGAP009368) were raised in rabbits against peptide epitopes (Plugin: NEHRDPQNHQLPSSC; AGAP009099:

CGSRYTDPMEKKYES) by a commercial supplier (GenScript Corp., Piscataway, NJ 08854, USA). 24.57mg Transglutaminase powder was dissolved (as per the manufacturer's instructions) in 17.30ml of distilled water to create a 1.420mg/ml concentration. Meanwhile, 11.44mg Plugin powder was dissolved in 11.20ml of distilled water to obtain a 1.021mg/ml concentration. Aliquots of the supernatant were stored in 1.5ml microcentrifuge tubes at under -20°C. One tube from each protein was kept at 4°C for use each day.

Fresh samples of 4-day old virgin male mosquitoes of KIL, Mopti 2003 and hybrid males were chilled and dissected in a drop of PBS solution. The last two abdominal segments were cut off using two fine needles. The lower male reproductive tract was taken and put into a microcentrifuge tube with a cap (1.5ml) containing 220µl of PBS with proteinase inhibitors (PI). Each tube was labelled with a unique number and the name of the strain. The tubes of samples were stored in a labelled Cryobox at -80°C until needed for use. Each tube was labelled with the first letter of the group and a unique number. The tubes were stored vertically in the Cryobox to make sure that the last abdominal segment was surrounded by the buffer (PBS+PI).

The amount of Plugin as well as Transglutaminase was estimated in 4 independent experimental groups. The purpose of this experiment was to discover whether or not production of proteins had a significant difference between groups. Elisa protocol followed the methods of Wigby *et al.*, 2009 with some manipulation. Two primary proteins were used in the current work: Plugin (AGAP009368) and Transglutaminase (AGAP009099) (Table 5.2). Each sample tube was subject to grinding for 30 seconds. All tubes were put into a Bioruptor sonicating water bath for 10 minutes at high speed. The tubes were kept in a -80°C freezer (Thermo Scientific, Revco Ultima II series) for 10 minutes. The tubes were

put in a cold centrifuge (Thermo Fisher Scientific, Heraeus, Fresco 21 centrifuge) for 15 minutes at 4°C at 13.000 rpm.

Table5.2. Summarized methods of sample dilution in both proteins

| Protein | Sample dilution | Number of males | Volume of PBS with PI in µl | MAGS equivalent in a whole volume | MAGS equivalent in 110µl | Volume taken from stock sample in µl | Volume of PBS with PI in µl add to sample tube |
|---|---|---|---|---|---|---|---|
| Plugin | 1/8 | 1 | 220 | 2 | 1 | 27.5 | 192.5 |
| Transglutaminase | 1/4 | 1 | 220 | 2 | 1 | 55 | 165 |

Samples were mixed, and 50µl of each sample (aliquots of the supernatant) was loaded into an Elisa plate of 96 wells (flat plate –Immunoplate maxisorp NUNC). The plate was covered with screen film (micro-Amp clear adhesive films). Two replicate wells for each sample were tested (on separate plates) for each protein. Plates were incubated overnight at 4ºC in a cold room by shaking (Rota-test shaker, model R-100/TW, Luckham). The next day, all the work was brought to room temperature. A multichannel pipette was used for all steps and pipette tips were changed for each row in the plate and for each next step to prevent sample contamination. The plate was taken and the wells emptied of their contents and washed three times with 200µl of washing solution, PBS Tween (0.05%). The plate was then forcefully placed down on paper tissue to discard all the fluid. The wells were then washed three times with 200µl PBS Tween (0.05%). Each well received 100µl of blocking solution (containing 0.05% Tween 20 and 5% non-fat dried milk). The plates were covered with screen film and subject to one hour's incubation, with shaking. The wells were then emptied into the sink, and the plates once more forcefully placed on paper tissue to discard all the fluid (with no washing). Blocking

solution prevents non-specific binding to plate surface. Therefore, free space on the plate surface was saturated by the blocking solution.

After this, in place of blocking solution, 50µl of primary antibody in a dilution of 1:1000 in blocking solution was used for both proteins (Transglutaminase and Plugin). The plates were covered with screen film and incubated for one hour with shaking. The plates were taken and the contents of the wells were flicked out into the sink. The plate was then drained forcefully onto paper tissue to discard all the fluid, and washed three times with 200µl PBS Tween (0.05%). 50 µl of secondary antibody was added (Goat anti rabbit HRP 1:2000 in blocking solution). Plates were covered with screen film and incubated for an hour with shaking. The plates were taken and the well contents were flicked out. The plate was then drained forcefully onto paper tissue to discard all the fluid, and washed three times with 200µl PBS Tween (0.05%). The HRP bottle was kept at room temperature for one hour before use it (as per the manufacturer's instructions). 100µl of HRP substrate (TMB liquid substrate system for Elisa Sigma – T0440- 100mls) was added to the plates, which were incubated in a dark place for between 20 to 40 minutes for Transglutaminase and 10 minutes for Plugin, covered with foil and shaken at a slow speed. The reaction was stopped by adding 100µl stopping solution (9%H3PO4) when colour gradually appeared. The plates were read using a plate reader (Labsystems multishan multi-soft) at 450nm which was connected to a printer (Hewlett Packard, DeskJet, 320). The data was transferred to an Excel file and standard curves, calculation was done using JMP software. Washing liquid was kept overnight in the fridge before using it the next day, to avoid variations in temperature between the plates, which were kept in the cold room overnight. Clean tissue was placed under the Elisa plates to keep the plate bottom clean and avoid finger prints which would impact upon the reading in the Elisa plate reader All solutions, including blocking solution, primary and secondary antibody and stopping solution, were

prepared just a few minutes before use. Each time a new screen film was used to prevent plate contamination.

To minimize confounding factors, each group's tubes were labelled with a different colour pen, with the first letter of each group plus a unique number. A separate foam lid filled with ice labelled with the name of each group was used to hold the tube samples during the first day working with Elisa. A unique number was also written on the top of each plate.

### 5.2.5. Capturing images of testes and accessory glands

Virgin males of super males, KIL and Mopti 2003 at 7 days old were used for capturing images using a digital camera (Olympus, E520) at a magnification of 6.3 of a dissection microscope, as described in Chapter Four (section 4.2.3).

### 5.2.6. Testes, accessory glands and body size measurements'

All images of virgin males at seven days of super males, KIL and Mopti 2003 were taken using a digital camera (Olympus, E520) under a dissection microscope. ImageJ software was then used to measure the length and size of testes and accessory glands as shown in Chapter Four (section 4.2.4).

After estimating the quantity of Plugin and Transglutaminase proteins in each experimental group, body size was determined to find out the significant difference between males in the 4 experimental groups. Wing length measurement was made as shown in Chapter Two (section 2.2.6).

### 5.2.7. Preparation of sperm slides and sperm length measurements

Sperm slides were prepared for super males, KIL and Mopti 2003 and examined under a Nomarski microscope (at 16x magnification as well as condenser II) which was connected to a digital camera (Lumenera s Infinity X) as explained in Chapter Four (section 4.2.6).

Sperm cells of hybrid males (Super males), KIL and Mopti 2003 were examined under a Nomarski microscope and photographs were taken using a digital camera (Lumenera s Infinity X) which was connected to a Toshiba computer. The same methods were used as those illustrated in Chapter Four (section 4.2.7).

### 5.2.8. Statistical analysis

All data was checked for deviations from normality and heterogeneity of variances. Based on these tests and visual inspection of the data distribution, it was either analysed by ANOVA and by Tukey`s test to compare group pairs, or alternatively by using Kruskal-Wallis (3+ groups) followed by pairwise 1-Way Wilcoxon tests (2 groups). Linear regression was used to analyze the relationship between sperm length, testes and accessory gland size, and MAG proteins estimates, and wing length. All statistical analysis was conducted using the software JMP9 (SAS Institute, Inc).

## 5.3. Results

### 5.3.1. Sperm length

The mean sperm length of the 1548 *An. gambiae* s.s. sperm cells across four experimental groups, Field Mopti, KIL, Mopti 2003 and Super male (♂ Mopti 2003 x ♀ KIL) was 0.220mm (0.215-0.225CI). The range of sperm length was 0.014-1.031mm across the four experimental groups (Fig. 5.1.A). Sperm measurements from the same mosquitoes are not independent points; hence the statistical unit used for most subsequent analyses was the mean sperm length per mosquito. Consequently, the mean sperm length in the 78 *An. gambiae* s.s. males studied was 0.220mm (0.203-0.236CI). The range of mean sperm length of the same mosquitoes was 0.051-0.531mm (Fig. 5.1.B). Across all groups, mean sperm length deviated significantly from a normal distribution (Shapiro-Wilkinson test: $n$= 78, W= 0.938, $P$ <0.001).

The distributions of sperm length and mean sperm length per individual were also studied within the experimental groups. Comparison of sperm length distributions overall and averaged by individual for each of the experimental groups (Fig. 5.2.AB) show that sperm length deviated significantly from a normal distribution in all experimental groups. (Shapiro-Wilkinson: $P$<0.001 in all cases) (Fig.5.2.A). Mean sperm length did not deviate from normality in the majority of groups (Shapiro-Wilkinson: $P$>0.776 in all cases) (Fig. 5.2.B).

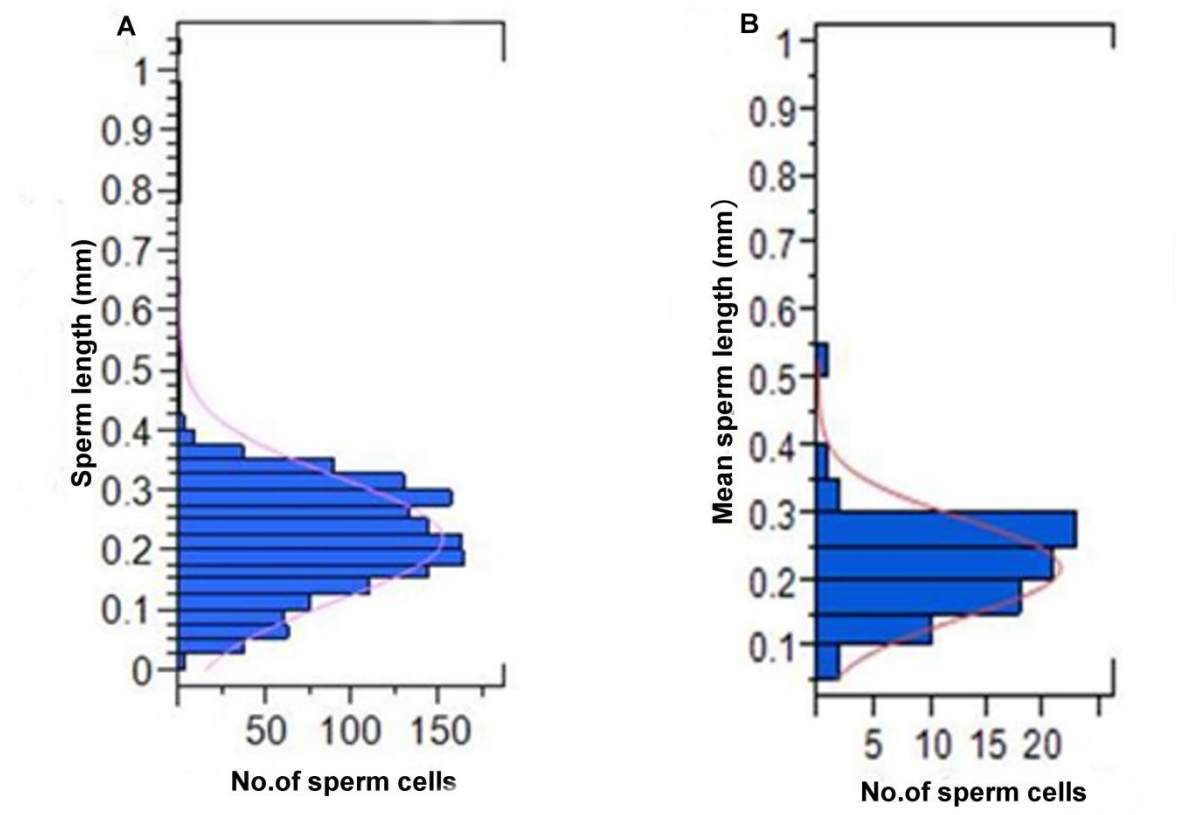


Fig. 5.1 (A) Frequency distribution of sperm length (mm) in *An. gambiae* males (all experimental groups combined), all sperm measured included, *n*= 1548. The pink line represents a normal distribution based upon the mean and standard deviation in the actual data. (B) Frequency distribution of mean sperm length for each individual, *n*= 78.

Mean sperm length (*n*= 1548) was 0.250mm (0.244-0.257CI) in Field Mopti, 0.190mm (0.184-0.195CI) in KIL; 0.169mm (0.154-0.185CI) in Mopti 2003, and 0.276 mm (0.271-0.281CI) in Super males (♂ Mopti 2003 x ♀KIL) (Fig. 5.2.A).

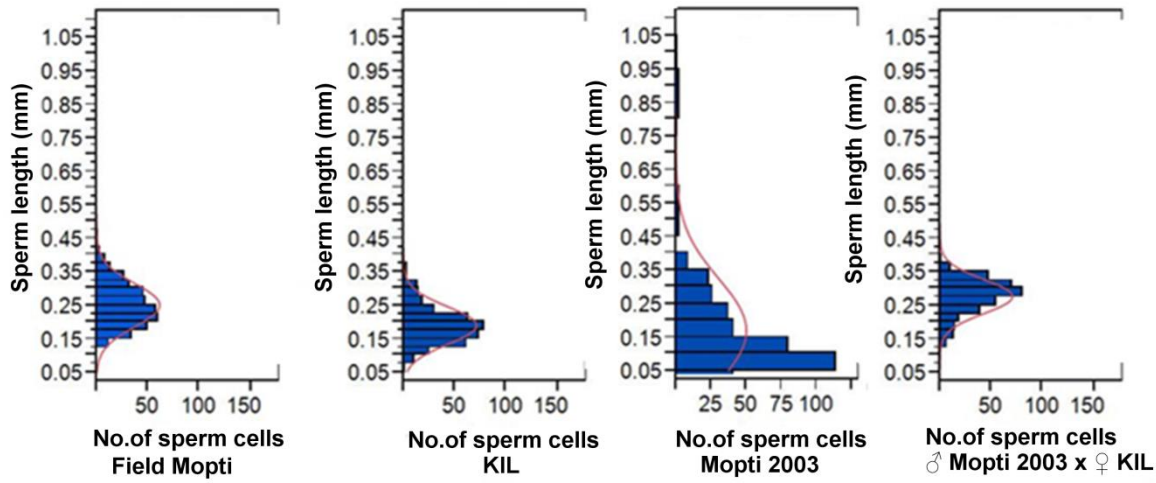


Fig. 5.2.A. Frequency distribution of sperm length (mm) of 1548 sperm cells in *An. gambiae* s.s males in Field Mopti, KIL, Mopti 2003 and Super male (♂ Mopti 2003 x ♀KIL).

Mean sperm length per individual (*n*= 78, 20 sperm per individuals) was 0.250mm (0.235-0.265CI) in Field Mopti, 0.190 mm (0.178-0.201CI) in KIL, 0.168 mm (0.118-0.219CI) in Mopti 2003, and 0.276mm (0.267-0.285CI) in. Super males (♂ Mopti 2003 x ♀KIL) (Fig. 5.2.B)

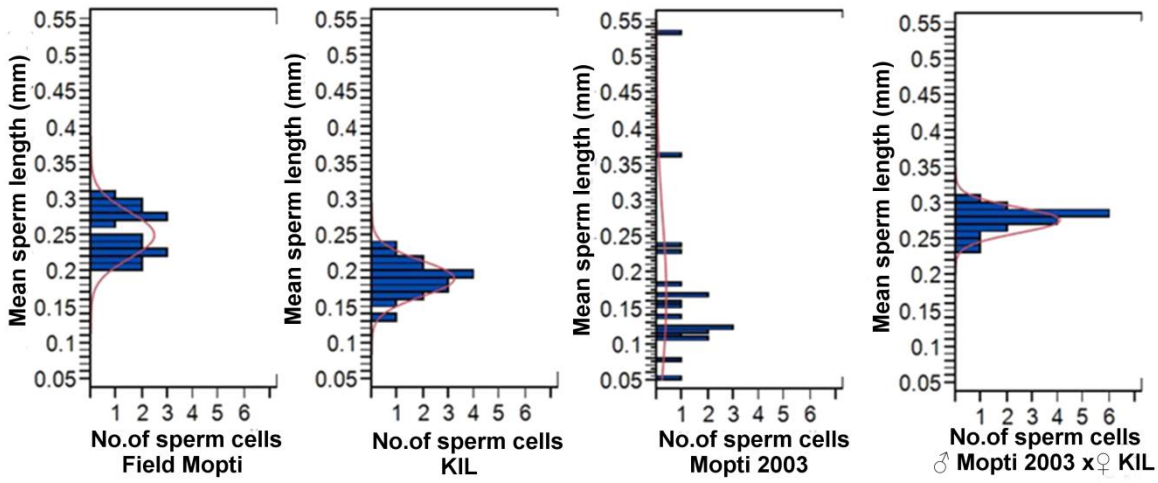


Fig. 5.2.B. Frequency distribution of mean sperm length (mm) of 20 sperm cells per male in 78 males in Field Mopti, KIL, Super male (♂ Mopti 2003 x ♀KIL) and Mopti 2003.

Body size (measured as wing length) was measured and recorded for males of each of the four groups. The average wing length of the 78 males of Field Mopti, KIL, Mopti 2003 and Super males was 2.778mm (2.727-2.828 CI). Male wing lengths showed significant difference between groups (ANOVA: $F_{3,78}$= 67.298; $r^2$= 0.731; $P$<0.001) (Fig. 5.3). Pair-wise comparisons of wing length between the four groups showed that there was a significant difference between KIL and Super males ($P$<0.001). Super males' body size at 2.540 (2.484-2.595CI) were significantly smaller than KIL at 3.070mm (3.017-3.122), Field Mopti, 2.769 (0.716-0.821CI) and Mopti 2003, 2.709 (0.657-0.725CI) ($P$<0.001 in all cases) (Fig. 5.3).

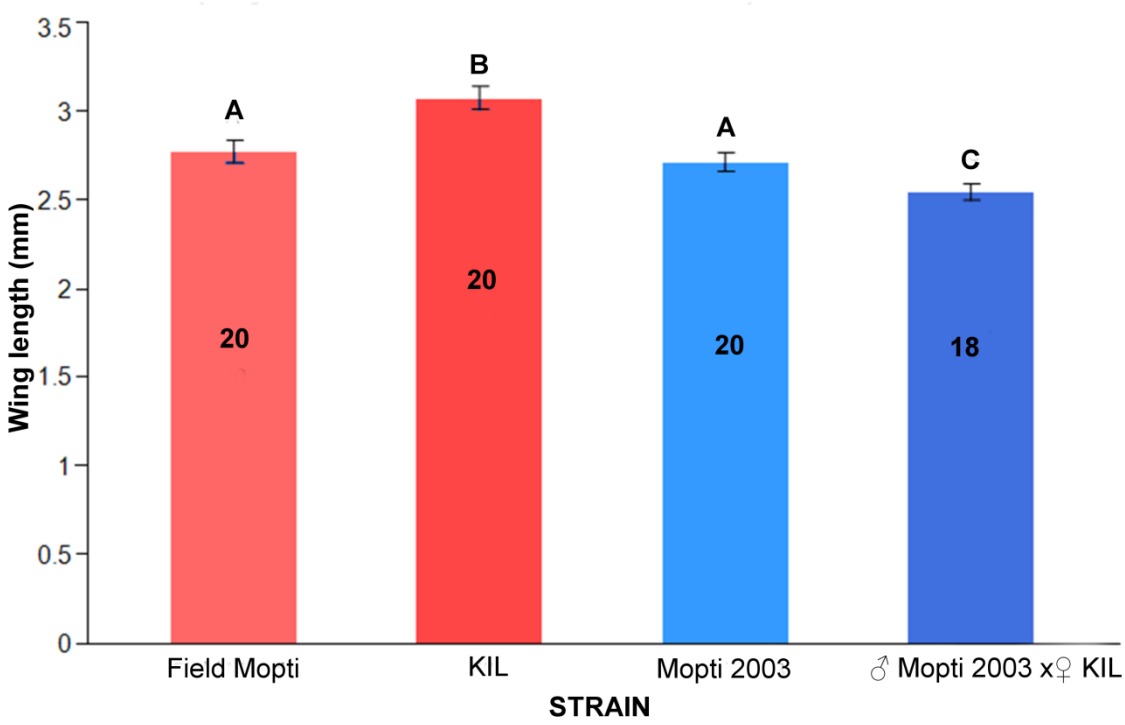


Fig. 5.3. Wing length (mm) (±95% CI) in Field Mopti, KIL, Mopti 2003and Super male (♂ Mopti 2003 x ♀KIL). Bars labelled with different letters differed significantly. Sample sizes are indicated.

The relationship between body size and sperm length was studied. A negative overall relationship was observed between mean sperm length and male body size amongst all combined groups (Fig. 5.4) (Linear regression: $n$= 78, $t$= *4.68*; $r^2$= 0.075; $P$=0.015).

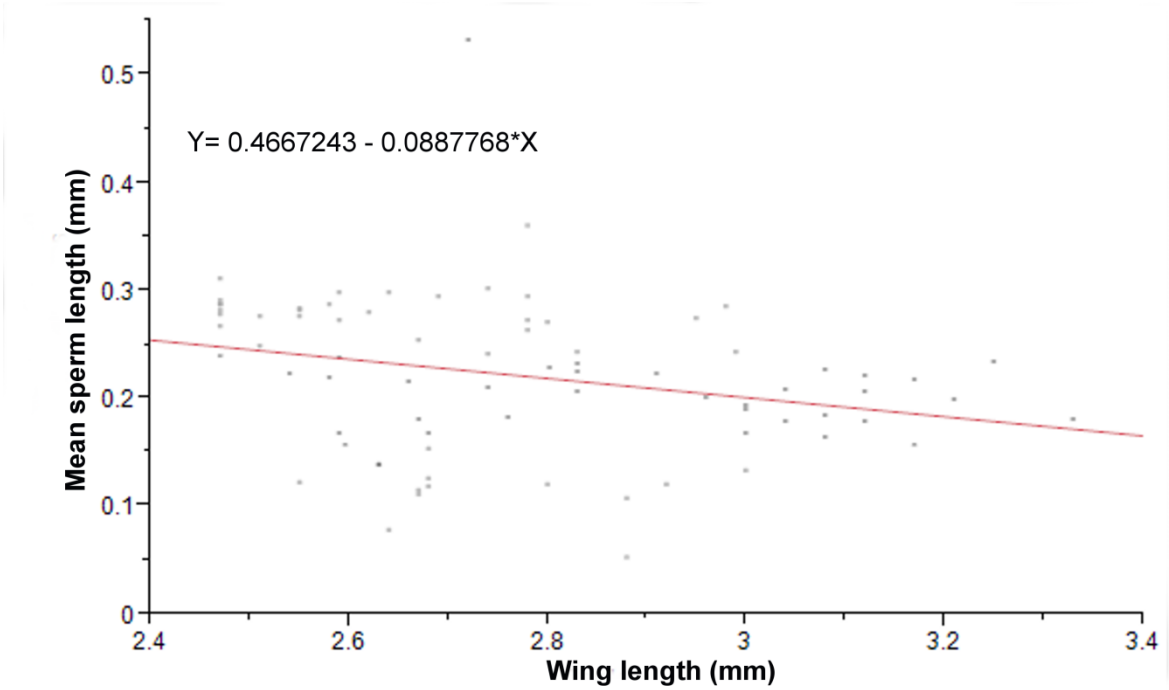


Fig. 5.4. Linear relationship between body size (wing length) and mean sperm length (All experimental groups; $n$= 78). The linear equation is indicated.

The relationship between body size (wing length) and sperm length was studied in each group separately. It was found that there was no relationship between body size and mean sperm length per mosquito in any of the groups (Linear regression: $P$>0.347 in all cases). Because of significant differences in body size (wing length) between groups, sperm length was corrected by dividing by body size in subsequent analyses. There was a significant difference between all combined experimental groups (ANOVA: $F_{3,78}$ = 21.149, $r^2$= 0.461, $P$<0.001) (Fig.5.5). Pair-wise comparisons between groups showed a significant difference between Super male and KIL as well as Mopti 2003 ($P$<0.001 in both cases),

between Field Mopti and KIL (*P*=0.005) and Mopti 2003 (*P*=0.006) (Fig.5.5). In fact, therefore, sperm length was significantly lower in old colonized strains than in Super males (♂ Mopti 2003 x ♀KIL) as well as in the Field Mopti individuals (Fig. 5.5).

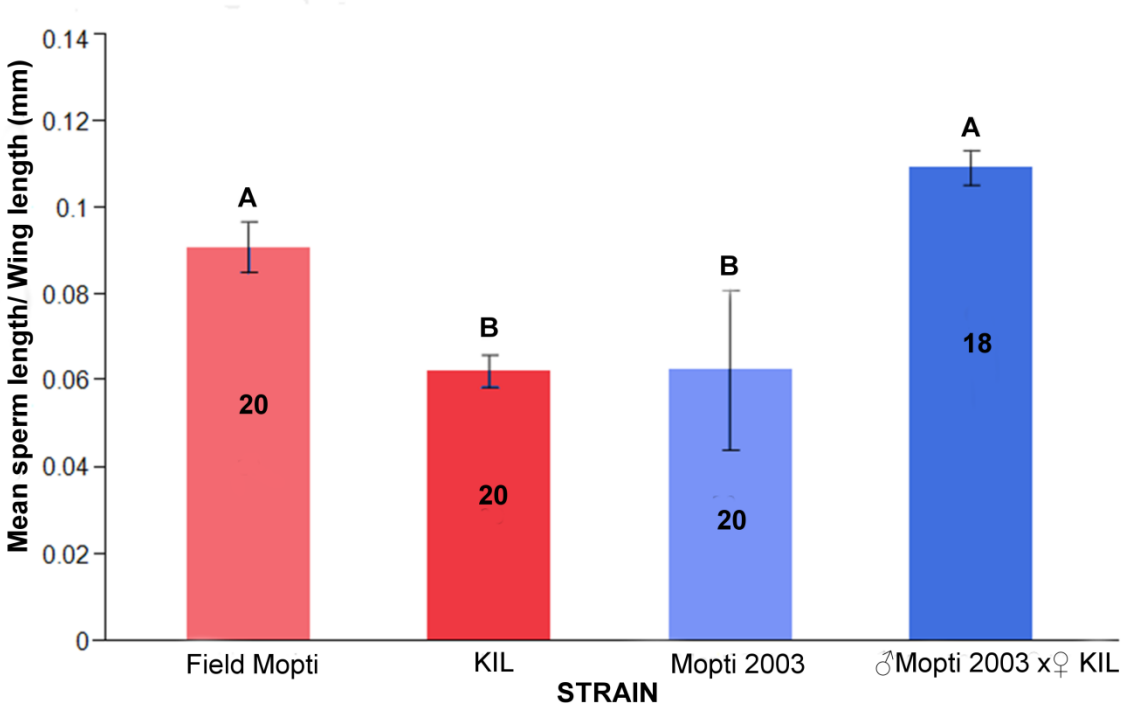


Fig. 5.5. Mean sperm length (mm) (20 sperm cells were measured in 20 individuals/group) corrected for wing length (±95% CI) in Field Mopti, KIL, Mopti 2003and Super males (♂ Mopti 2003 x ♀KIL). Bars labelled with different letters differed significantly.

### 5.3.2. Testes and accessory gland size (surface of image)

The mean of male accessory gland (MAG) size (surface of image) of 161 *An.gambiae* s.s. glands across four experimental groups, Field Mopti, KIL, Mopti 2003 and Super male (♂ Mopti 2003 x ♀ KIL) was 0.029mm (0.028-0.030CI), while the mean testes size of 157 males for the same mosquitoes was 0.034mm (0.033-0.035CI). The range

of MAG size was 0.018- 0.045 $mm^2$ while testes size range was 0.015- 0.053$mm^2$ (Fig. 5.6AB).

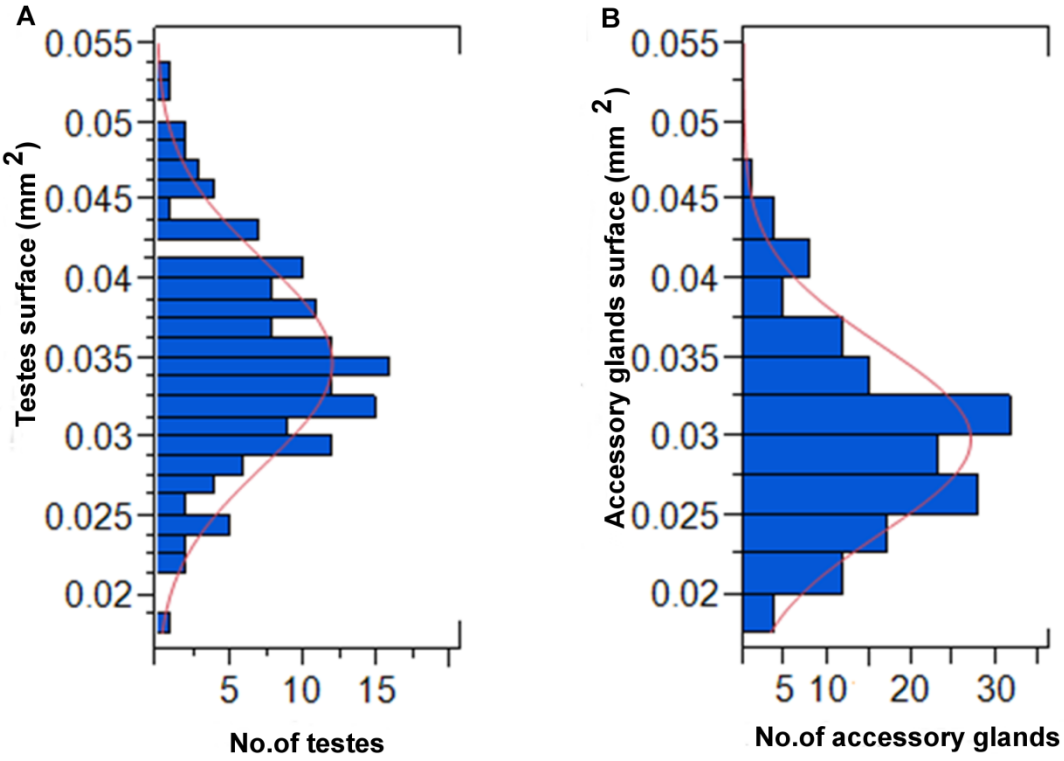


Fig. 5.6 (A) Frequency distribution of testes size ($mm^2$) (surface of testes image) (*n*= 157) and (B) accessory gland size ($mm^2$) (surface of accessory glands image) (*n*= 161) in *An. gambiae* s.s. males (all experimental groups combined). The pink line represents a normal distribution based on the mean and standard deviation of the actual data.

The mean testes size (surface of testes image) in Field Mopti was 0.031$mm^2$ (0.030-0.033CI), in KIL was 0.039$mm^2$(0.037-0.041CI); in Mopti 2003 was 0.033$mm^2$ (0.031-0.0335CI) and in Super males was 0.033$mm^2$ (0.031-0.036CI) (Fig. 5.7A).

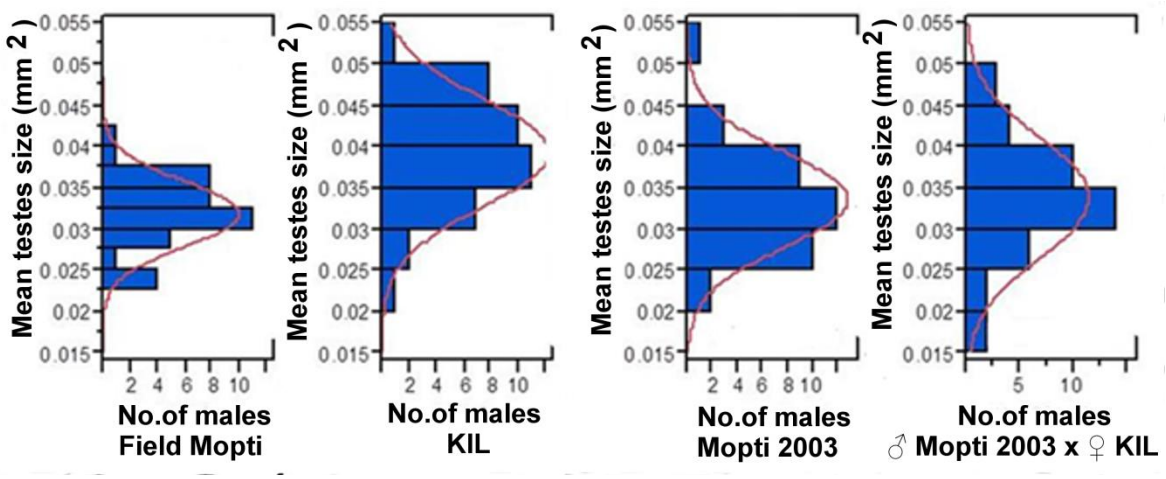


Fig. 5. 7 (A) Frequency distribution of testes size ($mm^2$) (surface of testes image) in *An. gambiae* s.s. males of four experimental groups: Field Mopti, KIL, Mopti 2003and Super males (♂ Mopti 2003 x ♀KIL).

The mean male accessory gland size (surface of MAG image) in Field Mopti was 0.036$mm^2$ (0.034-0.037CI), in KIL was 0.029$mm^2$ (0.027-0.030CI); in Mopti 2003 was 0.028$mm^2$ (0.026-0.029CI) and in Super males was 0.025$mm^2$ (0.024-0.026CI) (Fig. 5.7.B).

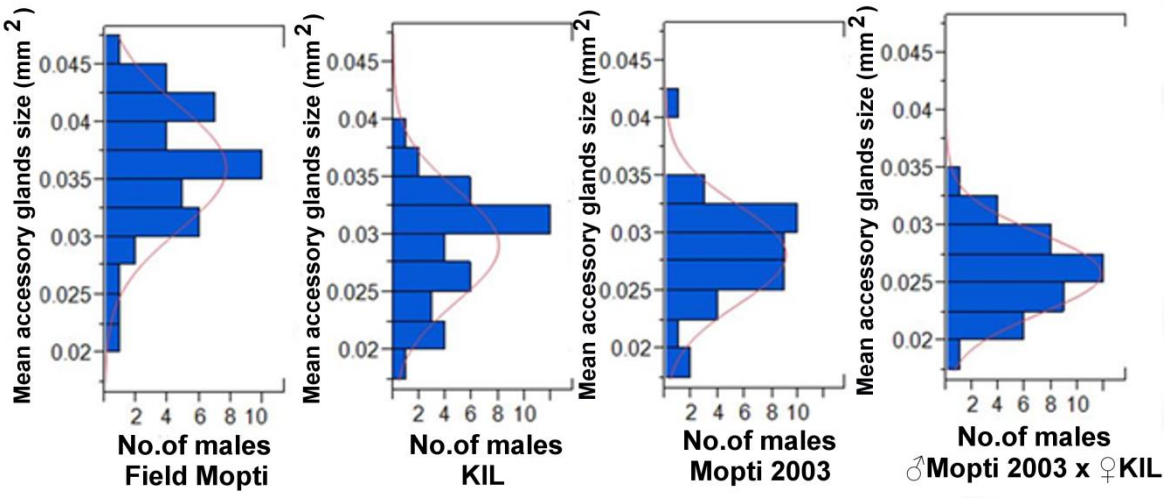


Fig. 5.7 (B) Frequency distribution of accessory gland size ($mm^2$) (surface of MAG image) in *An. gambiae* s.s. males of four experimental groups: Field Mopti, KIL, Mopti 2003and Super male (♂ Mopti 2003 x ♀KIL).

Body size (measured as wing length) was measured and recorded for males of each of the four groups. The average wing length of 166 males of Field Mopti, KIL, Mopti 2003 and Super males was 2.750mm (2.726-2.775CI). Overall, There was a significant difference in body size (wing length) between the four groups (ANOVA: $F_{3,166}$= 33.01, $r^2$= 0.379, $P$<0.001). Pair-wise comparisons showed a significant difference between Mopti 2003 and Super males ($P$<0.001) (Fig. 5.8). Because of that difference, testes size and AC size were corrected for effects of body size by dividing them by wing length$^2$.

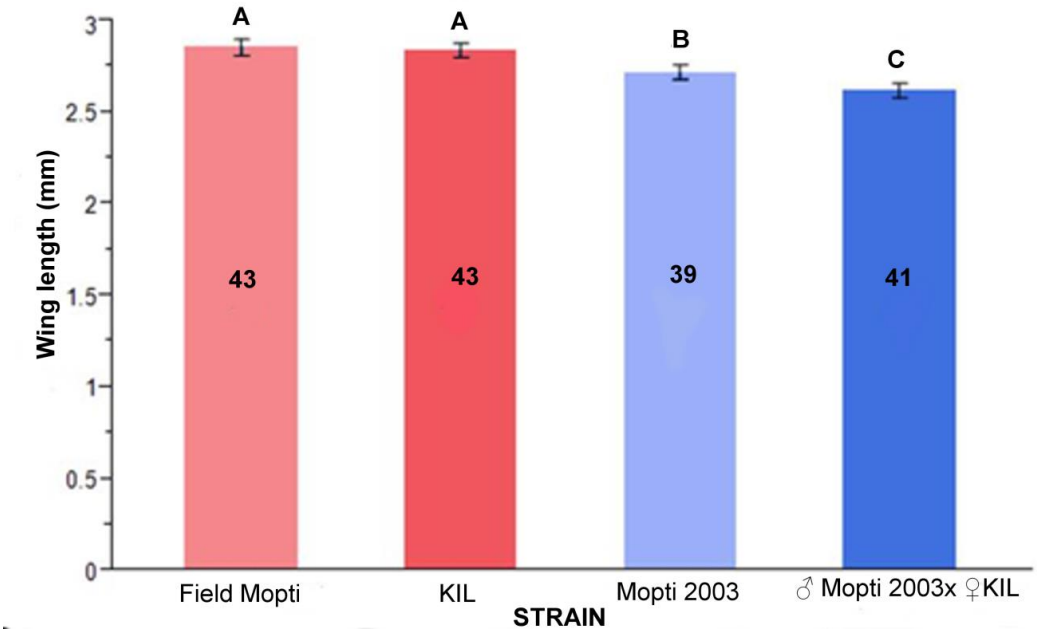


Fig. 5.8. Wing length (mm) (±95% CI) in Field Mopti, KIL, Mopti 2003and Super males (♂ Mopti 2003 x ♀ KIL). Bars labelled with different letters differed significantly. Sample sizes are indicated.

The relationships between body size and sexual organs size were studied to assess whether large males have large sexual organs or not. It was found that there was no relationship between body size (wing length) and testes size in any of the experimental groups (Linear regression: $P$>0.438 in all cases). However, a very strong relationship between body size and accessory gland size was found in two groups (Linear regression:

Field Mopti $n$= 42, $t$= -2.7, $r^2$= 0.433, $P$<0.001 and Mopti 2003 $n$=39, $t$=-0.18, $r^2$= 0.101, $P$=0.047) (Fig. 5.9 AC). In the other two groups, meanwhile, no significant relationship was found between body size and AC size (Linear regression: KIL $n$= 39, $t$= -0.15, $r^2$= 0.070, $P$=0.084; Super male (♂ Mopti2003 x ♀ KIL) $n$= 41, $t$= 0.34, $r^2$= 0.093, $P$=0.052) (Fig. 5.9.BD).

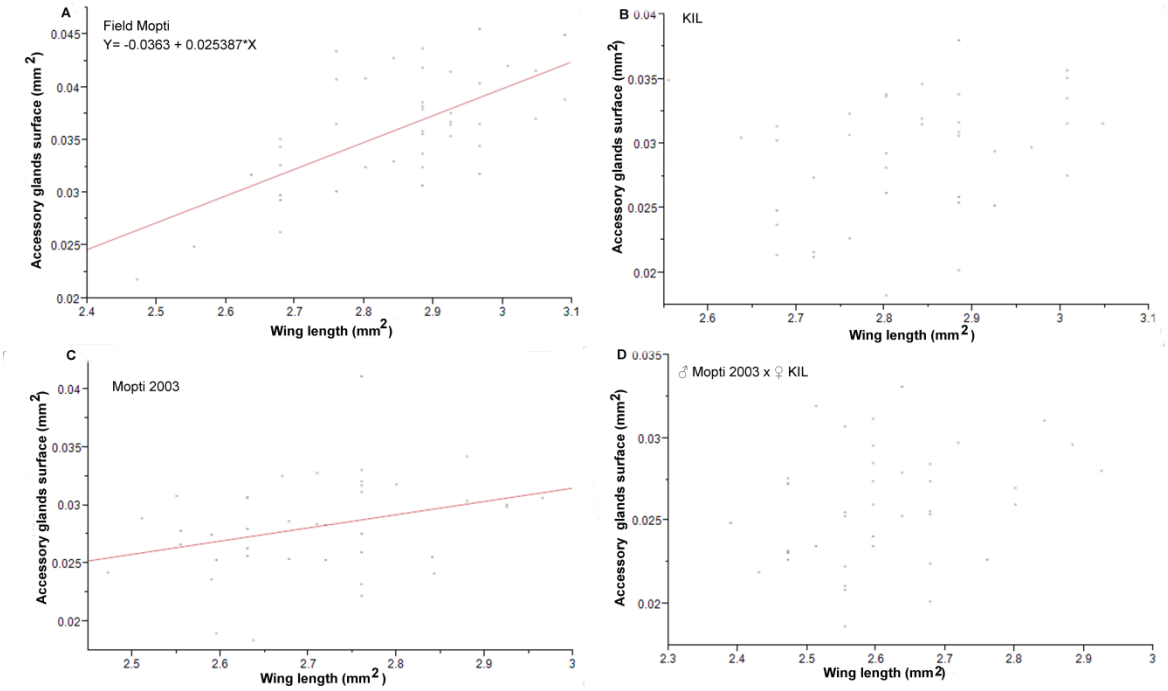


Fig. 5.9. Linear relationship between body size (wing length ($mm^2$)) and male accessory gland size ($mm^2$) (surface of AC image) in four experimental groups: (A) Field Mopti, (B) KIL, (C) Mopti 2003 and, (D) ♂ Mopti2003 x ♀ KIL (Super male).

Testes size and accessory glands size were corrected for the effects of body size by dividing them by wing length$^2$. The two variables were then checked for normality once more. Testes size and AC size distribution overall showed that the two variables did not deviate from normality in all four studied groups (Shapiro-Wilkinson: $P$>0.398 in all cases). Testes size (surface of testes image) showed a significant difference overall amongst the four studied groups (ANOVA: $F_{3,157}$= 10.759, $r^2$= 0.174, $P$<0.001) (Fig. 5.10).

Pair-wise comparisons showed that there was a significant difference between Field Mopti and Super males, KIL and Mopti 2003 ($P$<0.001 in all cases) (Fig. 5.10).

Accessory gland size (surface of MAG image) demonstrated a significant difference overall across the four studied groups (ANOVA: $F_{3,161}$ = 15.242, $r^2$ = 0.225, $P$<0.001) (Fig. 5.10). Pair-wise comparisons confirmed a significant difference between Field Mopti and KIL, Super males and Mopti 2003 ($P$<0.001 in all cases). Testes size was larger in colonized strains (KIL and Mopti 2003) and in Super males than in Field individuals In addition, the previous three groups had smaller accessory glands than the Wild group (Fig. 5.10).

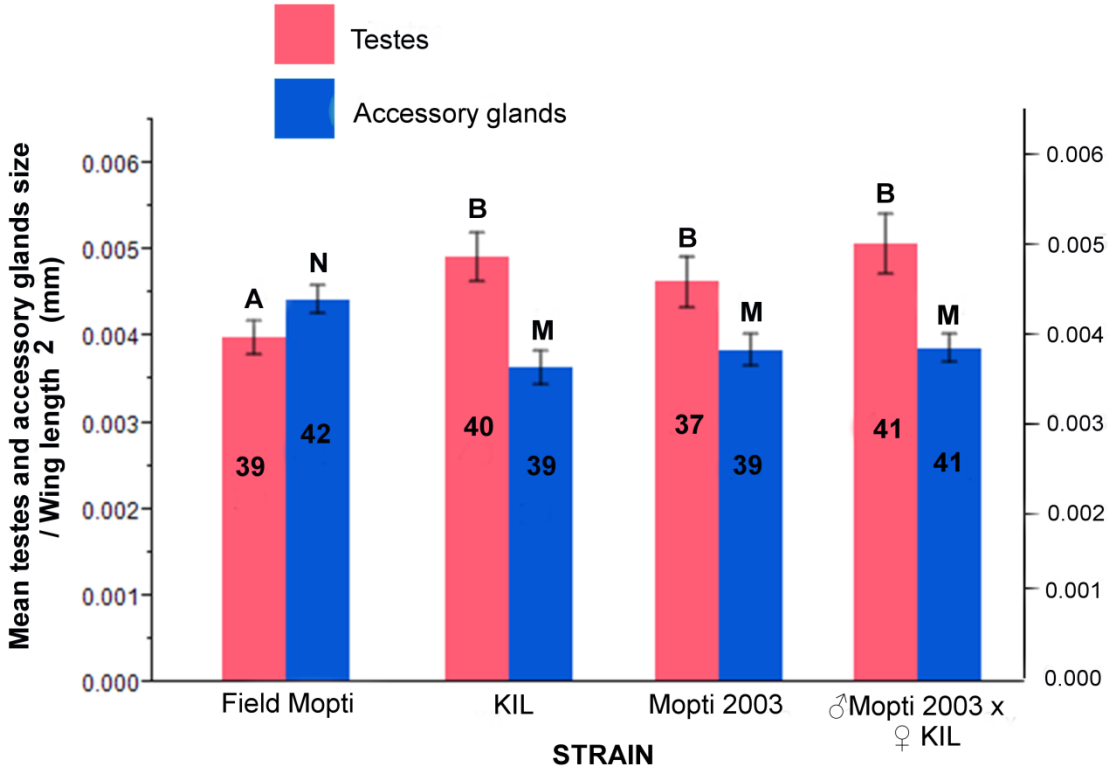


Fig. 5.10. Mean testes (pink) and accessory gland (blue) size ($mm^2$) (surface of image) corrected by wing length $^2$ (±95%CI) in Field Mopti, Mopti 2003, KIL and Super males. Bars labeled with different letters (A and B for testes; M and N for accessory glands) were significantly different (Tukey: $P<0.05$).

### 5.3.3. Production of proteins in *An. gambiae* male accessory glands (MAGs)

#### 5.3.3.1. Estimated Plugin protein

The amount of Plugin protein was estimated in the accessory glands of 4 day-old males of *An. gambiae* and quantified as MAGs equivalent (see methods, 5.2.4). Estimated Plugin protein in 142 males across four experimental groups (Field Mopti, KIL, Mopti 2003 and Super male (♂ Mopti 2003 x ♀KIL) was checked with the normality test by Shapiro-Wilkinson. The overall distribution of estimated Plugin deviated significantly from a normal distribution (Shapiro-Wilkinson: $n= 142$, $W= 0.893$, $P<0.001$) (Fig. 5.11). The median (25-75 quartiles) of estimated Plugin protein of 142 males for the same

mosquito groups was 1.742 MAG equivalent (0.799-3.018). The range for the 142 males was 0.125-8.136 MAGs equivalent (Fig. 5. 11).

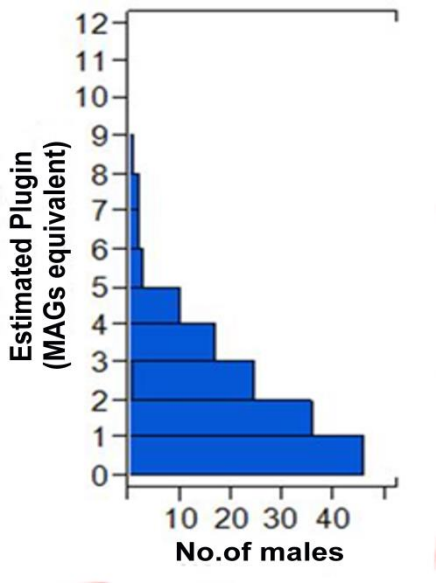


Fig. 5.11. Frequency distribution of estimated Plugin in *An gambiae* s.s. males (all groups combined, $n$ = 142).

The frequency of estimated Plugin was examined for each group separately to check for normality using Goodness Fit tests. Overall, all data deviated significantly from a normal distribution (Shapiro-Wilkinson Test: $P$<0.001 in all cases). The medians (25-75 quartiles) of the four groups were: Field Mopti, 1.776 (0.772-2.970); KIL, 1.979 (1.232-3.160); Mopti 2003, 1.414(0.531-2.320) and Super males, 1.807 (0.732-3.213) (Fig. 5.12).

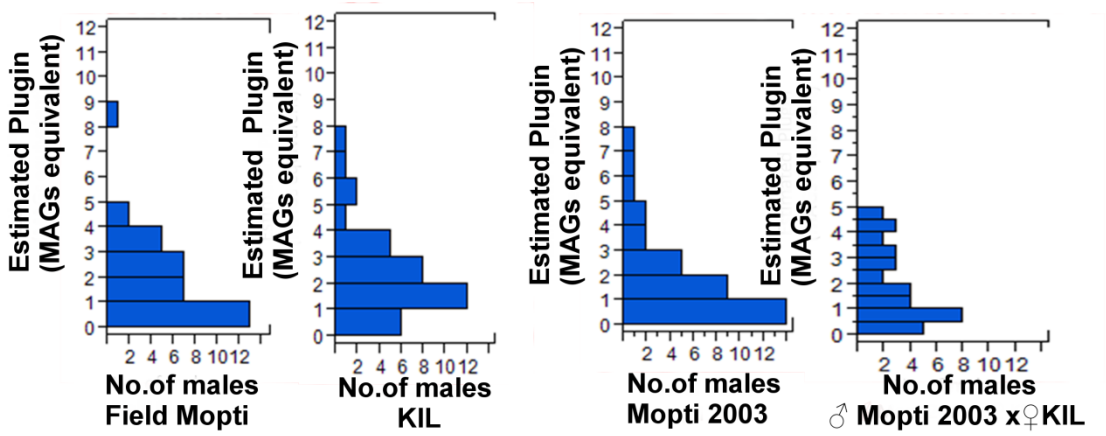


Fig. 5.12. Frequency distribution of estimated Plugin (MAGs equivalents) in Field Mopti, KIL, Mopti 2003 and Super males (♂ Mopti2003 x ♀ KIL ).

### 5.3.3.2. Relationship between male body size (measured as wing length) and quantity of Plugin

The mean wing length of 134 *An.gambiae* s.s. males (all four groups combined) was 2.662mm (2.634-2.689CI) (Fig. 5.13). Overall, there was no significant difference in the body size (measured as wing length) of 134 males across four combined groups (ANOVA: $F_{3,134}$= 2.063, $r^2$= 0.045, $P$=0.108).

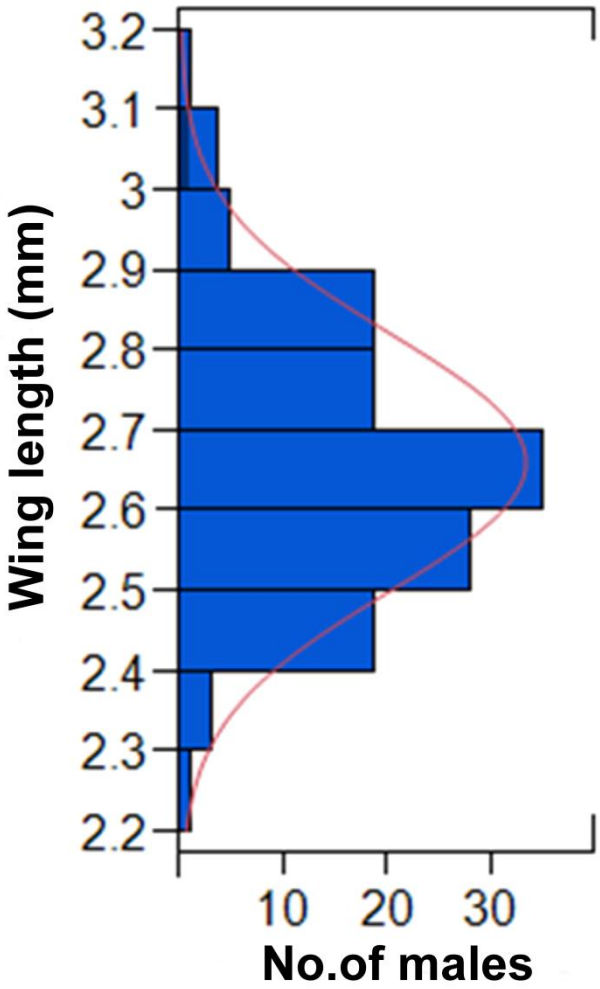


Fig. 5.13. Frequency distribution of wing length (mm) of *An. gambiae* s.s. males (all combined groups). The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

The correlation between wing length (body size) and estimated Plugin in 134 *An. gambiae* s.s. males across four experimental groups was investigated. An overall a significant linear relationship was found between body size and Plugin production in the MAGs (linear regression: $n$= 134, $t$= -1.3, $r^2$= 0.035, $P$=0.028) (Fig. 5.14). Large males produced more Plugin protein than small males.

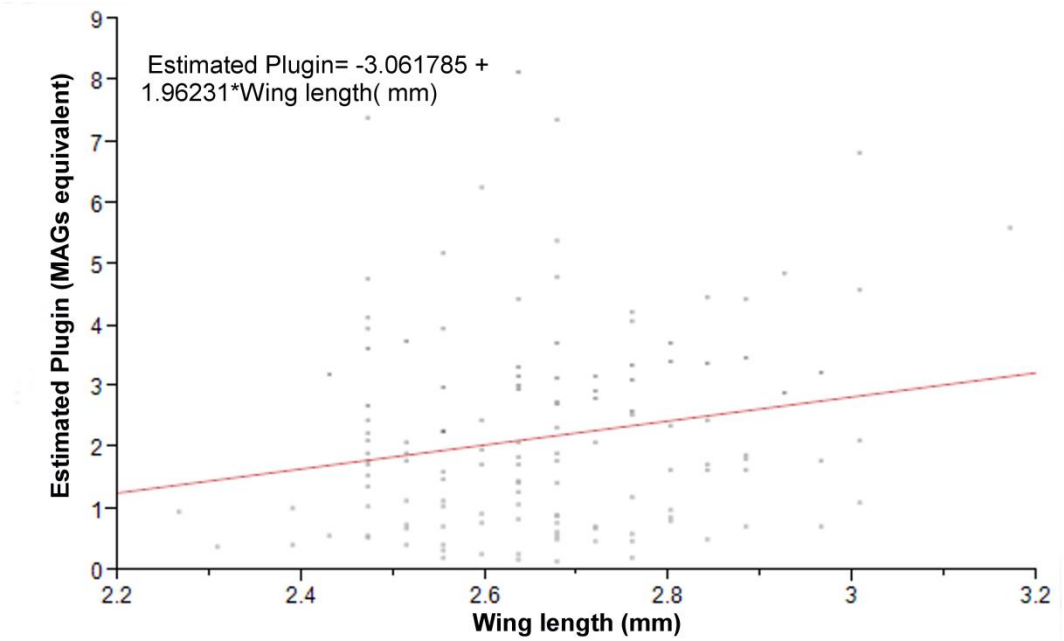


Fig.5.14 Linear relationship between body size (wing length) and estimated Plugin in *An. gambiae* s.s. males (all experimental groups combined).

Linear regressions of estimated Plugin and body size (wing length) performed within each of the four studied groups showed that there was no relationship between the two variables in three groups (linear regression: Field Mopti $n$= 31,$t$= -0.01, $r^2$= 0.002, $P$=0.780; KIL $n$= 36, $t$= 0.74, $r^2$= 0.003, $P$=0.737; Super males $n$= 35, $t$= -0.78, $r^2$= 0.053, $P$=0.182) but that there was a significant relationship in Mopti 2003 ($n$= 32, $t$=-1.95 $r^2$= 0.165, $P$=0.02) (Fig. 5.15).

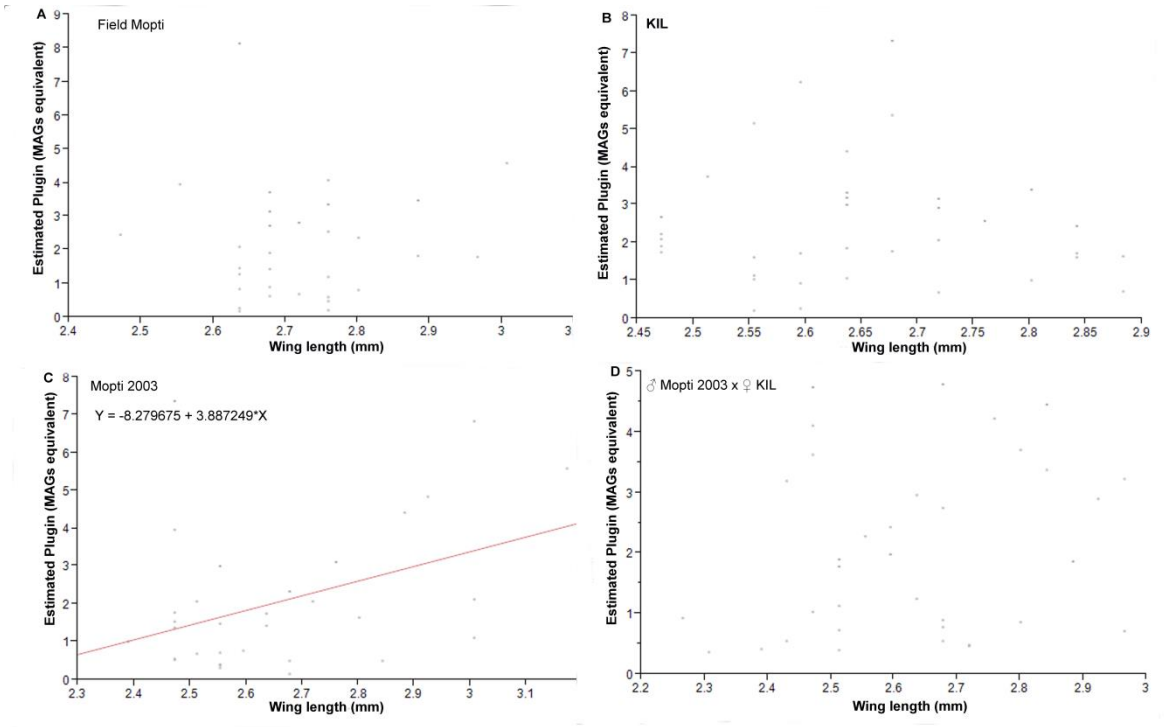


Fig. 5.15. Linear relationship between body size (wing length) and estimated Plugin in four groups: (A) Field Mopti; (B) KIL; (C) Mopti 2003, and (D) Super males (♂ Mopti 2003 x ♀ KIL).

As a result, the estimated amount of Plugin was corrected by body size and the data reanalyzed. The normality test was made again. The overall distribution of Plugin estimates (MAG equivalents) was divided by wing length$^3$. Since all groups deviated significantly from normality (Shapiro-Wilkinson: $P<0.005$ in all cases), the data was analyzed non-parametrically. Overall there was no significant difference of Plugin estimated/wing length$^3$ (mm) in male *An. gambiae* s.s. across the four studied groups (Kruskal-Wallis: $n= 134$, $df= 3$, $\chi^2= 2.10$, $P=0.391$) (Fig. 5.16).

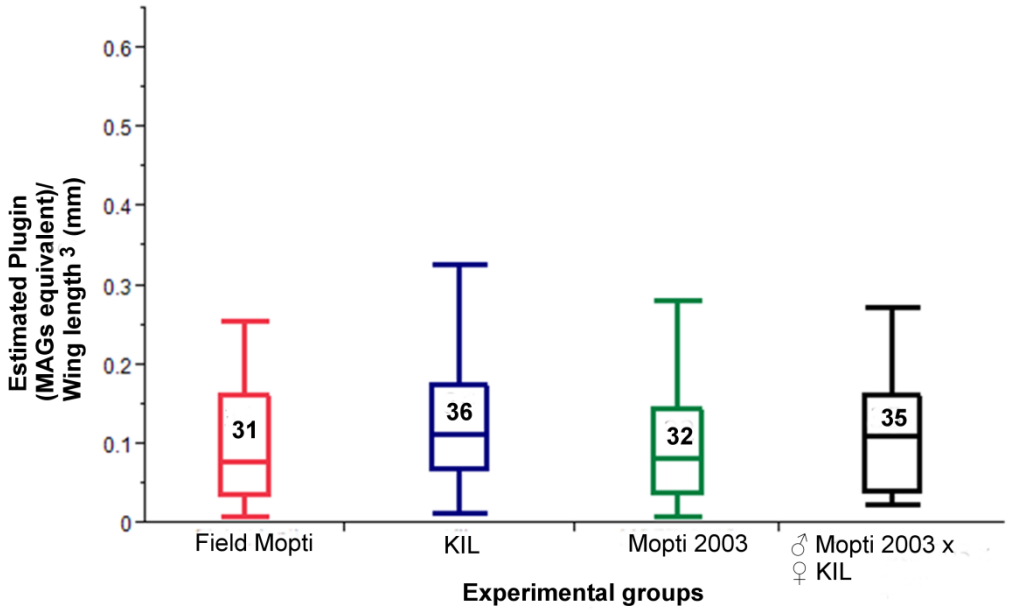


Fig. 5.16. Estimated Plugin (MAGs equivalent) corrected for wing length$^3$ (mm) in Field Mopti, KIL, Mopti 2003 and Super males (♂ Mopti 2003 x ♀ KIL). Plugin estimated followed a non-normal distribution: therefore, bars represent the median. Boxes are in the interquartile range as a box (75- 25%). The whiskers display upper and lower parameter values, excluding outliers. Sample sizes are indicated.

### 5.3.3.3. Transglutaminase protein estimated in MAGs

The frequency distribution of estimated Transglutaminase protein in 146 *An.gambiae* s.s. males across four experimental groups, Field Mopti, KIL, Mopti 2003 and Super male (♂ Mopti 2003 x ♀KIL) was checked for normality by a goodness of fit test. Overall, all data deviated significantly from a normal distribution (Shapiro-Wilkinson: $n$= 146, W=0.848, $P$<0.001). The median (25-75 quartiles) of estimated Transglutaminase protein of 146 males for the same groups was 1.481 MAGs equivalent (0.663-2.872). The range of Transglutaminase estimated in 146 males across the four studied groups was 0.042-10.679 MAGs equivalent (Fig. 5.17).

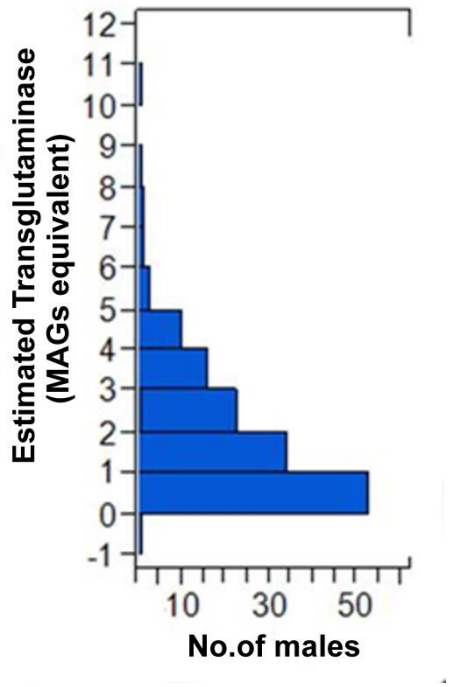


Fig. 5.17. Frequency distribution of estimated Transglutaminase in *An. gambiae* s.s. males (all combined groups, *n*= 146)

The distribution of estimated Transglutaminase was examined in each group for normality. It was found that the majority of the groups did not follow a normal distribution (Shapiro- Wilkinson: $P<0.0001$ in all cases) (Fig. 5.18). The minimum amount of estimated Transglutaminase in male accessory glands among the four groups was found in Field Mopti, as the range of estimated protein was 0.110-2.184 MAGs equivalent. Meanwhile, the maximum amount of estimated Transglutaminase was seen in KIL males, as the range was 0.328-10.679 MAGs equivalent (Fig. 5.18).

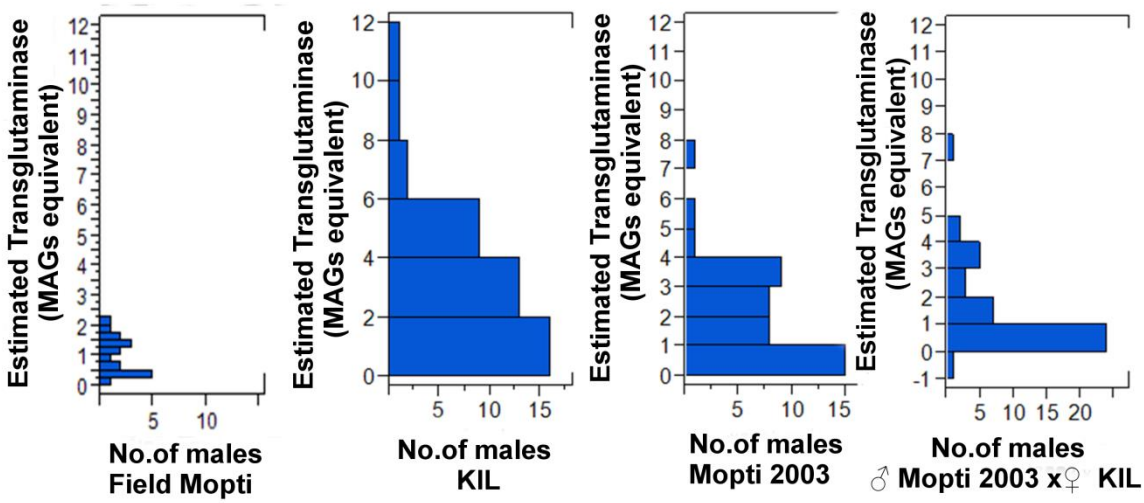


Fig. 5.18. Frequency distribution of estimated Transglutiamnse (MAGs equinelant) in Field Mopti, KIL, Mopti 2003 and Super males (♂Mopti 2003 x ♀ KIL).

### 5.3.3.5. Relationship between male body size (measured as wing length) and quantity of Transglutaminase

The mean wing length of 138 *An.gambiae* s.s. males (all four groups combined) was 2.661mm (2.633-2.689CI) (Fig. 5.19).

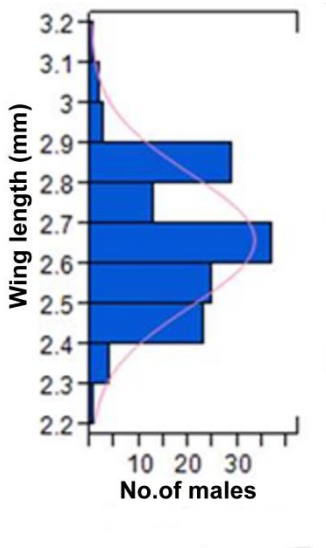


Fig.5.19. Frequency distribution of the wing length (mm) of males for which Transglutaminase in the MAGs was estimated. The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

Overall, there were significant differences in body size (wing length) of the 138 males across the four studied groups (ANOVA: $F_{3,138}$= 6.953, $r^2$= 0.134, $P$=0.002). Pair-wise comparisons showed there was a significant difference between Super males and KIL ($P$<0.001), between Field Mopti and Super males ($P$=0.030), and between Mopti 2003 and Super males ($P$=0.046). Super males were slightly smaller than the other three groups (mean= 2.575mm) (2.528-2.622CI).

The relationship between body size (wing length) and estimated Transglutaminase was examined. A positive relationship was found between body size and Transglutaminase production in the MAGs in two groups (Linear regression: KIL $n$= 40, $t$= -2.00, $r^2$=0.134, $P$=0.019; Super males $n$= 41, $t$= -2.42, $r^2$= 0.165, $P$=0.008) (Fig. 5.20 BD). However, there was no relationship between body size (wing length) and the amount of Transglutaminase protein in the other two groups (Linear regression: Mopti 2003 $n$ = 41, $t$= -0.62, $r^2$ = 0.037, $P$=0.225; Field Mopti $n$= 16, $t$= 0.09, $r^2$ = 0.003, $P$=0.832) (Fig. 5.20 AC).

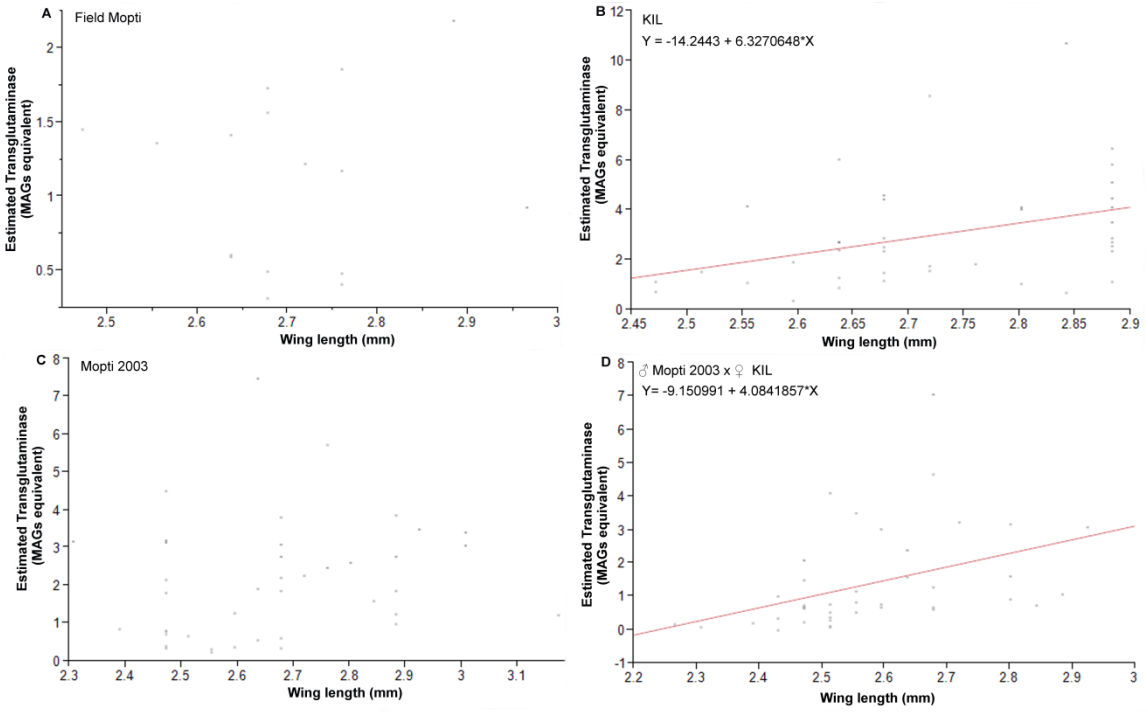


Fig.5.20. Linear relationship between body size (wing length) and estimated Transglutaminase in four experimental groups: (A) Field Mopti, (B) KIL, (C) Mopti 2003 and (D) Super males (♂ Mopti2003 x ♀ KIL).

As a result, the estimated Transglutaminase amount was corrected by body size. Overall, there was a significant difference in the corrected Transglutaminase estimates between groups (Kruskal-Wallis: $n$= 138, $df$= 3, $\chi^2$= 22.2, $P$<0.001). Pair-wise comparisons between the four studied groups showed a significant difference between KIL and Field Mopti ($P$=0.002), between Mopti 2003 and Field Mopti ($P$=0.028), between Super males and Mopti 2003 ($P$=0.026), and between Super males and KIL ($P$<0.001) (Fig. 5.21)

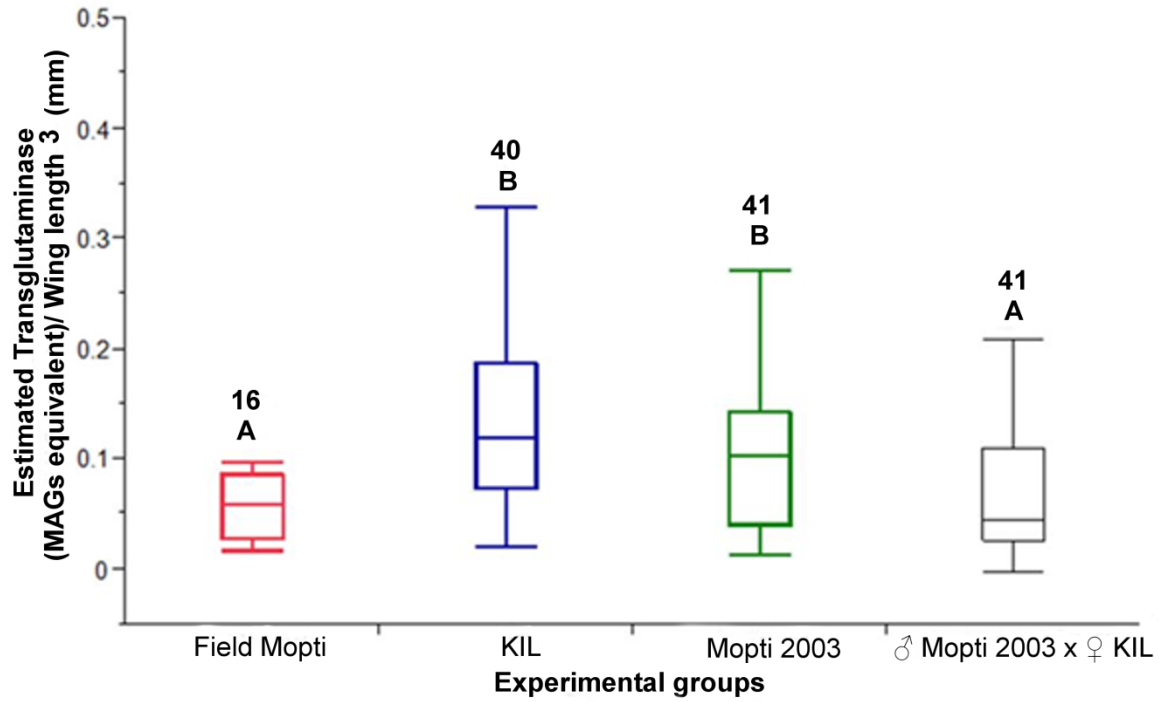


Fig. 5.21. Estimate Transglutaminase (MAGs equivalent) corrected with wing length $^{3}$ (mm) in Wild males, KIL, Mopti 2003 and Super males (♂ Mopti 2003 x ♀ KIL). Transglutaminase estimated followed a non-normal distribution: therefore, bars represent the median. The median is represented by the horizontal lines within the boxes, and the interquartile range as a box (75- 25%). Whiskers display upper and lower parameter values, excluding outliers. Boxplots labelled with different letters differed significantly (Wilcoxon: $P<0.026$). Sample sizes are indicated.

Comparison between Plugin and Transglutaminase production in male accessory glands showed that the amount of both proteins varied across the four groups studied (Table.5.3). Field Mopti and Super males produced more Plugin in their MAG than Transglutaminase (Plugin estimated was 1.77 and 1.80 respectively) but, there was no significant difference between these two groups. Meanwhile, KIL and Mopti 2003 produced more Transglutaminase in their MAG than Plugin (Transglutaminase estimated was 2.58 and 1.84 respectively) (Table 5.3).

Table 5.3. Comparison between Plugin and Transglutaminase proteins generated in male accessory glands across the four studied groups.

| Strain | Median estimated Plugin (25-75% quartile) | Sample size | Median estimated Transglutaminase (25-75% quartile) | Sample size |
|---|---|---|---|---|
| Field Mopti | 1.776 (0.772-2.970) | 35 | 1.048 (0.458-1.477) | 18 |
| KIL | 1.979 (1.232-3.160) | 36 | 2.589 (1.390-4.149) | 42 |
| Mopti 2003 | 1.414 (0.531-2.320) | 35 | 1.840 (0.767-3.064) | 43 |
| ♂ Mopti 2003 x♀ KIL | 1.807 (0.732-3.213) | 36 | 0.752 (0.497-2.063) | 43 |

## 5.4. Discussion

The state of knowledge regarding both the form and function of sperm is surprisingly scant given the importance of this topic (Morrow and Gage, 2000). In light of this, work on sperm length, with its utility in determining fitness in the male, can be seen as an important step in countering this situation (Voordouw *et al.*, 2008). The frequency distribution of sperm length in *An. gambiae* s.s. in this study found a variation of sperm length in all four groups as well as in mean sperm length per mosquito. Within each group this variation was in large part due to differences between individual males. Indeed, *An. gambiae* s.s. is noted by Klowden and Chambers, (2004) to demonstrate greater variation in sperm length in comparison to other Anophelines. Moreover, Voordouw *et al*, 2008 demonstrated intra-specific variation of sperm length in *An. gambiae.* s.s. The different methodology used by the two studies, however, makes comparison problematic, as Klowden and Chambers (2004) measured 100 sperm on three to five males, whereas Voordouw *et al.* (2008) measured 10 sperm per individual in twenty-five *An. gambiae* s.s. and 17 *An. stephensi* males of unknown age and mating history to study intra-specific variation of sperm length

in both strains. Meanwhile, in the present study, 20 sperm per individual in 78 virgin males at seven days old from four independent groups were used. The mean sperm length (mean ±SD) per mosquito across the four experimental groups was 0.220mm ± 0.070 and the range was 0.051-0.531mm. Klowden and Chambers (2004) reported a variation of between 26µm and 500 µm. Voordouw *et al.* (2008) recorded a range of sperm length between 100 to 250 µm, the mean sperm length (mean± SE) was 197 ± 7.2µm. In the present study, a large variation in sperm length was found across the studied groups. Eupyrene and apyrene sperm showed significant variation in length in a study of moths (Morrow and Gage, 2000). The sperm variation between groups which reported here may have a fitness consequence on male mosquitoes whether, is a positive or negative effect is, at present, unclear and further studies are needed. One explanation of variation in sperm length is that sperm is created in testes at different times (Harris *et al.*, 2007) or there is developmental plasticity in production of the sperm lengths (Green, 2003). Another explanation holds that sperm length depends on the female's sperm storage organ length (Miller and Pitnick, 2002; Baer *et al*, 2003). In moths, a link was found between mating habits and length of sperm (Morrow and Gage, 2000). However, there is no definitive link between size of the reproductive tract in the female nor in the numbers of sperm produced or numbers transferred to the female (Pitnick, 1996). In terms of sperm length, distribution is heavily subject to selection pressure (Miller and Pitnick, 2002; Green, 2003; Malo *et al*, 2006). However, in studies across 83 mammal species, evidence for a link between sperm competition and length is absent (Gage and Freckleton, 2003). Green (2003) observed that sperm length increased as a function of male age and not by limits placed on a dietary regime. In the current study however, male age was not important for variation of sperm length, as all males used were seven days old. This age is considered to be the point at which *An.gambiae* s.s. reach sexual maturity, as assessed by Huho *et al* (2006) from the

number of spermatocysts with immature spermatozoa, viewed in comparison with measurement of the sperm reservoir which contains mature spermatozoa. Huho *et al.* (2006) found that the mean spermatocyst count in old males (> 4 days) age was less than 2, while the sperm reservoir had grown to make up ~80% of testis volume, with this occurring more rapidly in virgins.

Across various species of *Drosophila*, body size, sperm length and age of sexual maturity for each sex were observed to differ significantly. From 11 *Drosophila* species, differences in energy used for production of sperm as assessed by comparison of the dry mass of the testes, were attributable to sperm length rather than sperm numbers. Among species with comparatively long sperm, testes comprised 8-11% of dry body mass, compared with 1-5% for species with lesser sperm length, irrespective of the fact that the species producing longer sperm had greater body sizes (Pitnick *et al*, 1995). In this study, body size (wing length) was positively correlated with sperm length (in all combined groups: 78 males). This is consistent with the other datasets in *An.gambiae* s.s. (Voordouw *et al*, 2008) and in *Bombus hypnorum* (bumblebees) (Baer *et al*, 2003), in butterflies (Gage, 1994) and in *Drosophila* (Pitnick *et al*, 1995). In contrast, no significant relationship between sperm length and body size was found in each group separately (20 males per group). This is in agreement with Sturup *et al*. (2011), in a study of the leafcutter ant *Atta colombica*, as there was no relationship between body size (head width and mesosoma width) and sperm length, nor with numbers of sperm produced and stored. (Sturup *et al*, 2011). In *Drosophila* meanwhile, a positive association was found between the volume of sperm production and the dry mass of the testes (Pitnick, 1996). A significant relationship between body size and sperm length was found in all combined studied groups. However, there was no relationship between the two previous variables in

each group separately. While the cause of the relationship is not known, it possibly stems from sperm competition (Gage, 1994).

There was a significant difference in body size (wing length) between the four experimental groups. This variation depended upon the amount of food provided during larval stages, as shown by Nayar (1969), who demonstrated a positive link between increased food availability and larger body size in *Aedes taeniorhynchus*. Further, in *Drosophila melanogaster,* nutrients gained from parents were found to strongly influence phenotype (Valtonen *et al*, 2012). In the current work, a significant difference between all experimental groups was observed in sperm length, which was corrected by dividing it by the body size. Sperm length was significantly lower in colonized strains (KIL and Mopti 2003) than in Super males (M-form crossing) or in Field Mopti individuals. The KIL strain had been reared under laboratory conditions for more than 35, years while Mopti 2003 had been so for seven years. Laboratory selection may present a reason for the lack of a strong hereditary influence upon mean sperm length, as genetic variation is restricted by colonization in *Anopheles* (Voordouw *et al*., 2008). Differential fitness loss might take place as a result of inbreeding creating a greater vulnerability to environmental stressors. It is thought that this vulnerability results from the greater expression of deleterious recessive alleles when under stress, or from reduced cellular capacity to tolerate stressors (Fox & Reed, 2011).

In this study, frequency distribution analysis confirmed variation in testes and accessory gland size (surface of image) between individuals. The mean accessory gland size (surface of image) of 161 males at seven days old was 0.029mm (0.028-0.03CI). Meanwhile, the mean testes size in 157 males (surface of image) of the same mosquitoes was 0.034mm (0.033-0.035CI). The size of both organs in all experimental groups fell between 0.02 and .05 mm. Mahmood and Reisen (1982) recorded the length and width of

testes (mean ±CI) (327±17 and 121±5 µm respectively) at seven days, and also measured 238±13 µm for the length of the accessory glands and 127±5µm for its width at the same age. It was not possible to compare the previous study with the current study because the former used measurements of length and width, while in the present study we used size measured by the surface image of testes and accessory glands. Unfortunately, similar studies of sexual organs' surface measurements in *Anopheles* species are rare.

Accessory glands develop from imaginal disks located in the ninth segment of the abdomen, beginning in the larval phase and growing further in both pupal stages and adulthood (Clements, 1999). Mahmood and Reisen (1982) observed that in *An.stephensi*, males emerged with small accessory gland and testes size (length and width) and gradually became larger with age. Meanwhile, testes length and width both showed notable variation over adult life, showing the greatest size upon maturation at three days and least at 32 days old males (Mahmood and Reisen, 1982). Again, in the current study no impact of age on the size of sexual organs was observed because seven day-old males were used across the four experimental groups. Accessory gland size in males seems to be pivotal in determining the reaching of sexual maturity. In the stalk-eyed fly, while testes and accessory glands are small upon emergence, testes size quickly increases, followed by a phase of increased growth in the accessory glands, at which time sexual maturity is reached. It appears that it is this growth in accessory glands which signals sexual maturity, as sperm bundles appear at 12 days and motile sperm at 21 days, before sexual maturation is achieved (Baker *et al,* 2003). Not only did male age have an impact on sexual organ size, another factor was mating status, as in mated males the size of accessory glands and testes was smaller than in unmated individuals. The length of the accessory glands was shown to be markedly reduced in males who had mated, compared with those of the same age who had not. This change was accompanied by notably reduced testes length

(Mahmood and Reisen, 1982). In *Aedes aegypti*, virgin males at three days old were shown to have reached maximum maturity, as well as being replete with secretions (Ramalingam, 1983). In the current study however, no impact of mating status was observed, as virgin males at seven days old were used.

There was no relationship between body size and testes size (surface of image) in each of the four experimental groups. This is in agreement with Wigby *et al.* (2009), who found no relationship between body size (using wing area as a proxy) and testes size (the averages of the testes size of the left and right of these organs) in male *D. melanogaster*. However, Pitnick (1996) observed a positive correlation between body mass and relative testis mass in *Drosophila* species. In *D. hydei*, both the length and thickness of testes were found to be related to body size. The reason for this may be the comparatively limited resources of energy or space in smaller males for testis growth. Differences in the width of testes was associated with sperm production, which showed a 50% increase in sperm bundles in large males in comparison to the smallest (Pitnick and Markow, 1994b). In contrast with this, in the current study, a very strong correlation between body size and accessory gland size was found in Field Mopti and Mopti 2003 groups, meaning that large males had large accessory glands. The reason for this is not clear, but it may be that genetic factors caused this difference in each group.

In contrast, an interesting result was observed that testis size was larger in colonized strains (KIL and Mopti 2003), as well as in Super males, than in the progeny of Field individuals. In addition, KIL, Mopti 2003 and Super male groups had smaller accessory glands than in the wild group. A comparison between groups showed that field males were significantly different from other groups. Those males with larger accessory glands were found to mate more often (Baker *et al*, 2003; Rogers *et al*, 2005a). It is also suggested that in males who mate frequently, greater testis size is advantageous in allowing sperm

numbers in the ejaculate to remain steady over later matings (Rogers *et al*, 2008). In the stalk-eyed fly, a link was observed between the size of the male accessory glands in adult males and their mating frequency (Baker *et al*, 2003). The cause for this link has not been established; however it may be that either spermatophore numbers or production capacity for spermatophores are restricted by the size of the accessory glands (Baker *et al*, 2003). Neither body size nor testis size were found to be related to frequency of mating. In addition, based upon the findings for size of testis, it is suggested that, in this species, either production of sperm does not restrict the frequency of mating, or some other measurement should be used for sperm production other than testis length. The authors of the study emphasise the connection between mating frequency in the male and the size of accessory glands (Baker *et al*, 2003). In the stalk-eyed fly, *Cyrtodiopsis dalmanni*, various observations by Baker *et al*, (2003) support the greater influence of accessory gland size rather than testis size on the frequency of copulation (Rogers *et al*., 2005b). Firstly, while testis size was not found to notably decrease upon mating compared with unmated males, the accessory glands show a reduction, and mating activity in the field resumed as accessory gland size recovered. Baker *et al*. (2003) also demonstrate a correlation phenotypically between the frequency of copulation and the length of accessory glands, while no such correlation exists with testis length. Thirdly, there was an associated response in accessory gland length to bidirectional artificial selection on male mating frequency, while no such response was observed with testis length (Rogers *et al.*, 2005b). Large accessory glands are therefore beneficial in terms of mating frequency and sperm competition (Rogers *et al*., 2005a). In my opinion, mosquitoes reared under laboratory conditions have a different lifestyle than those individuals who live in the natural field. For example, field males need to search for food and for females for mating reasons: also, all environmental factors are varied in the field, including temperature, daylight and humidity,

while in the laboratory, conditions such as temperature and photoperiod are constant. Therefore, insects try to adapt to their new, limited environment or space (cage). It is suggested here that small accessory glands and large testes in inbred strains may be a kind of adaptation to the laboratory environment.

Male reproductivity is determined by not just sperm numbers in each testis but by a combination of sperm with seminal fluid, and is therefore dependent on the amount and potency of accessory gland secretion. Studies on male accessory glands (MAGs) in insects have not covered all parts of these important organs in male reproductive success. Studies of MAGs have paid attention to physiology (Jones, 1973; Ramlingam, 1983; Linklater *et al*, 2007; Reinhardt *et al*, 2011), structure of the organs (morphology and histology) (Clements, 1999; Mahmood and Reisen, 1982, 1994; Happ, 1984; Mahmood, 1997) chemical components (Giglioli and Mason, 1966; Gillott, 2003) and function (Tram and Wolfner, 1999; Wolfner, 2002; Lung and Wolfner, 2001; Lung *et al.*, 2001). Studies have also paid attention to the identification of MAGs proteins in several insects. For example, such studies have been conducted in grasshoppers (Muse, 1993); in moths (Nagalakshmi *et al.*, 2004); in fruit flies (Wigby *et al.*, 2009) and in mosquitoes (Rogers *et al.*, 2009). The current study showed that Plugin and Transglutaminase proteins were found in the MAGs in 4-day-old unmated *An.gambiae* s.s. males. This is in agreement with Rogers *et al.*, 2009 in that they identified fifteen MAGs proteins in *An. gambiae*, both in the MAGs which were transferred to females during copulation. They found that Plugin and Transglutaminase were the most two important proteins in MAGs, as they played an important role in generating the female mating plug. They did not, however, measure the quantity of the two proteins in the MAGs.

In the current study, it was found that the quantity of each protein was different. Plugin's estimated median (25-75 quartiles) in 142 *An. gambiae* s.s. males was 1.72

(0.799-3.018) MAGs equivalent. Meanwhile, Transglutaminase's estimated median (25-75 quartiles) in 146 males was 1.481 (0.663-2.872) MAGs equivalent. At all times Plugin was estimated to be present in greater amounts than was Transglutaminase, and this was true across the four groups studied. Wigby *et al* (2009) used ELISA methods to determine the quantity of two MAGs proteins, sex peptide (SP, aka Acp 70Aa) and Ovulin (aka Acp 26 Aa), in *D. melanogaster* males of two different sizes: large and small. They observed that there was a notably greater amount of SP protein in large males, while no marked difference was identified for Ovulin. The difference in the amount of the two proteins may be due to the fact that they are produced in different synthesis sites within the MAGs (Wigby *et al.*, 2009). It appears that in insects, the epithelial cells of the accessory glands are responsible for the production of secretory proteins (Happ, 1984). This secretory epithelium appears to contain multiple strata, while being histologically simple (Happ, 1984). Production of secretory products occurs within each cell individually, (Happ, 1984). Within the accessory glands, two areas can be identified: a large anterior one which accounts for 75% of the glands' volume and contains large yellow granules; and a smaller section with smaller granules which are white in colour (Mahmood and Reisen, 1982; 1994; Mahmood, 1997). Ramalingam (1983) studied the histology of accessory glands in *Aedes aegypti*, showing that the glands contain two types of secretory cells, each type differing in the nature and release of its products.

The positive relationship between body size and estimates of both proteins was shown in the current study. Wigby *et al.* (2009) observes that large males have large accessory glands and produce more proteins in their MAGs than small males. In *An. freeborni*, large males were more successful in terms of the numbers of females inseminated than were their smaller counterparts (Yuval *et al*., 1993). Further, in *Ae. aegypti*, large males were found to transfer greater volumes of sperm to the female

(Ponlawat and Harrington, 2009). In *An. gambiae* s.s. moreover, it was found that large females are preferred by males (Okanda *et al*., 2002).

All males across the four studied groups produced a similar amount of Plugin estimated in their MAGs, while the quantity of Transglutaminase was different across the experimental groups. However, Super males produced the equivalent amount of Transglutaminase as did Field Mopti. Also, Mopti 2003 generated the equivalent amount of Transglutaminase as did KIL. In the current study, the assumption was made that hybrid males were better than the colonized lines. This seems to be true for sperm length because hybrid males had a long sperm length as did the field samples. However, the levels of Transglutaminase in the colonized lines were higher than in the hybrids. In my view, not all the fitness qualities were affected by inbreeding. For example, the quantity of Plugin showed no fitness difference between hybrids, colonized lines and field individuals. Mopti 2003 and KIL were colonized strains, which had been reared under laboratory conditions for more than seven years. Field Mopti was the progeny of wild-caught females. It is possible that the difference was based on chromosome levels (Bijlsma *et al.*, 1999), because there were few or no deleterious recessive alleles in Field Mopti and Super males relative to the two colonized strains. When chromosome balancers were utilised in a field population to obtain samples of chromosomes, approximately half of all chromosomes contained mutations which were either lethal or sub-lethal, and this was found in homo- and heterozygous conditions (Bijlsma *et al.*, 1999). Through selection only of chromosomes with similar viability to heterozygotes under homozygous conditions, deleterious recessive alleles were then mainly eliminated (Bijlsma *et al.*, 1999). Fitness may be adversely affected by inbreeding via two mechanisms. The over-dominance hypothesis suggests that favourable combinations of heterozygotes are lost with inbreeding, and that this leads to inbreeding depression (Wright *et al.*, 2007). The partial

dominance hypothesis meanwhile states that deleterious recessive alleles find more widespread expression under conditions of inbreeding, thus causing inbreeding depression (Wright *et al.*, 2007). Life history, as opposed to morphological traits, would thus be expected to show inbreeding depression to a greater extent, because dominance variance is central to both mechanisms (Wright *et al.*, 2007). Inbreeding can be caused by depleted heterozygosity due to the two alleles at a locus becoming homozygous. In addition, allele fixation can also occur through genetic drift (random variations in allele frequencies) (Lacy *et al*, 1997). In an outbred population, if there are slightly or moderately deleterious alleles found, individuals homozygous for these deleterious alleles will incur a fitness cost (Charlesworth and Willis, 2009). Different genes can work in many different ways, fitness reductions as a result of homozygosity for deleterious alleles (mutant alleles or alleles at loci with overdominance) act roughly multiplicatively (Charlesworth and Willis, 2009). This has been observed to occur when different deleterious mutations act independently of the trait (Charlesworth and Willis, 2009).

The history of inbreeding and any limiting experimental conditions will impact on male mating behaviour (Joron & Brakefield, 2003). For example, Kuriwada *et al* (2011) found that there was a significant effect of inbreeding depression on male mating performance (number of mating frequency per night) in West Indian sweet potato weevil, *Euscepes postfasciatus.* Nevertheless, the effect was low as fitness loss was 6.3%. In addition, Nakahara and Tsubaki (2008) studied the impact of inbreeding on mating in damselfly *Ischnura senegalensis* (Rambur) by counting the number of dead and live sperm in female sperm storage organs to discover whether inbred males transferred more dead sperm to females than wild male during copulation. They found inbred pairs had significantly low fertility and there was no significant difference in dead and live sperm numbers in the female's sperm storage organs between inbred and wild individuals. The

low inbred fertility was explained as depending on female choice at the insemination stage to store sperm in their organs and/or use them, rather than the sperm quantity or quality measured by live-to-dead ratio (Nakahara and Tsubaki, 2008). Similar results were found by Okada *et al*, (2011) in *Drosophila simulans* showing that male fertility was negatively affected by inbreeding depression while sperm viability was not.

In Super males, alterations in the genome may occur swiftly with hybridisation with expansion of the genome, rearrangement of chromosomes, differential gene expression, and gene silencing (Baack and Rieseberg, 2007). The other explanation of MAGs activity is that this depends on corpora allata hormones (Regis *et al.*, 1985). In *Tribolium castaneum* beetles which were given hydroprene, a juvenile hormone analogue, without RH-2485, the ecdysteroid analogue, MAGs were observed to grow larger and contain greater quantities of protein and total RNA, as well as greater expression of Acp genes (Parthasarathy *et al.*, 2009). By contrast, knock-down in the expression of the JHAMT gene mediated by RNAi led to smaller MAGs and reduced Acp expression. JH appears to be central to male reproductive fitness, as males lacking in this performed poorly in mating, with less activity, decreased transfer of sperm, and comparatively low production of eggs and offspring in the females with which they mated (Parthasarathy *et al.*, 2009).

**Conclusion**

The quality of males is an important factor in male reproductive success. The present study suggests that hybrid individuals (Super males) could represent a way to improve the males' value under laboratory conditions. Sperm length in super males was found to be similar to the field individuals, whereas it was lower in colonized strains. The impact of colonization was observed, as all colonized strains as well as Super males had a larger testes size and smaller accessory glands size whereas field Mopti had small testes and large

accessory glands. The amount of both proteins, Plugin and Transglutaminase, was measured in male accessory glands. Across the four groups studied there was no difference in estimated amounts of Plugin, as all males produced a similar amount of it in their MAGs. However, Transglutaminase production could differ between groups. The amount of estimated protein for Plugin was consistently higher than the amount of Transglutaminase.

# Chapter 6

# Quantity of accessory glands proteins that manipulate *An gambiae* s.s. female reproductive behavior and male mating success

## 6.1. Introduction

Male accessory glands (MAGs) secrete a seminal fluid which is transferred to the female insect during copulation. When the female receives it, she changes her behaviour. MAG secretions or seminal fluid may make her unable to re-mate for a while, which allows sperm to be stored, as well as protecting the legacy of the male, as his sperm will be used for fertilization if any eggs are laid (Gillott, 2003; Ravi Ram *et al.*, 2006; Sirot *et al.*, 2009). The secretions achieve this, in addition to their role in allowing sperm movement, by creating a physical barrier to re-mating or by preventing the retention of any sperm which is introduced later (Lung and Wolfner, 2001). In addition, these secretions positively influence both the number of eggs produced and the rapidity with which they develop, possibly affecting both ovulation and oviposition (Gillott, 2003). Craig (1967) established that female mosquitoes of *Aedes aegypti* (L.), after receiving MAGs substances during copulation, then became more refractory to further insemination.

MAGs of numerous insect species produce and secrete a number of reproductive proteins together called accessory gland proteins (Acps) (Dottorini *et al.*, 2007). Sirot *et al.*, (2008) identified sixty-three new proteins in *Ae. aegypti.* They used two methods; mass spectrometry of proteins from *Ae. aegypti* MAGs, ejaculatory ducts and female reproductive tracts, plus bioinformatic comparisons to *D. melanogaster* accessory gland proteins. Twenty one of these proteins were found in mated females and not in virgins. Sirot *et al.* (2009) improved this method by using enzyme-linked immunesorbent assay (ELISA) using MAGs antibodies (Ovulin and sex peptide) in *D. melanogaster*. They discovered that the quantity of both proteins found in mated females dropped as the time

period since mating increased. They also observed that mated males refilled their glands and transferred the same amount of proteins as did virgin males after three days of sexual inactivity (Sirot *et al.*, 2009).

Ravi Ram and Wolfner (2007) investigated the function of twenty-five Acps in *Drosophila* males by using RNA interference (RNAi) to reduce the amounts of selected Acps. They found that five proteins were responsible for egg production, storage of sperm in the female spermatheca, and accessibility of the female to re-mating. Also, another four proteins were thought to influence any long-term response, with effects upon physiological changes as well as upon behaviour after mating. Exhibition of the post-mating response over a longer period depends upon the storage of sperm. A number of Acps working in conjunction were found to be necessary in order to reduce the mated females' mating receptivity, as well as for prolonged production of eggs and release of sperm stored within the seminal receptacle.

MAG secretions have been identified as playing an essential part in post-mating changes in female behaviour in *An. gambiae*. MAGs are introduced into the female atrium in the form of a solid 'mating plug', which over the next 24-48 hours is then digested (Gillies, 1956). This body was observed only in mated wild females immediately after a blood meal, and whose ovaries were at stage II or III according to Christophers's classification (Gillies, 1956). The mating plugs were not present in virgin mosquitoes (Gillies, 1956). Under laboratory conditions, females mated with more than one male, and as a result, double plugs were sometimes created (Gillies, 1956). Tripet *et al* (2005), in their study of the effects of cross-mating in *Anopheles gambiae* s.s. between M and S molecular forms, did not find any difference in the effect of mating with either a male of the same form or an alternative form in preventing further female mating and stimulating egg-production, indicating that the proteins from the male accessory gland secretions

involved in these processes do not differ between the two forms, in contrast to previous research on the topic (Tripet *et al.* , 2005). Hatching rates and numbers of larvae surviving to adulthood were affected by cross-mating: a finding which the authors suggest may be due to the hybrid form rather than any effect of MAG secretions. Shutt *et al*. (2010) injected MAG secretions into the intra-thoracic region in both *Anopheles stephensi* Liston and molecular forms M and S of *An. gambiae* s.s., as well as crossing substances to be injected between these two forms of *An. gambiae*. It was found in all cases that further mating by females did not occur, thus indicating that the substances involved are the same in both forms. The authors further suggest, as a result of these findings, that where remating occasionally occurs in *An. gambiae* s.s., this is not as a result of mating incompatibility between M and S forms (Shutt *et al*, 2010). When tethered, the majority of *An. gambiae* females were found to allow mating three or four times within a short period, receiving a mating plug from each male: thereafter they tended to attempt to prevent copulation by kicking, although normal copulation still appeared to take place in some cases (Charlwood and Jones, 1979).

The mating plug in *An. gambiae* s.s. is responsible for storing sperm, because if the mated female has failed to receive a plug, she cannot store sperm in her spermathecae (Rogers *et al.*, 2009). While the exact significance of the proteins of the seminal fluid in the mating processes of *An. gambiae* s.s. remains unclear, Rogers *et al*. (2009) identified the source of the proteins in the mating plug, MAGs and atria of virgin females by using mass spectrometry and reverse transcription PCR (RT-PCR). They discovered twenty seven proteins in the plug mass which comprised 15 proteins originally from the male, 6 proteins from the female and the rest of the proteins from both sexes. One special protein, termed Plugin, AGAP009368, was established in both MAGs and mating plug specimens. Plugin protein found an intensive signal in the front part of the MAGs, as well as being of

importance for the mating plug. Another main protein in the MAGs was Transglutaminase, AGAP009099, the key role of which is in the activity of TGase enzymes. These two proteins were found only within the MAGs in males and within the atria in females (Rogers *et al*, 2009). The protein AGAP009099 was found to be essential in forming the mating plug. Mating plugs are found in many insect species such as the bumblebee *Bombus terrestris* (Baer *et al*, 2001), in the fruit fly *D. melanogaster* (Lung and Wolfner, 2001) and in ant worker *Dinoponera quadriceps* (Monnin and Peeters, 1998). *An. gambiae* females have been found to differ significantly from *D. melanogaster* in their reproductive mechanisms and associated genes. Certain structural and molecular elements of the female atrium in *An. gambiae* s.s. cease to function after the first copulation, which may mean that further insemination is not supported. Further, it was discovered that certain genes in the sperm storage organ responded to copulation, probably being involved in maintaining and utilising the sperm stored there. These discoveries have implications for possible vector control, and specifically for fertility manipulation (Rogers *et al.*, 2008).

There are other functions of MAGs secretion. The males of the fruit fly *D. melanogaster*, transfer antimicrobial peptides to females during mating. These peptides offer antimicrobial defence to the male's and then the female's reproductive tract during this process, and thereafter to the eggs. It is also possible that this defence is extended to the sperm that transferred after the seminal fluid (Lung *et al.*, 2001). The steroid hormone 20-hydroxyecdysone, which plays a main role in stimulating vitellogenesis, one procedure necessary for egg maturation, is also transferred to the female from the male in *An.gambiae* through male accessory gland secretions (Pondeville *et al.*, 2008).

There are several environmental factors that impact on the fecundity and fertility of adult female mosquitoes: for example, successfully taking a blood meal, searching for a partner for mating and finding a host (Onyabe *et al.*, 1997). No link is found between

blood-feeding patterns and mating status, with the likelihood of taking a full blood meal in *Anopheles gambiae* Giles females after mating being the same irrespective of whether they took a blood meal prior to mating or not (Detinova and Gillies, 1964). In *An. gambiae* s.s. and *An. funestus,* biting activity is initiated irrespective of mating status (Detinova and Gillies, 1964). Klowden and Russell (2004) found that the amount of blood ingested was directly related to the likelihood of eggs maturing (Klowden and Russell, 2004). Oogenesis was found to occur only above a wing length of 2.8mm in *An. gambiae* s.s. females which were provided with single blood meals, while this lower threshold was not seen in sugar-fed mosquitoes, in all of which eggs with the synthesis of maternal deposits were matured (Fernandes and Briegel, 2005). Greater amounts of lipid were found in the yolk with sugar-feeding, despite the interference of this type of feeding with blood meals (Fernandes and Briegel, 2005). In *An. stephensi*, oogenesis has been found to depend upon raised levels of haemolymph amino acid concentration: it is also seen that ovarian development, particularly in poorly fed females, is boosted by mating (Uchida *et al*, 2003).

In *An. gambiae* s.s., multiple blood meals were consistently taken, and 120 eggs were produced per female (Fernandes and Briegel, 2005). The number of eggs per mosquito was seen to vary widely from individual to individual, based upon body size, breeding site and the time of year (Shannon and Hadjinicalao, 1941), in The The number of Anopheline species under field conditions was also affected in some cases by blood taken, as females who had taken a second meal post-breeding, a common occurrence in rainy conditions, produced eggs in greater numbers (Shannon and Hadjinicalao, 1941). Additionally, larger batches were found to consist of smaller eggs (Shannon and Hadjinicalao, 1941). The direct connection between a female's weight and the number of eggs produced, and the weight of the blood meal taken is even more directly connected, and thus it was found to

be more accurate in the estimate of number of eggs matured by mated females in *An. triseriatus* fed to satiety (Cochrane, 1972).

Keeping insects under captive conditions or in small populations causes some genetic problems such as inbreeding depression (Futuyma, 2009), as shown under laboratory conditions by Wolska (1980), in zoos (Lacy, 1993) and in the wild (Crnokrak and Roff, 1999). Inbreeding happens when related individuals mate together rather than with nonrelatives (Futuyma, 2009). As a result, homozygosity increases leading to inbreeding depression (Futuyma, 2009). The causes of this are two-fold: the over-dominant interaction of alleles; and the expression of deleterious recessive alleles. The effects of the two causes differ, and the second may be avoided via selection (Dudash and Carr, 1998; Futuyma, 2009; Charlesworth and Willis, 2009). Inbreeding depression is reported by numerous studies in various insect species. For example, Rutledge and Piper (1984) reared several strains of *Ae. aegypti* and *An. stephensi* under laboratory conditions for more than six generations. They observed that an inbreeding process of a deleterious recessive character appeared over the time of inbreeding. Deleterious traits were noted in decreased female fertility and lowered adult longevity, as well as greater mortality at the immature stages. Under laboratory conditions, investigation was made of the effect of inbreeding on the Copra mite, *Tyrophagus putrescentiae* (Acarina, Agabidae). It was observed that the impact of inbreeding increased rapidly after keeping the insects for more than 16 generations causing extended egg development time, increased mortality percentages, especially at the larval stage, and a reduction in adult longevity, especially of males (Wolska, 1980). Hunt and Drummond (1983) studied the effect of inbreeding on lone star ticks, *Amblyomma americanum (L.)* by comparing inbred and wild groups. They noticed that the strain which had been reared in a colony for over twenty years had slighter body mass, spent a longer time for pre-oviposition and oviposition, and females laid smaller

numbers of eggs with a lower hatching rate than field females. Reciprocal crosses between wild and colonized insects produced progeny which showed significant improvement in reproduction characteristics similar to wild females (Hunt and Drummond, 1983). In the tsetse fly, *Glossina* spp. *morsitans morsitans,* inbreeding depression had only a slight impact on this species (Jordan, 1980). Over forty generations, female fecundity, female lifespan, the number and weight of pupae created as well as the adult sex ratio, presented little difference between cohorts (Jordan, 1980). The existence of inbreeding depression may be thought to render kin-to-kin mating maladaptive. This must be balanced, however, against enhancements to fitness which may in some situations occur through such mating. In species not seriously affected by inbreeding depression, kin mating may therefore be considered to be adaptive behaviour. For example, in the West Indian sweet potato weevil, *Euscepes postfasciatus,* which was found to display little inbreeding depression, apart from a reduction in mating performance in the male which was not significant, mating was found to occur more frequently between full-sibling pairs than between non-related pairs (Kuriwada *et al*, 2011).

Inbreeding is likely to occur in mosquito colonies because of the colonization process. It was observed in chapter 4 that colonization has a strong effect on sperm length. In addition, the offspring of field collected females and recently colonized strains tended to have smaller testes than those in long established strains, suggesting potential selection on this trait. The accessory glands of the progeny of wild-caught females were significantly larger than all colonized strains. This impact of colonization may cause problems for mosquito releases in an SIT programme. For that reason, one easy solution which does not involve complicated back-crossing schemes, is to create heterozygous males from two inbred strains in order to take advantage of hybrid vigour, as found in Chapter 4 & 5.

To date, in *An. gambiae* s.s. the quantity of male accessory gland proteins transferred to females during copulation has not been investigated in previous studies, and neither has the impact of inbreeding depression. In the current study, the quantity of two MAG proteins (Plugin & Transglutaminase) in the female reproductive tracts was estimated in two 25+ year old and 7-year old inbred strains and in highly heterozygous males resulting from a cross between the two long established strains, referred to as Super males. The number of eggs, larvae and the hatching rate were recorded in order to compare the fecundity and reproductive success of Super males versus the inbred parental lines.

## 6.2. Material & methods

### 6.2.1. Set-up of mating cages

Blood feeding took place for KIL as well as Mopti 2003 strains. Two days post blood feeding, mature female mosquitoes laid eggs into white polystyrene pots lined with filter paper and half filled with distilled water. After hatching out, 200 first instar larvae were relocated into 6 trays (33 x 23 x 5 cm) for each strain, including one litre of distilled water. Larvae go through four stages and a pupal stage within 8-10 days after hatching (see section 2.2, chapter 2). To minimize variance, all trays were trayed out at the same time for each strain. To reduce the impact of the environment, the trays were moved once a day on the shelf. More than 600 pupae of both sexes for each strain from the same generation were used to set up mating in the following cages: 100♂ Mopti 2003 x 50♀ KIL, 100♂ KIL x 50♀ KIL and 100♂ Mopti 2003 x 50♀ Mopti 2003. After 48h post mating, blood feeding took place for all (see section 2.2.5, chapter 2). Two days later, oviposition pots were added to each cage. After a further two days, the eggs hatched and the larvae were trayed out, with 7-8 trays for each experimental group (Super males, KIL and Mopti 2003), making a total of 23 trays.

### 6.2.2. Larval feeding regime

The larval feeding regime was designed to range over nine days to create approximately similar body sizes of adult mosquitoes, and the previous feeding regime was manipulated to produce normal adult body size (see section 2.2.3, chapter 2). Here, the amount of food was changed to create a new feeding regime different from the one described in chapter 2. The feeding regime took place in plastic trays for newly hatched eggs. For each experimental group, 7-8 trays were trayed out to each feeding regime, where each group per tray was fed one drop of liquifry (Interpet Ltd., Dorking, UK), once during the first day. 10mg of ground flakes of baby fish food (Tetra werk, Mulle, Germany) was added for the next two days. 30mg of fish food was added once each day on days four and five. From days six to nine (until pupation) 30mg was added per tray twice a day (Table 6.1). To minimize any variation between the quantity of food, the fish food was measured by using a balance (30mg/tube) and then stored in individual micro centrifuge tubes (1.5ml). The tubes were stored in plastic bags (50 tubes per bag) at room temperature. Also, to minimize the impact of environmental conditions including temperature, humidity and light under artificial conditions, which might affect larval and adult growth, the trays on the shelves, were moved to a different location once a day. Four larval trays from each combination were put together as one set and another set were put together to reduce the impact of environment on each single tray. In addition, to minimize confounding factors, the trays were labelled using a unique pen colour and the name of the combination for each larval group.

Table 6.1. A summary of larval feeding regime used to produce the same adult body size. Different amounts of ground fish food (Tetra werk, Mulle, Germany) were used to feed larval trays, which included 200 larvae, from day one until pupation.

| **Post-tray out of 1st instars** |
|---|
| **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 & evening) in total, 60 mg |
| **Day 7:** 30 mg twice daily (morning & evening) in total, 60 mg |
| **Day 8:** 30 mg twice daily (morning & evening) in total, 60 mg |
| **Day 9:** 30 mg twice daily (morning & evening) in total, 60 mg |

### 6.2.3. Hybrid males created

More than three thousand pupae were sexed to set up mating cages in four different combinations. After the pupae emerged as adults, mating cages were set up as follows in Table 6.2.

Table 6.2. Mating combinations of the adult male and female mosquitoes in mating cages. Mating cages with the same number had the same combination in two replicates.

| Replicate 1 | Replicate 2 |
|---|---|
| 200 ♂ KIL/Mopti2003 (super males) x 100♀ KIL 1a | 200 ♂ KIL/Mopti2003 (super males) x 100♀ KIL1b |
| 200 ♂ KIL/Mopti 2003 (super males) x 100 ♀ Mopti 2003 2a | 200 ♂ KIL/Mopti 2003 (super males) x 100 ♀ Mopti 2003 2b |
| 200♂Mopti 2003x 100 ♀ Mopti 2003 3a | 200♂Mopti 2003 x100 ♀ Mopti 2003 3b |
| 200 ♂ KIL x 100 ♀ KIL 4a | 200 ♂ KIL x 100 ♀ KIL 4b |

A mouth aspirator was used to transfer adult mosquitoes to the mating cages because this allowed for the sex to be checked more easily and for the mosquitoes to be counted. To start with, four cages were set up by transferring 50 males each time to each cage until 200 males were located in each cage. The males were transferred a night before adding the females, to ensure those males adapted to the cages and started swarming. The next day, 50 females were transferred to each cage until 100 females were located in each cage. The same methods were repeated with the second set of 4 cages as a second replicate. Distilled water on moistened cotton wool was provided on the top of each cage at all times and mosquitoes were fed on 5% glucose and 0.05% para-amino benzoic acid (PABA). All mating cages were used to assess the quantity of male accessory gland proteins transferred to females during copulation, using the Elisa method. The super male fertility rate was assessed by counting eggs and and recording the hatching rate. To minimize the impact of environmental conditions including temperature, humidity and light under artificial conditions, which might have an effect on adult growth, the cages, on shelves, were moved once a day. Also, to minimize confounding factors, the cages were labelled using a unique number and symbols.

### 6.2.4. Assessment of male accessory gland proteins in female reproductive tracts via Elisa.

After less than 24h post overnight mating, females of 3-5days old were collected from each cage to ensure that the mating plug was present in the female tract as it dissolves after 24h post mating (Gillies, 1956). Female adults were randomly picked by shaking the cage before collecting by using a mouth aspirator from various heights in the cage. After overnight mating, all the males were removed. Fifty females from each combination were dissected to assess the amount of male accessory gland proteins transferred to the female

during copulation. The last two abdominal segments of the females, containing the spermatheca, and mating plug were cut, transferred to 1.5ml microcentrifuge tubes containing 220 µl PBS+PI and frozen at -80C until used for the Elisa work. This was carried out using the same procedure as that described in section 5.2.5, Chapter five.

The mating rate across the four studied groups was determined by real-time quantitative PCR (by the researcher's colleague, Nkiru Esther Ekechukwu). It was found that the mating rate in 192 females was more than 95% across the four groups (Male Mopti 2003 x Female Mopti 2003 was 97.9%, Super male x Female Mopti 2003 was 96.9%, while Male KIL x Female KIL and Super male x Female KIL were 95.8%).

### 6.2.5. Estimation of female fecundity

The fecundity of both sexes is measured by how many gametes, sperm cells and ova (usually eggs), are produced in the gonads by an individual. The fertility of males however, is measurable by counting the number of viable young fathered within a given duration (Lincoln *et al.*, 1998). Two day post blood feeding, another set of forty-eight females (depending on the number of alive adults) were collected from each combination. The females were transferred by mouth aspirator to individual falcon tubes (30ml) as an oviposition site. Oviposition tubes designed as the methods of Shannon and Hadjinicalao, (1941) with some manipulation. The tubes were prepared as oviposition sites by using filter paper strips (2cmx28cm) which were rolled and put into each tube and 5ml of distilled water was added to provide a surface for egg-laying. The top of each tube was covered with net which had a small hole that was closed with a piece of cotton, through which to insert a female into the tube. The next day, the tubes were checked for eggs. The filter paper was taken with forceps from the tube and transferred to a funnel with filter

paper and a squeezy water bottle was used to wash the strip of paper as well as the oviposition tube to make sure all the eggs were transferred to a new filter paper. Care was taken to avoid losing the eggs. The filter paper which contained the eggs was then carefully transferred to a petri dish and the eggs were counted by eye under a magnifying glass. The number of eggs for each combination group was counted by counter twice for more accurate results and recorded on a data sheet.

### 6.2.6. Estimation of male reproductive success

After counting the number of eggs, they were transferred to small plastic trays containing 200ml of distilled water. One drop of liquifry fish food was added to each tray. According to Impoinvil *et al*, (2007) mosquito eggs of *An. gambiae* s.s. held under moist conditions at 22-27C° had the highest overall mean hatching rate of between one to three days of incubation time. Therefore, the trays were left to stand for four days to allow for the hatching of eggs present, and the resultant larvae were recorded by putting the tray on ice to reduce larval movement and counting from one corner to the other using a manual counter under a magnifying glass, to make sure that no larvae were counted twice. The number of larvae for each combination was counted twice for more accurate results and recorded on a data sheet. All trays were labelled with the name of the combination and the date using a unique pen colour for each one. The experimental processes are summarized as a design shown in Fig. 6.1.

### 6.2.7. Wing length measurement

All female bodies used for Elisa work or to estimate male fertility were kept in microcentrifuge tubes (1.5ml) at -4C° for wing length measurement. The wing length was measured as shown in section 2.2.6, Chapter two. Tubes were labelled with the name of the group and a unique number.

### 6.2.8. Statistical analysis

Because the data of estimated Plugin and Transglutaminase were found to be deviated from a normal distribution, they were analyzed using Kruskal-Wallis Tests (3+groups) and 1-Way Wilcoxon test (2 groups) were used in examination for difference between groups. The number of eggs and larvae did not deviated significantly from normality. So, ANOVA tests followed by Tukey`s test for pair comparisons were used. All statistical work was carried out using JMP software.

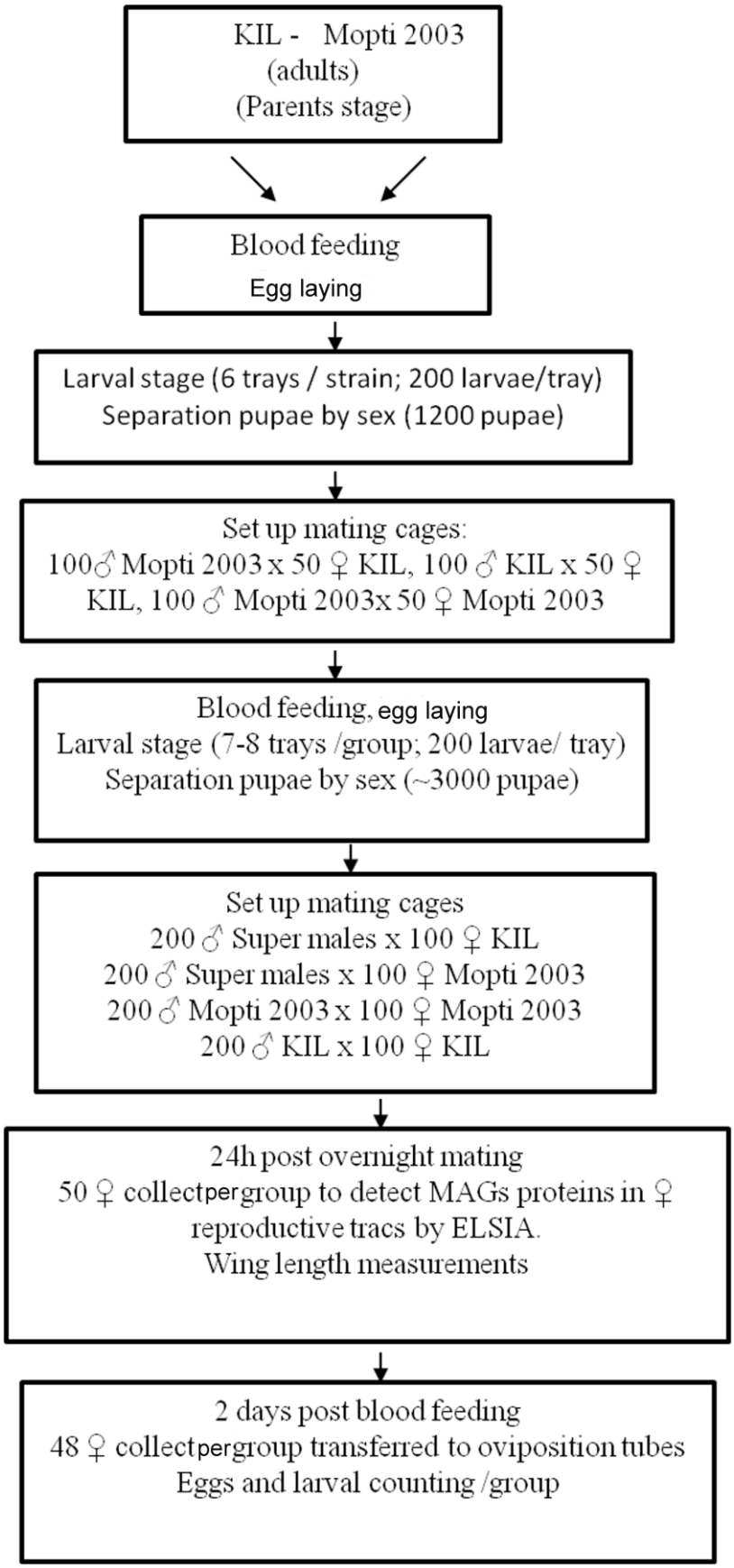


Fig.6.1. Design of experiment to investigate super male reproductive success and female fecundity.

## 6.3. Results

### 6.3.1. Quantity of male accessory gland proteins in female reproductive tracts

#### 6.3.1.1. Estimated Plugin protein

The quantity of Plugin protein transferred to females during copulation and stored in their reproductive tracts (mating plug and spermatheca) was estimated in females of *An. gambiae* s.s. following a single night of mating and quantified as MAGs equivalent (see methods, sections 6.2.4 and 5.2.5). The estimated Plugin protein in 193 female reproductive tracts across four mating combinations, (Super male x ♀ Mopti 2003; Super male x ♀ KIL; ♂ Mopti 2003 x ♀ Mopti 2003; and ♂ KIL x ♀ KIL) was checked using the normality test by Shapiro-Wilkinson. The overall distribution of Plugin estimated deviated significantly from a normal distribution (Shapiro- Wilkinson: $n$= 193, W= 0.766, $P$<0.001). The median (25-75 quartiles) of estimated Plugin protein of 193 females for the same mosquito groups was 0.600 (0.257-2.153). The range of estimated Plugin was 0.063-6.123 (Fig. 6. 2).

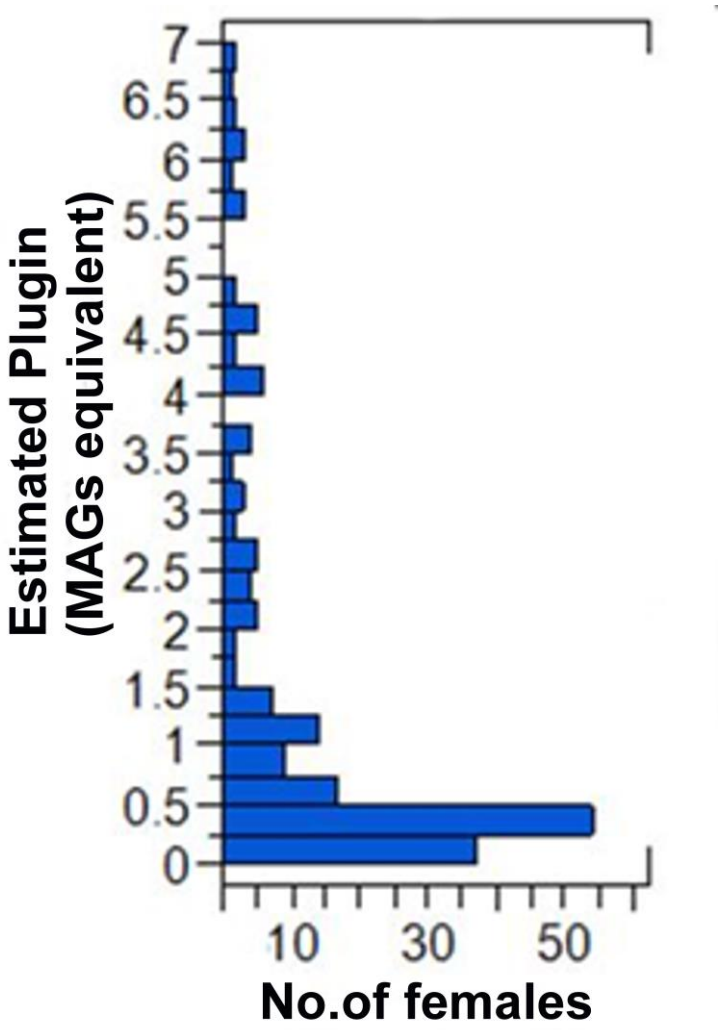


Fig.6.2. Frequency distribution of estimated Plugin in female reproductive tracts of *An. gambiae* s.s. (all combined groups, *n*= 193).

The frequency distribution of estimated Plugin in the female tracts was examined for each group separately to check for normality using Goodness Fit tests. Overall, all data deviated significantly from a normal distribution (Shapiro - Wilkinson: $P<0.001$ in all cases). The medians (25-75 quartiles) of four groups were as follows: Super male x ♀ KIL was 0.633 (0.255-2.727); ♂ KIL x ♀ KIL was 0.924 (0.267-2.557); Super male x ♀ Mopti 2003 was 0.595 (0.239-1.906); Mopti 2003 x ♀ Mopti 2003 was 0.447 (0.258-1.428) (Fig. 6.3).

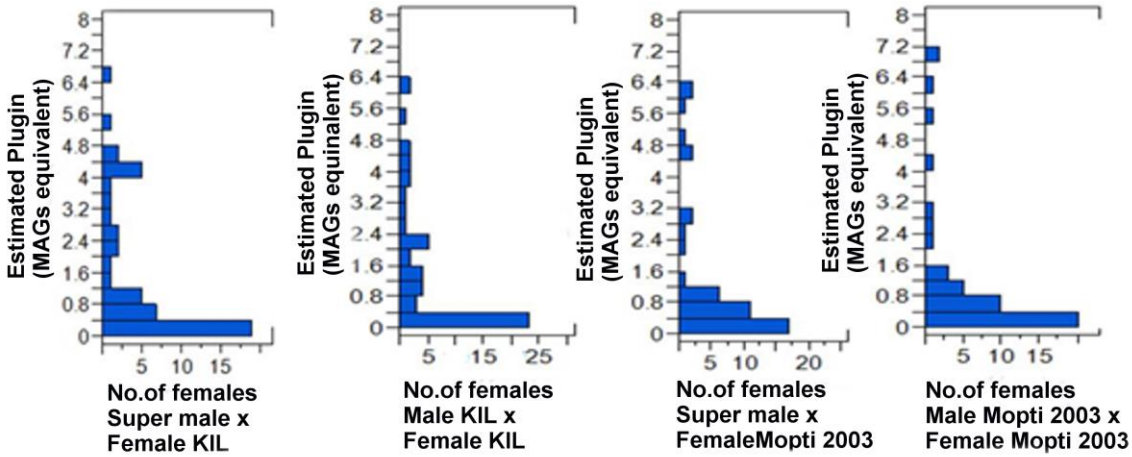


Fig.6.3. Frequency distribution of estimated Plugin (MAGs equivalant) in female reproductive tracts in the four studied groups (Super male x ♀ KIL; ♂ KIL x ♀ KIL; Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003).

### 6.3.1.2. Relationship between female body size (measured as wing length) and quantity of Plugin in female reproductive tracts

The mean wing length of 191 *An. gambiae* females (all four groups combined) was 2.991mm (2.976-3.006CI) (Fig.6.4). Overall, there was significant difference in body size (measured as wing length) of the 191 females across the four combined groups (ANOVA: $F_{3,191}$= 6.683, $r^2$= 0.096, $P$=0.003). Pair wise comparisons between the groups showed a significant difference between Super male x ♀ Mopti 2003 and Super male x ♀ KIL ($P$=0.002), and between ♂Mopti 2003 x♀ Mopti 2003 and Super male x♀ KIL ($P$=0.011). However, there was no significant difference between all other comparison pairs ($P$>0.051 in all cases).

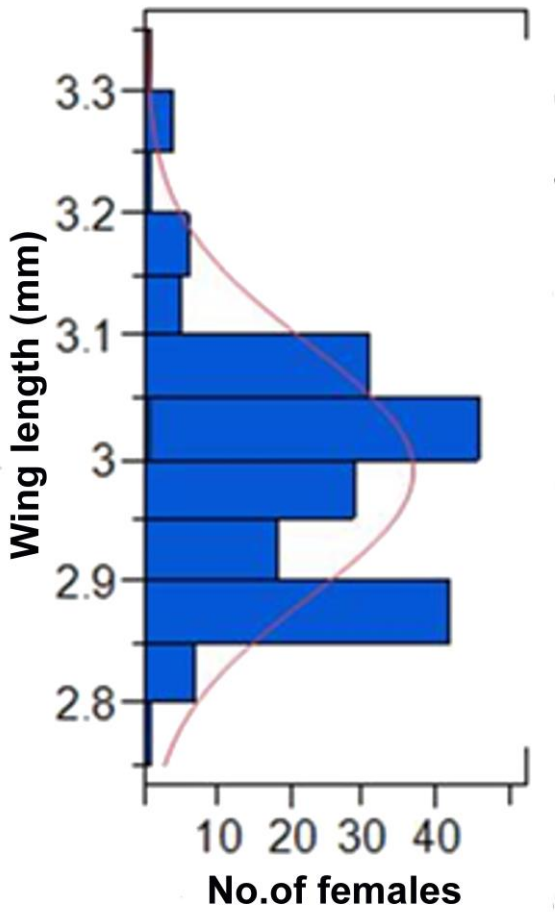


Fig.6.4. Frequency distribution of wing length (mm) of *An. gambiae* s.s. females (all combined groups, $n$= 191). The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

The correlation between wing length (body size) and estimated Plugin in 191 *An. gambiae* s.s. females across the four experimental groups was investigated. Overall, no linear relationship was found between body size and Plugin estimated in the reproductive tract of females (Linear regression: $n$= 191, $t$= 1.58, $r^2$= 0.007, $P$=0.242) (Fig. 6.5).

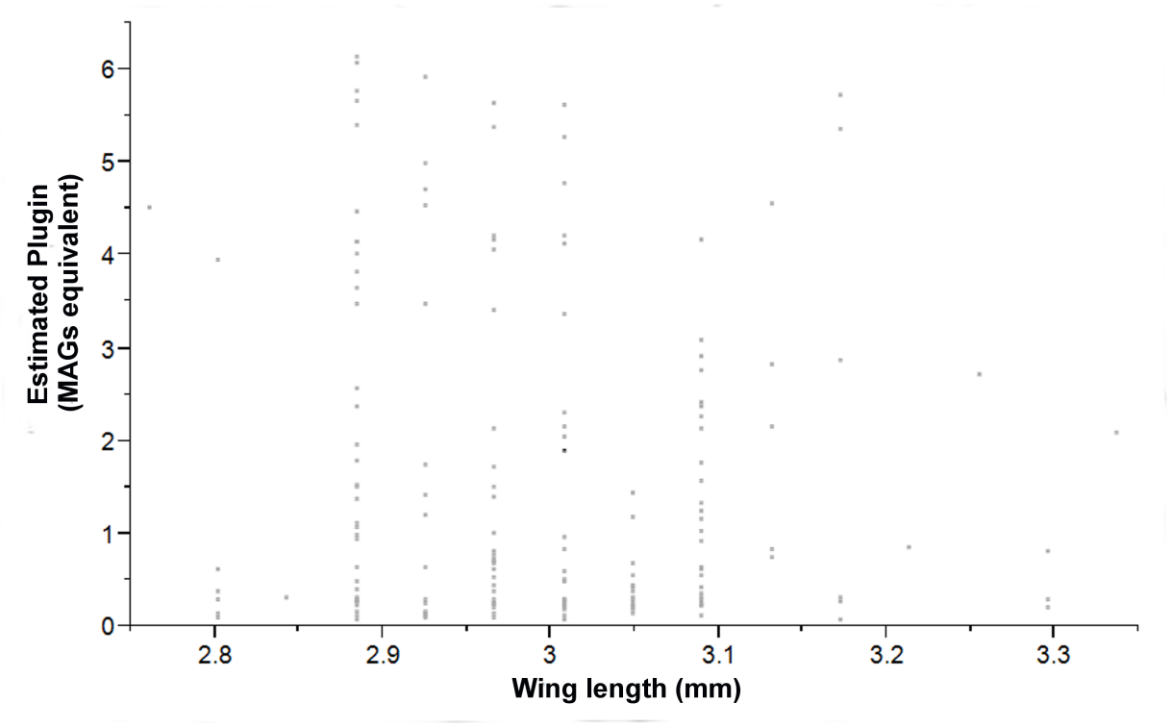


Fig.6.5. Linear relationship between body size (wing length) and estimated Plugin in *An. gambiae* s.s. females reproductive tracts (all combined experimental groups, *n*= 191).

Linear regressions of estimated Plugin and body size (wing length) performed within each of the four studied groups showed that there was no relationship between these properties in any of the groups (Linear regression: ♂KIL x ♀ KIL $n$= 53, $t$= 2.13, $r^2$= 0.067, $P$=0.059; Super male x ♀KIL $n$= 49, $t$= 0.25, $r^2$= 8.249, $P$=0.950; Super males x ♀ Mopti 2003 $n$= 45, $t$= -0.63, $r^2$= 0.015, $P$=0.411), and ♂ Mopti 2003 x ♀ Mopti 2003 ($n$= 44, $t$= 1.10, $r^2$= 0.019, $P$=0.892) (Fig.6.6).

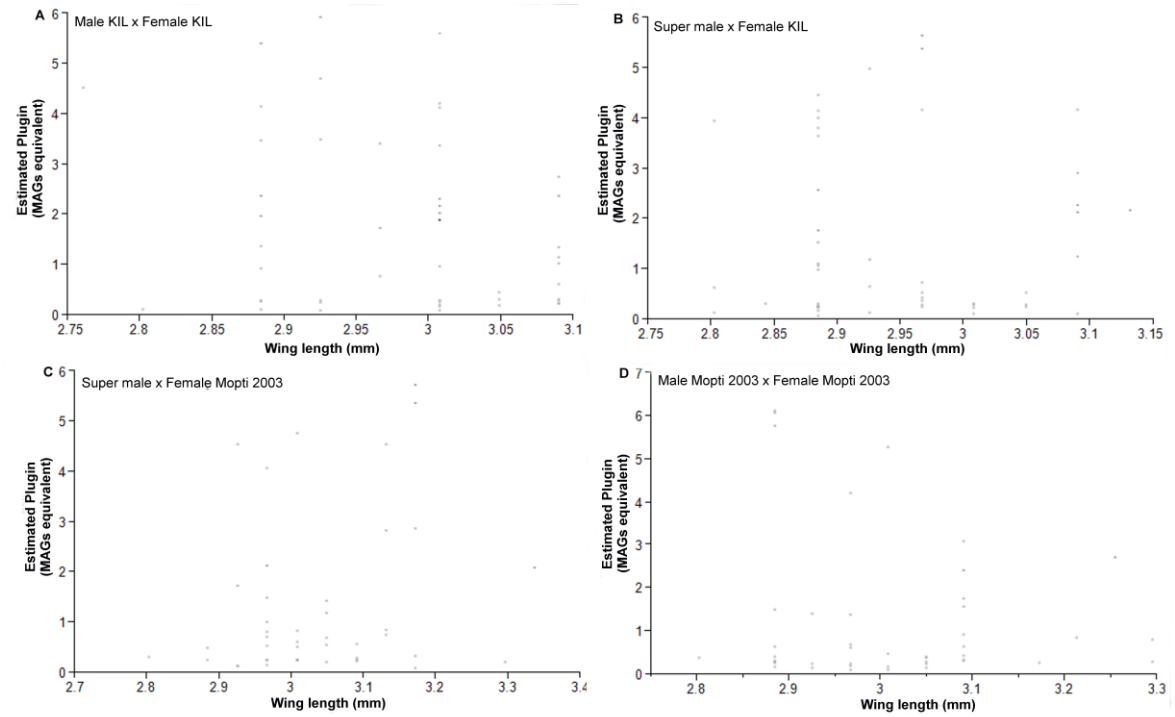


Fig. 6.6. Linear relationship between female body size (wing length) and estimated Plugin in four groups: (A) ♂ KIL x ♀ KIL, (B) Super male x ♀ KIL, (C) Super males x ♀ Mopti 2003, and (D) ♂ Mopti 2003 x ♀ Mopti 2003.

Because of the significant differences in body size (wing length) found between groups, the estimated amount of Plugin was corrected by body size by dividing the estimated Plugin (MAG equivalent) by wing length$^3$, and the data reanalyzed. The overall distribution of corrected Plugin estimates deviated significantly from a normal distribution (Shapiro-Wilkinson: $P<0.001$ in all cases). Overall, there was again no significant difference in Plugin estimated/wing length$^3$ (mm) in female *An. gambiae* s.s. across the four studied groups (Kruskal-Wallis: $n= 191$, $df= 3$, $\chi^2= 1.602$, $P=0.658$) (Fig. 6.7).

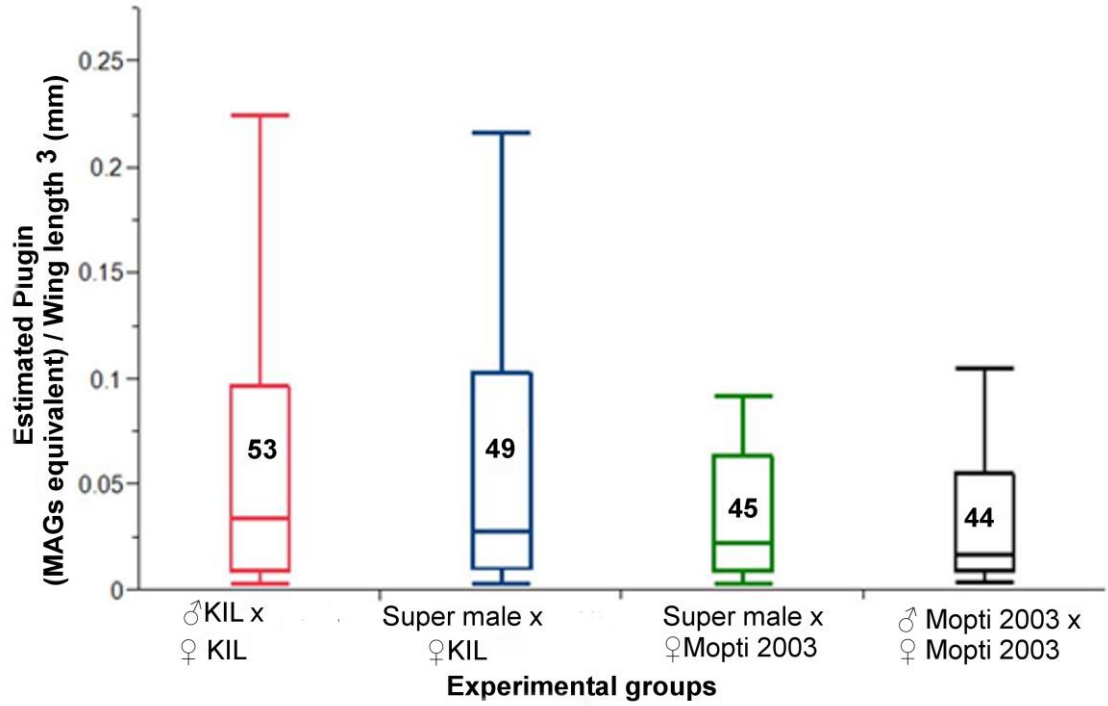


Fig.6.7. Estimated Plugin (MAGs equivalent) in the reproductive tracts of female *An. gambiae* s.s. corrected for wing length$^3$ (mm) in four mating combinations: Super male x ♀ Mopti 2003; Super male x ♀ KIL; ♂ Mopti 2003 x ♀ Mopti 2003; and ♂ KIL x ♀ KIL. Plugin estimated followed a non-normal distribution: in the diagrams, the bars represent the median. The boxes show the interquartile range (75- 25%). The whiskers display the upper and lower parameter values, excluding outliers. Sample sizes are indicated.

### 6.3.1.3. Estimated Transglutaminase protein

The frequency distribution of estimated Transglutaminase protein in the reproductive tract of 143 *An. gambiae* s.s. females across four experimental groups, Super male x ♀ Mopti 2003; Super male x ♀ KIL; ♂ Mopti 2003 x ♀ Mopti 2003; and ♂ KIL x ♀ KIL were checked by a normality test for goodness of fit. Overall, the data deviated significantly from a normal distribution (Shapiro-Wilkinson: $n$= 143, W= 0.601, $P$< 0.001). The median (25-75 quartiles) of estimated Transglutaminase protein of 143 reproductive tract females for the same groups was 0.413 MAGs equivalent (0.273-0.659).

The range of Transglutaminase estimated in 143 reproductive tracts of females across the four studied groups was 0.184-4.840 MAGs equivalent (Fig. 6.8).

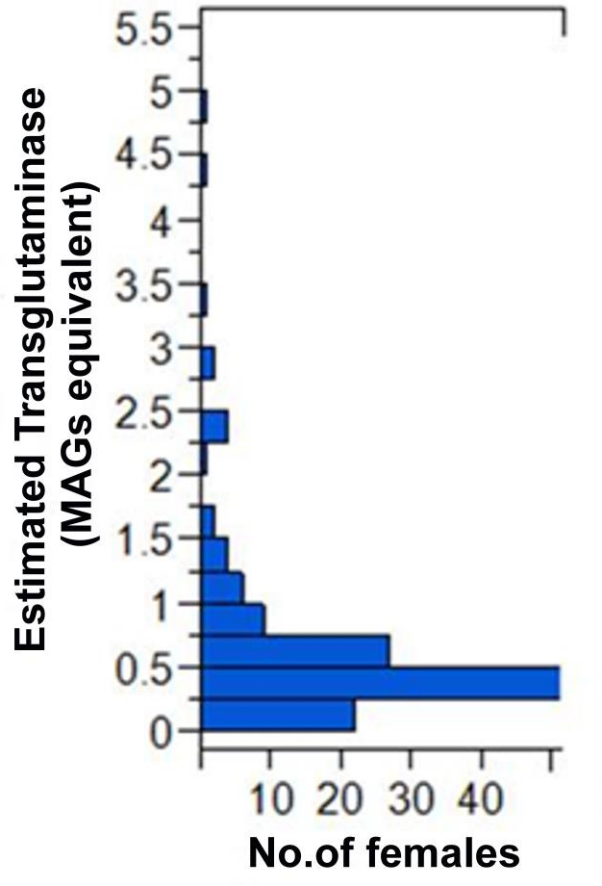


Fig.6.8. Frequency distribution of estimated Transglutaminase in *An. gambiae* s.s. reproductive tracts of females (all combined groups, *n*= 143)

The distribution of estimated Transglutaminase was examined for normality within each group. It was found that the four groups deviated significantly from a normal distribution (Shapiro- Wilkinson: $P< 0.001$ in all cases). The medians (25-75 quartiles) of the four groups were 0.383 (0.27-0.65) in Super male x Female KIL, 0.493 (0.27-0.66) in Super male x Female Mopti, 0.331 (0.27-0.50) in Male KIL x Female KIL, and 0.49 (0.33-1.53) in Male Mopti 2003 x Female Mopti 2003 (Fig. 6.9).

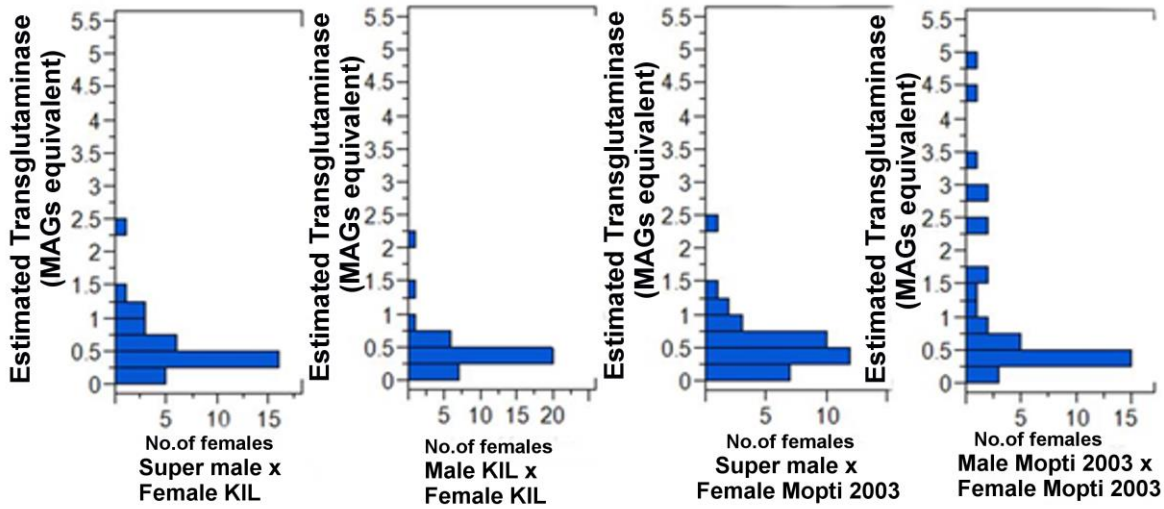


Fig.6.9. Frequency distribution of estimated Transglutaminase (MAGs equinelant) of *An.gambiae* s.s. reproductive tract females in the four studed groups: Super male x ♀ KIL, ♂ KIL x ♀ KIL, Super male x ♀ Mopti 2003; and ♂ Mopti 2003 x ♀ Mopti 2003.

### 6.3.1.4. Relationship between female body size (measured as wing length) and quantity of Transglutaminase in female reproductive tracts

The mean wing length of 143 *An. gambiae* s.s. females (all four groups combined) was 2.989mm (2.972-3.006 CI) (Fig. 6 .10).

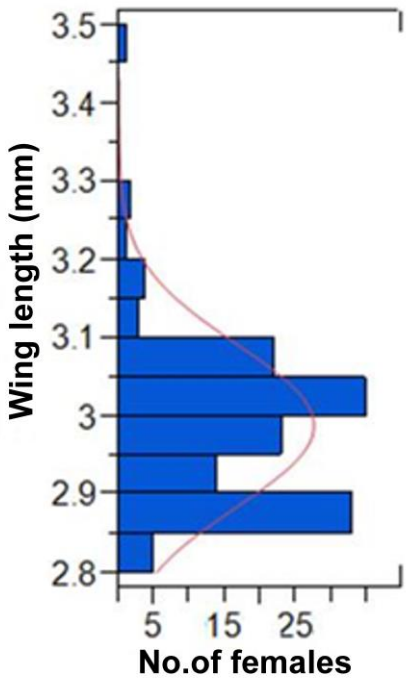


Fig. 6.10. Frequency distribution of the wing length (mm) of females for which Transglutaminase estimated was measured in the reproductive tracts. The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

Overall, there was a significant difference in body size (wing length) of the 143 females across the four studied groups (ANOVA: $F_{3,143}$= 5.406, $r^2$= 0.104, $P$<0.001). Pair-wise comparisons showed that there was a significant difference between Super males x ♀ Mopti 2003 and Super male x♀ KIL ($P$<0.001), and between Super male x ♀ Mopti 2003 and ♂ Mopti 2003 x ♀ Mopti 2003 ($P$=0.022). Super male x ♀ Mopti 2003 were slightly larger than the other three groups (mean= 3.040) (3.008-3.073CI). However, there was no significant difference between all the other comparison pairs ($P$>0.155 in all cases).

The relationship between body size (wing length) and estimated Transglutaminase in 143 females was examined. There was no relationship between body size and the amount of Transglutaminase transferred to females by males during copulation (Linear regression:

*n*= 143, *t*= 1.70, $r^2$= 0.012, *P*=0.185) (Fig. 6.11). Linear regressions of estimated Transglutaminase and body size (wing length) performed within each of the four studied groups showed that there was no relationship between the two variables in all groups (linear regression: ♂ KIL x ♀ KIL *n*= 36, *t*= -0.23, $r^2$= 0.005, *P*=0.666; Super male x ♀ KIL *n*= 35, *t*= 018, $r^2$=3.145, *P*=0.975, Super males x ♀ Mopti 2003 *n*= 36, *t*= 0.47, $r^2$= 0.007; *P*=0.877 and ♂ Mopti 2003 x ♀ Mopti 2003 *n*= 36, *t*= 1.54, $r^2$= 0.051, *P*=0.183) (Fig. 6.12).

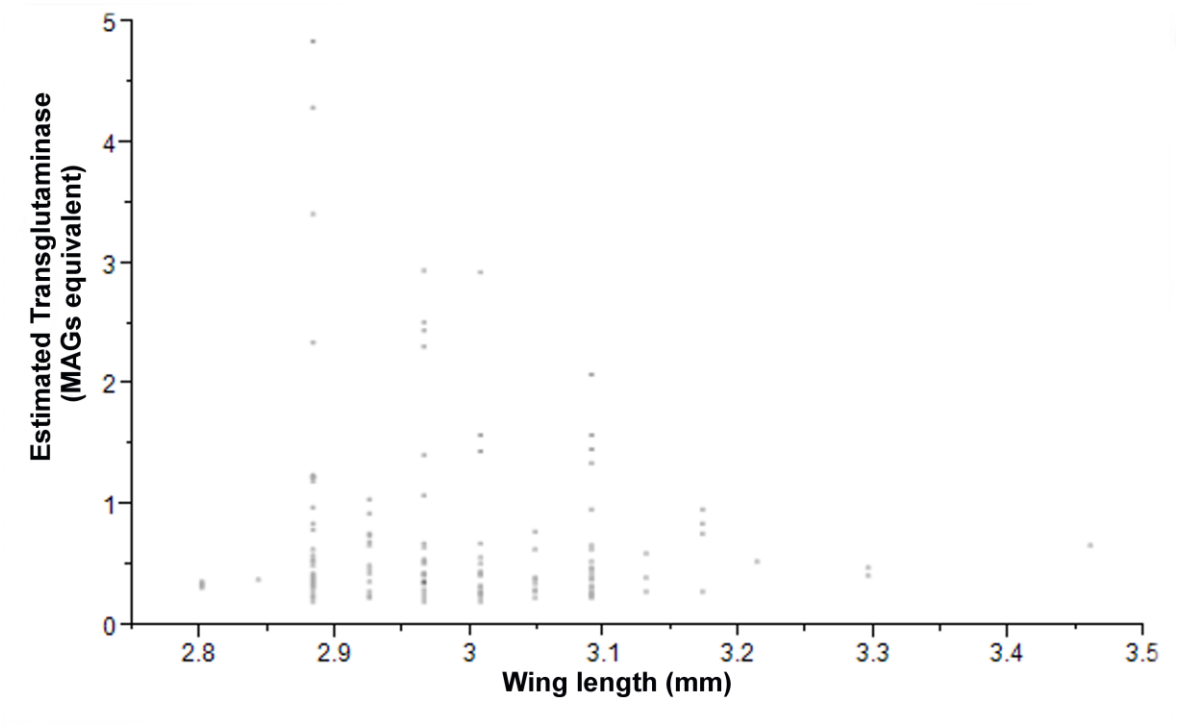


Fig. 6.11. Linear relationship between body size (wing length) and estimated Transglutaminase in *An. gambiae* s.s. females (all combined experimental groups, *n*= 143).

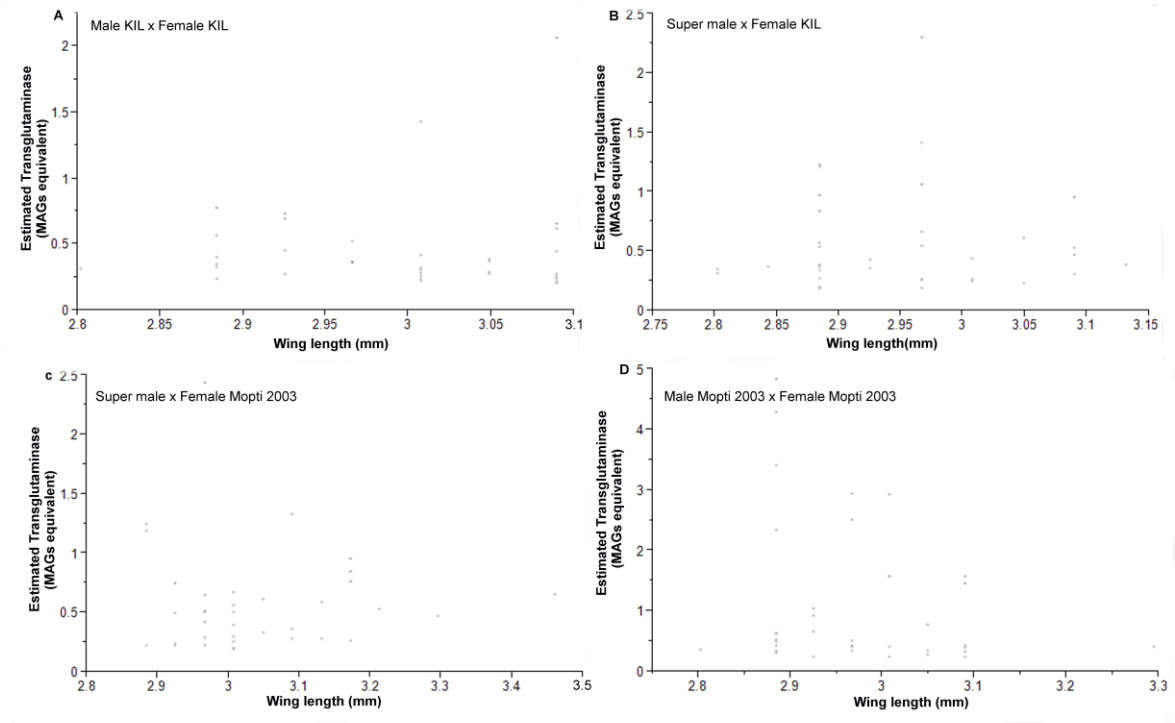


Fig.6.12. Linear relationship between female body size (wing length) and estimated Transglutaminase in four groups: (A) ♂ KIL x ♀ KIL, (B) Super male x ♀ KIL, (C) Super male x ♀ Mopti 2003; and (D) ♂ Mopti 2003 x ♀ Mopti 2003.

Because of the significant difference in body size (wing length) observed between groups, the estimated Transglutaminase was divided by wing length$^3$ and the data reanalyzed. The overall distribution of Transglutaminase estimates (MAG equivalents) deviated significantly from normality (Shapiro-Wilkinson: $n$= 143, W= 0.576, $P$<0.001). This was also true within groups (Shapiro-Wilkinson: $P$<0.001 in all cases). Overall, there was a significant difference in the corrected Transglutaminase estimates between groups (Kruskal-Wallis: $n$=143, $df$=3, $\chi^2$= 10.07, $P$=0.018). Pair-wise comparisons between the four studied groups showed a significant difference between Male Mopti 2003 x Female Mopti 203 and Male KIL x Female KIL ($P$=0.0.002), and Super male x Female Mopti 2003 ($P$=0.038) (Fig. 6.13).

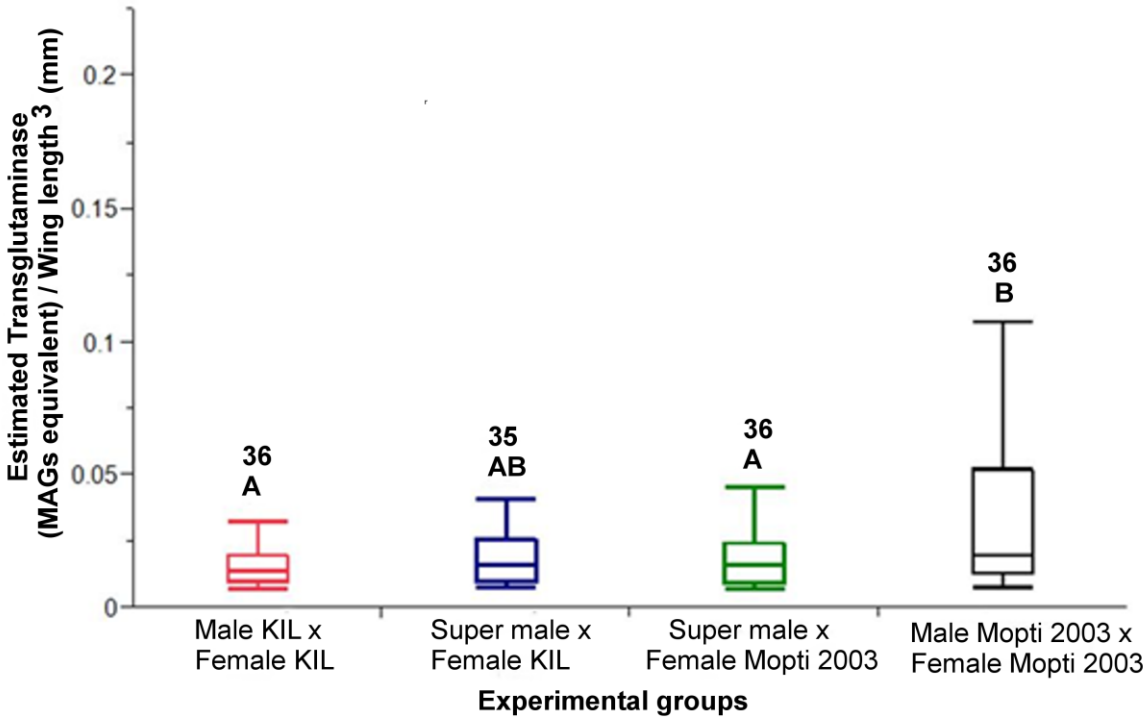


Fig. 6.13. Estimated Transglutaminase (MAGs equivalent) in female reproductive tracts corrected for wing length$^3$ (mm) in four groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003. The Transglutaminase estimated followed a non-normal distribution: here the bars represent the median, and the boxes the interquartile range (75- 25%). The whiskers display the upper and lower parameter values, excluding outliers. Boxplots labelled with different letters were significantly different (Wilcoxon, $P$<0.001). Sample sizes are indicated.

In Plugin, the lowest median (25-75 interquartile) was 0.439 (0.261-1.06) in Male Mopti 2003 x Female Mopti 2003, while the highest median was 0.899 (0.255-2.31) in Male KIL x Female KIL. There was no significant difference across all groups. However, there was a significant difference in Transglutaminase between Male Mopti 2003 x Female Mopti 2003 (median (25-75 interquartile =0.497 (0.339-1.533)) and Super male x Female Mopti 2003(median (25-75 interquartile= 0.493 (0.271- 0.661)) and Male KIL x Female KIL (0.331 (0.270-0.500)) (Table 6.3)

Table.6.3. Comparison of estimated Plugin and Transglutaminase proteins (Median (25-75% quartile)) in female reproductive tracts after less than 24h overnight mating across the four studied groups: Super male x ♀ Mopti 2003; Super male x ♀ KIL; ♂ Mopti 2003 x ♀ Mopti 2003; and ♂ KIL x ♀ KIL.

| Combination | Median estimated Plugin (25-75% quartile) | Sample size | Median estimated Transglutaminase (25-75% quartile) | Sample size |
|---|---|---|---|---|
| Male KIL x Female KIL | 0.899 (0.255-2.31) | 53 | 0.331 (0.270-0.500) | 36 |
| Super male x Female KIL | 0.626 (0.255-2.86) | 49 | 0.382 (0.270-0.659) | 35 |
| Super male x Female Mopti2003 | 0.545 (0.264-1.31) | 45 | 0.493 (0.271- 0.661) | 36 |
| Male Mopti 2003 x Female Mopti 2003 | 0.439 (0.261-1.06) | 46 | 0.497 (0.339-1.533) | 36 |

### 6.3.2. Fecundity and reproductive success

#### 6.3.2.1. Number of eggs

After 24 h overnight mating with subsequent blood feeding, 48 females from each mating combination in the four experimental groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003; and ♂ Mopti 2003 x ♀ Mopti 2003, were used to measure the fecundity of females across the four groups. The number of females who laid eggs varied for each group. In ♂ KIL x ♀ KIL and in Super male x ♀ Mopti 2003, only 12 females out of 48 laid eggs, while in Super male x ♀ KIL 7 females out of 48 laid eggs, and the same was found in ♂ Mopti 2003 x ♀ Mopti 2003 (Table 6.3, Fig.6.14). Overall, the mean number of eggs per 40 females across the four studied groups was 61.7 (54.323-69.076 CI). The range of number of eggs for the same mosquitoes was 19-125 across the four groups (Fig. 6.15). Across all groups, the mean number of eggs did not deviate from normality (Shapiro-Wilkinson: $n$= 40, W= 0.970, $P$>0.377). A comparison of number of egg distributions overall for each of the experimental groups showed that the number of eggs laid did not deviate from normality in any of the groups (Shapiro-Wilkinson: $P$>0.382 in all cases) (Fig.6.16). Overall, There was no significant difference in the number of eggs laid between the four groups (ANOVA: $F_{3,40}$= 0.586, $r^2$= 0.046, $P$=0.628) (Fig. 6.19).

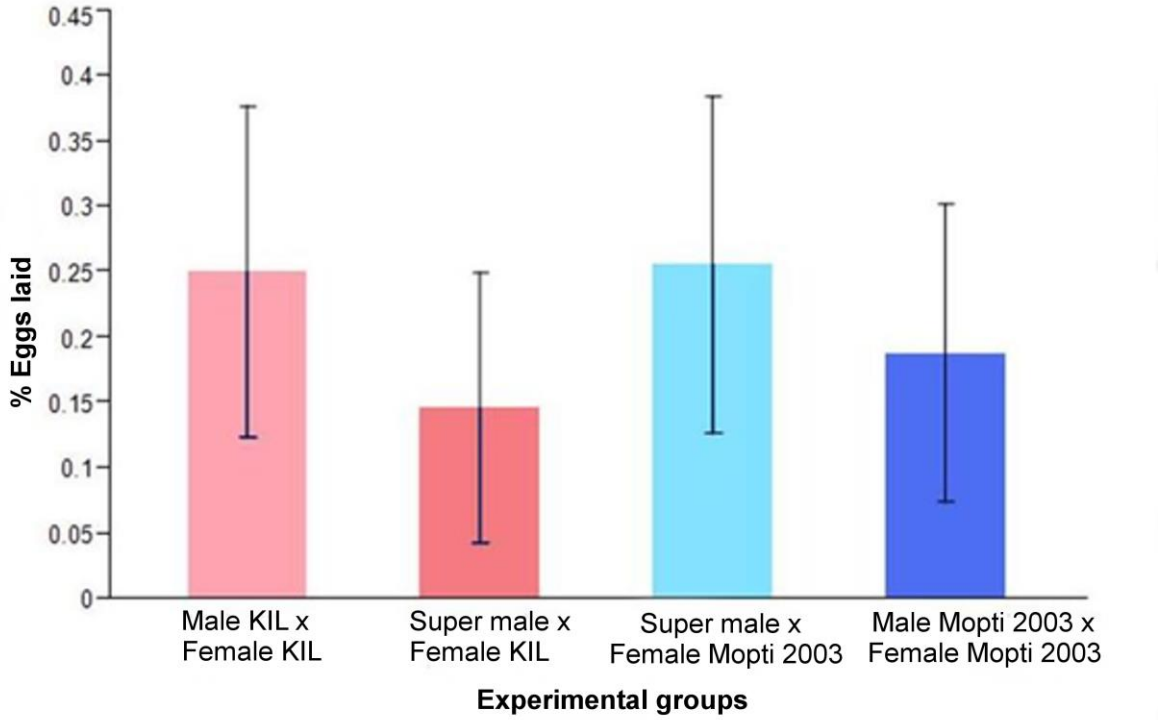


Fig. 6.14. The percentage of eggs laid (±95% CI) by females across four experimental groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003.

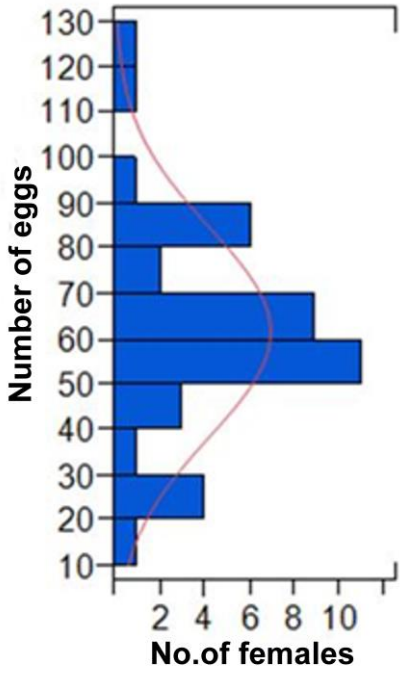


Fig. 6.15. Frequency distribution of the number of eggs laid by females across the four studied groups (all combined groups, *n*= 40). The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

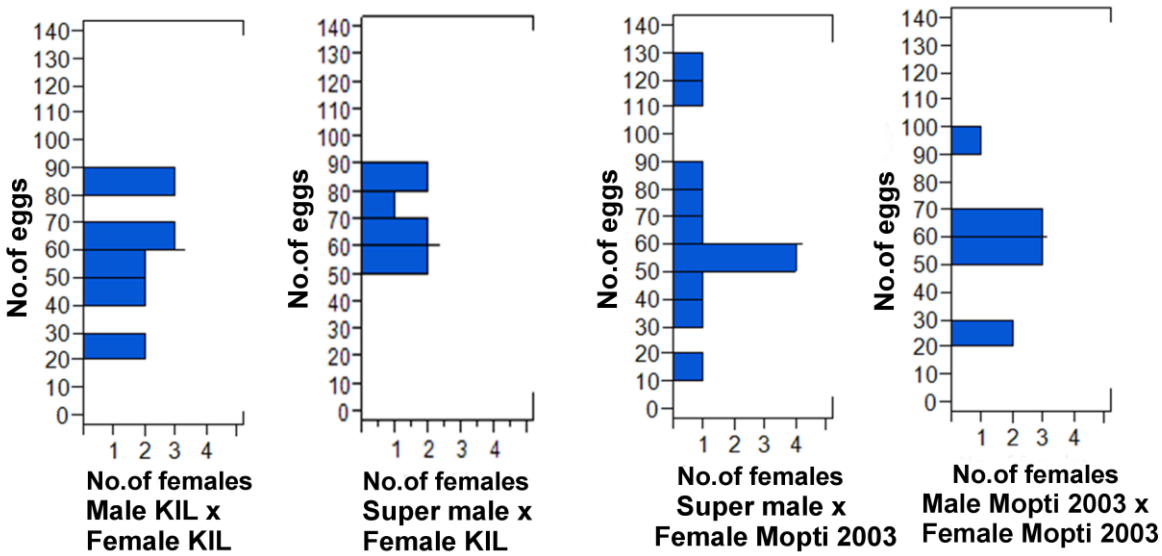


Fig. 6.16. Frequency distribution of the number of eggs laid by females across the four studied groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003.

### 6.3.2.2. Number of larvae and larval hatching rate (Male reproductive success).

After 4 days post egg-laying eggs; larvae from each mating combination in the four experimental groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003; and ♂ Mopti 2003 x ♀ Mopti 2003, were counted to measure male and female reproductive success across the four studied groups. Overall, the mean number of larvae in 40 females across the four groups was 37.25 (30.905-43.594 CI). The range of numbers of larvae for the same mosquito was 0-83 across the four groups (Fig.6.17). Across all groups, the mean number of larvae did not deviate from normality (Shapiro-Wilkinson: $n$= 40, W= 0.962, $P$=0.210). A comparison of the number of larvae distributions overall for each of the experimental groups showed that the number of larvae did not deviate from normality in any group (Shapiro-Wilkinson: $P$>0.060 in all cases) (Fig. 6.18).

Overall, there was no significant difference in the number of larvae between the four groups (ANOVA: $F_{3,40}$= 0.361, $r^2$= 0.029, $P$=0.781).

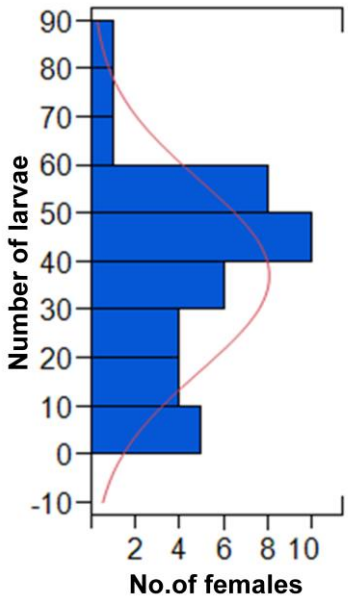


Fig. 6.17. Frequency distribution of the number of larvae across the four studied groups (all combined groups, *n*= 40). The pink line represents a normal distribution based upon the mean and standard deviation of the actual data.

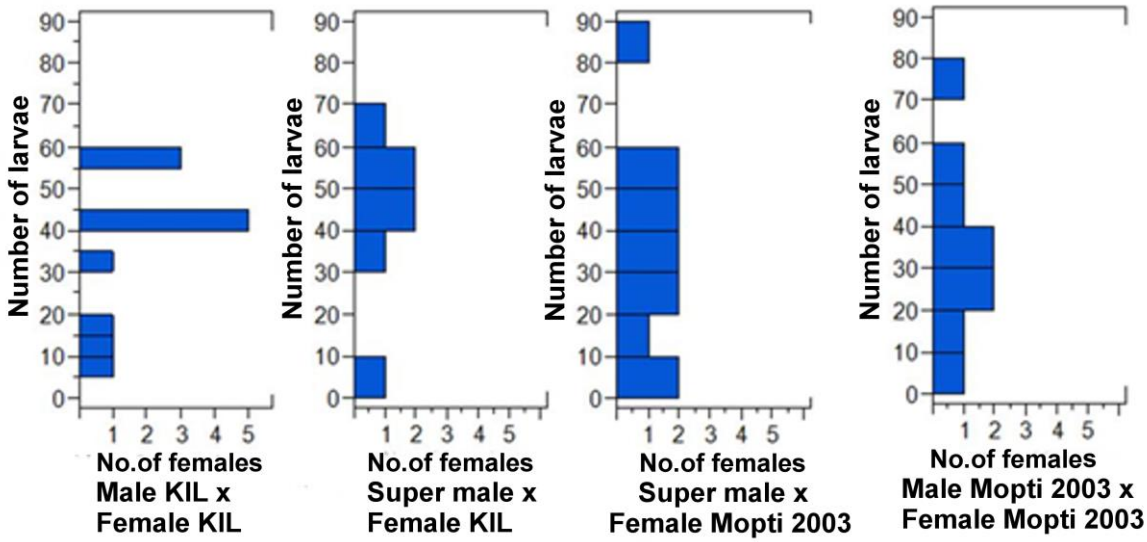


Fig. 6.18. Frequency distribution of the number of larvae across the four studied groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003; and ♂ Mopti 2003 x ♀ Mopti 2003.

There was also no significant difference in the number of larvae across the four studied groups as mentioned earlier (Fig. 6.19). The highest hatching rates (number of larvae/number of eggs x 100) was 63.68% in Male KIL x Female KIL and in Super male x Female KIL, it was 62.3%, while the lowest hatching rates were 60.57% in Male Mopti 2003 x Female Mopti 203 and 55.23% in Super male x Female Mopti 2003 (Fig. 6.20 & Table 6.4).

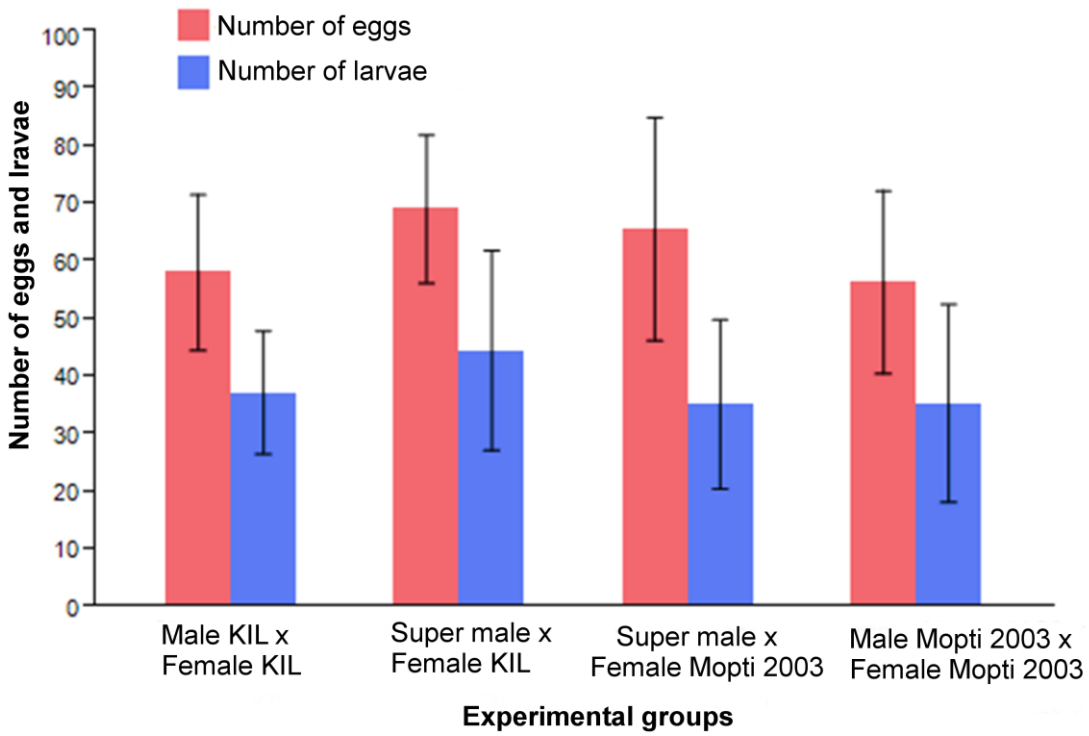


Fig.6.19. Number of eggs and larvae laid by females across the four studied groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003; and ♂ Mopti 2003 x ♀ Mopti 2003.

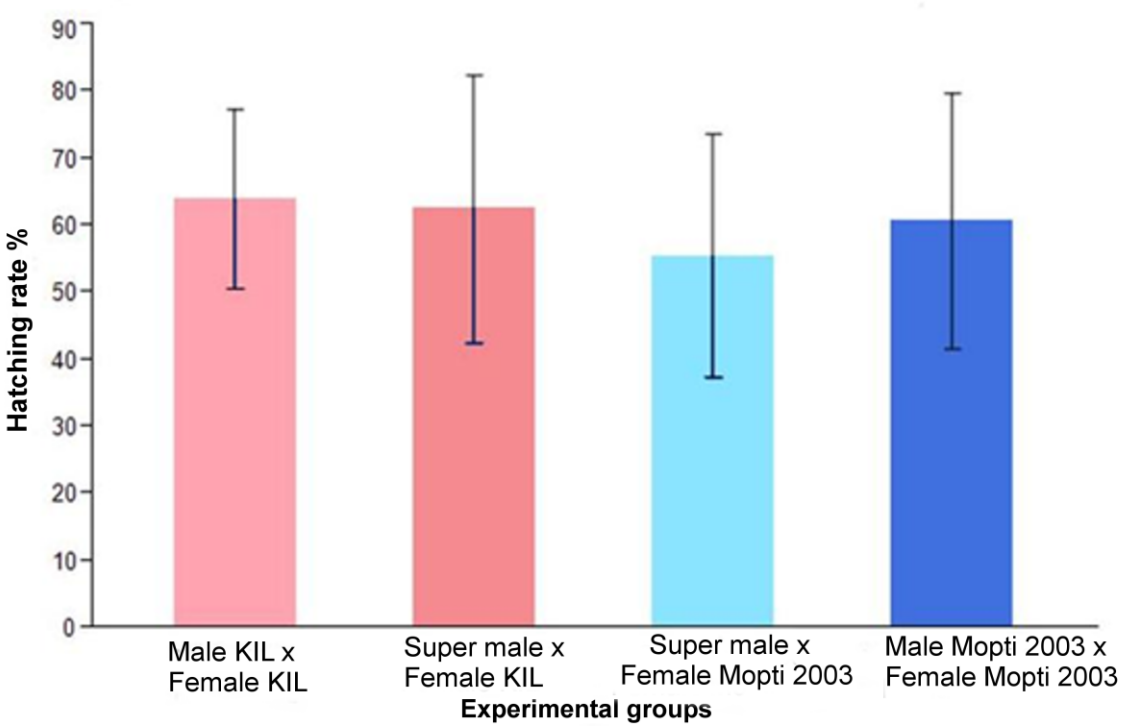


Fig.6.20. Hatching rate % (number of larvae/number of eggs laid x 100) of *An. gambiae* s.s. females in the four studied groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003.

Table 6.4. *An. gambiae* males' fertility and female fecundity as measured by the number of eggs (fecundity) and larvae (fertility) (Mean ±SD) laid by 48 females (hatching rate & % eggs laid) across the four studied groups: ♂ KIL x ♀ KIL, Super male x ♀ KIL, Super male x ♀ Mopti 2003, and ♂ Mopti 2003 x ♀ Mopti 2003.

| combination | % eggs laid | No. of females who laid eggs | Mean No. of eggs ±SD | % Hatching rate | Mean No. of larvae ±SD |
|---|---|---|---|---|---|
| Male KIL x Female KIL | 25 | 12 | 57.92± 21.25 (44.42-71.42 CI) | 63.68 | 37 ±16.64 (26.42-47.57 CI) |
| Super male x Female KIL | 14.58 | 7 | 68.85± 13.77 (56.12-81.59 CI) | 62.3 | 44.28±18.78 (26.91-61.65.65 CI) |
| Super male x Female Mopti 2003 | 25.53 | 12 | 65.42±30.50 (46.03-84.80 CI) | 55.23 | 35± 22.93 (49.57-20.42 CI) |
| Male Mopti 2003 x Female Mopti 2003 | 18.75 | 9 | 56.22±20.74 (40.28-72.16 CI) | 60.57 | 35.11±22.21 (18.03-52.18 CI) |

## 6.4. Discussion

In general, both males and females of *An.gambiae* mosquitoes must be over one day old before copulation takes place, and in the male, the terminalia rotate during this period (Clements, 1992, Service 2012). In terms of the time of mating, males and previously unmated females are most active in the hour following dusk (Rowland 1989, Charlwood and Jones 1979). In the present experiment, the female to male ratio at 3-5days old was 1:2 (100 females: 200 males in two replicates) to ensure a sufficient insemination rate. It was found that the insemination rate was more than 95% in all groups (see Methods). This fits with another laboratory study by Gary *et al.* (2009) which used the sex ratio 1:2 (female: male) to study the effect of sugar availability on male insemination success in *An.gambia.* This ratio was chosen to allow those groups where males might display poor mating success rates to provide adequate data for analysis. Gary *et al.* (2009) used 30 females to 60 males in four replicates for each experiment. An 82% insemination rate was achieved after 5 days where males had access to sugar. In the current work, sugar solution was available at all times in the mating cages and a fixed age for males of 3-5days was chosen for mating reasons. According to Gary *et al.* (2009), where sugar was made available, males showed a sharp rise in capacity for insemination three days post-emergence (this study was carried out in a semi-natural environment). This significant increase at 3-days old where sugar is provided is supported by Charlwood and Jones (1979) and Reisen *et al.* (1979) in other Anopheline species. A similar rate of 80% was found by Charlwood and Jones (1979) in a study using four replicates of groups with 50 females to 100 males, finding that the rate of insemination peaked at three days old across both sexes. The current work found that this number of males and females is sufficient to achieve a good mating rate, and ensured that almost all females had MAGs proteins in their female reproductive tracts.

In the current study, after overnight mating, males at 3-5 days old transferred their MAGs proteins to the same age females. It was found that there was no significant difference in the Plugin estimated across the four groups studied. Males from all treatment groups transferred a similar amount of Plugin protein to females during copulation. There was no evidence of any impact of either super males or males from parental or old established strains on the estimated amount of Plugin. There was a significant difference, however, in the amount of Transglutaminase protein across the same groups. When male Mopti 2003 mated with female Mopti 2003, they transferred more Transglutaminase protein than the other groups studied. It was observed from the data presented in Chapter 5 that Mopti 2003 and KIL males produced a greater amount of Transglutaminase in their glands than Super males and Field individuals. Mopti 2003 was reared for seven years under laboratory conditions, while KIL was an old strain that had been reared for more than 30 years. Consequently, it is possible that Mopti 2003 males attempted to transfer more Transglutaminase proteins to females to encourage them to store more sperm and produce progeny. Rogers *et al.* (2009) report that Transglutaminase in *An. gambiae* s.s. plays a significant role in stimulating sperm storage rather than as a mechanical barrier to further insemination. Moreover, this finding could be a result of adaptation to the laboratory environment or to avoid the impact of inbreeding depression (as discussed in Chapters 4 and 5). Variation was observed between the groups in terms of the quantity of secretions transferred to the female during copulation. Possible reasons for this include differences in the growth process during sexual maturity (Ramalingam, 1983), or differences in the quantity held within the glands and renewal rates (Ramalingam, 1983). These findings might be attributed to genetic variations in combination with the specific conditions provided to mosquitoes during the larval and adult stages and for mating (Ramalingam, 1983). This author suggests that the capacity to renew secretions for

subsequent mating depends to a great extent on the following factors: maturity of the glands; the quantity of secretion utilised per incidence of mating; how far cells incur damage during copulation; and JH activity after mating (Ramalingam, 1983).

The present study detected Plugin and Transglutaminase as MAGs in the female reproductive tract that included a mating plug. This in agreement with Roger *et al*, (2009) who identified Plugin and Transglutaminase, out of 15 MAGs proteins transferred to females and responsible for creating a female mating plug, as being influential in limiting further mating activity in the female, as well as in the storage of sperm and in oviposition (Lung and Wolfner, 2001; Ram and Wolfner, 2007). Rogers *et al.* (2009) suggest that the creation of the mating plug may be the result of interactions between proteins from both male and female. They further posit that possibly, coagulation occurring within the male accessory glands themselves may be needed for the transfer of secretions, as before the plug is put in place, sperm are transferred into the spermatheca (Rogers *et al.*, 2009).

As expected, there was no relationship between female body size (measured as wing length) and estimates of either protein, because females did not produce those proteins, but rather received these from the males and stored them in the female reproductive tract. Here, an equal amount of larval food was used for all experimental groups: however, there remained a significant difference in female body size across the four groups studied for both proteins. This could reflect the ability of the larvae to take in and digest more food, and also the way in which that food is invested during the developmental and growth process.

Copulation and transfer of seminal fluid causes great changes to occur in the female in many mosquito species (Craig, 1967). In *Drosophila*, a mated female‘s attractiveness, receptivity for up to eleven days post-mating, and movement in response to potential mates

are all reduced, while oviposition activity increases for up to nine days, and stored sperm is used (Wolfner, 1997). Mating also shortens the lifespan of the female in *Drosophila* (Wolfner, 1997). Tram and Wolfner (1999) point to the action of the seminal fluid on the female as providing the maximum chance of offspring from the copulation and thus assisting reproductive success in the male. The secretions of the male accessory glands are seen to stimulate increased oviposition in the female. This condition is stated by Herndon and Wolfner (1995) to consist of a one-day first phase followed by a further phase of between seven and nine days' duration.

Seminal fluid and sperm also have an important function in sperm competition (Reinhardt *et al*, 2011). The role of seminal fluids in ejaculate allocation over the lifespan of the bedbug *Cimex lectularius* was examined by Reinhardt *et al* (2011). The lowest volume of seminal fluid and sperm at which mating activity started was estimated by using age–related changes. They observed that sexually mature males transferred 19% of their seminal fluid and 12% of their sperm capacity per mating. Also, seminal fluids not sperm were depleted in each mating. This led to males stopping mating and the authors concluded that the male mating rate is inhibited by the availability of seminal fluids (Reinhardt *et al*, 2011). Seminal fluids but not sperm also declined after 5 successful matings in *D. melanogaster* (Lefevre and Jonsson, 1962). Larger male accessory glands, not testes size, were found to increase the male mating rate (Wigby *et al*, 2009); and seminal fluid production, not sperm controls and stimulates males to mate at a higher mating rate (Fedorka *et al*, 2011). These studies demonstrate that seminal fluid is a limiting factor in lifetime mating success in multiple insect species. Thus males that produce more seminal fluid may have a fitness advantage. In the stalk-eyed fly, *Cyrtodiopsis dalmanni*, there was a positive correlation between mating rate and the replenished size of accessory glands, but not testes (Baker *et al*, 2003). In addition, mating frequencies were only affected by

accessory gland size, not testes size (Rogers *et al*, 2005). Reinhardt *et al*, (2011) mentioned that the low mating rate due to the low seminal fluid availability occur in three situations. First, over the male adult lifespan, there was a correlation between seminal fluid reserves (not sperm reserves) and the mating rate. Secondly, sexually mature males stopped mating when there was a decline in seminal fluid not sperm volume. Thirdly, male seminal fluids in inbred family groups limited the future mating rate. A recent study by Thailayil *et al* (2011) examined the role of sperm in female postmating behaviour without affecting the function of male accessory glands by creating spermless males via the RNAi—mediated silencing of *zpg*, a gene responsible for germ cell development. Study results demonstrated that *An. gambiae* s.s. females do not depend on male sperm to stimulate oviposition or the induction of refractoriness to further insemination (Thailayil *et al*, 2011). *An. gambiae* s.s. females lay infertile eggs without sperm being transferred suggesting that, in contrast to conclusions from previous work (Klowden, 2001), signaling is controlled by the male accessory gland secretions in the spermathecae rather than transferred sperm. All the previous studies which have been discussed referred to the importance of male accessory glands secretions rather than sperm in males mating success. No studies were found which discussed the effect of inbreeding depression on sperm quality and seminal fluid proteins in *An. gambiae* s.s. mosquitoes. Further studies are needed to determine the impact of inbreeding depression on both.

In the current work, after overnight mating and subsequent provision of a single blood meal, only a quarter of females (25%-14.5% of eggs laid) laid eggs despite having a high mating rate. Overall, the mean number of eggs in 40 females across the four studied groups was 61.7 (54.323-69.076 CI). The range of the number of eggs for the same mosquito was 19-125 eggs across the four groups. There are growing numbers of laboratory studies which have reported the number of eggs in *An. gambiae* s.s. For

example, the mean fecundity was 81.6±2.9 in 134 in *An. gambiae* s.s. females studied by Hogg *et al.* (1996). Takken *et al.* (1998) studied the impact of female body size and blood meal size on egg production. They found that the average number of eggs in small females (mean wing length =2.63mm) was 14 at 1.5µl blood size and 24.3 eggs at 2.0µl blood meal size, while in large females (mean wing length =3.02mm), the average number of eggs was 39.4 at 1.5µl blood meal size and 59.4 eggs at 2.0µl. Neither intake of a blood meal prior to mating, nor age in the female are observed to influence egg-laying in *An.gambiae* (Chambers and Klowden, 2001). In my opinion, the blood meal size and female body size are the main factors that control the number of eggs laid, as females with a large body size with a small blood meal laid only a few eggs. Mating is responsible for an increase in egg maturation that is associated with the transfer of male accessory gland substances. *Ae. aegypti* females display greater rates of egg maturation after mating: this is thought to be an effect of the secretions transferred during mating (Klowden and Chambers, 1991). By contrast, a similar increase occurs in *An. gambiae* but not linked to MAG secretions (Klowden and Russell, 2004).

From the observations reported here, there was no evidence of an impact of male or female genotypes across the four studied groups on the number of eggs and larvae, as there was no significant difference in the number of eggs and larvae produced across the four experimental groups. This showed that there was no difference in the Super males and males from parental or older established strains in terms of fecundity and reproductive success in either sex. This may be because the Super males had similar genetic components inherited from their parents. Moreover, it was found that there was no difference in the amount of sperm transferred to females by Super males in comparison with other studied groups (Ekechukwu, Nkiru Esther, unpublished data). This could be due to a negative effect of colonization as seen in Chapters 4 and 5. The impact of inbreeding depression is

reported in several insect species. For example, in the mite *Schizotetranychus miscanthi*, Saito *et al*. (2000) determined deleterious recessive genes that caused decreased female fecundity over time. Those genes produced as an impact of inbreeding on haplo-diploid (hemizygous) males were purged generation by generation. The authors found that there was no depression in egg hatchability, nor in the larval survival of progeny over four generations in experiments where the mother was crossed with her son. However, fecundity showed a notable impact from inbreeding depression. Two lineages were created during inbreeding, only one of which negatively affected fecundity. Deleterious recessives were found via backcrosses to be the cause of the depression. The findings are taken as evidence of deleterious genes among wild haplo-diploid populations and show their effects through the adult female. In a seed-feeding beetle, *Stator limbatus* Horn (Coleoptera: Chrysomelidae: Bruchinae), inbreeding caused a decrease in the production of adults as a percentage of eggs laid from over 80% where there was no inbreeding to 54% where mating was with close kin. Furthermore, adults produced through inbred matings required an additional 1.5 days (>5%) to achieve adult mass, although the eventual body size was unaffected. The effect of inbreeding upon mortality was not uniform across the three populations studied, with the most isolated group showing the least effect, This suggests that in *S. limbatus,* those recessive deleterious alleles which impact upon the development and hatching of eggs and mortality among larvae vary in their distribution among populations (Fox and Scheibly, 2006). Inbreeding had a greater effect on fitness characteristics such as fecundity and development time than on morphological traits (Roff, 1998). In a tree-hole-breeding mosquito, *Aedes geniculatus,* Armbruster *et al.* (2000) estimated the effects of inbreeding on fitness traits (Fertility (% egg hatch), larval survivorship and female pupal weight) under field and near optimal laboratory conditions. They found that fitness characteristics were lower in the field than under laboratory

conditions due to inbreeding impacts in both environments. However, there were no significant interactions between environmental conditions and inbreeding depression. These findings indicate that inbreeding depression is not likely to be greater in extent in the wild than under laboratory conditions.

So far, all the previous studies showed that inbreeding depression had a significant impact on female fecundity. In the current work 12 or fewer females across the four studied groups out of 48 in each experimental group laid eggs. Females fecundity were low could be an impact of inbreeding depression.

**Conclusion**

Male accessory gland (MAG) secretions are the fundamental product transferred to female mosquitoes during copulation. In the current study, Plugin and Transglutaminase MAGs proteins were found in the females' reproductive tracts across the four different mating combinations and there was no significant difference in the estimated amount of Plugin between all groups studied. However, there was a significant difference in the Transglutaminase estimated across the four studied groups. There was no relationship between female body size and the estimated amount of both proteins. In addition, the amount of Plugin estimated in the female reproductive tracts was higher than that of Transglutaminase across three of the groups, while in Male Mopti 2003 x Female Mopti 2003, the Transglutaminase estimated was higher than Plugin estimated in the female reproductive tracts. The number of eggs and larvae were counted to determine the fertility and fecundity of males across the four studied groups. It was found that there was no significant difference in the number of eggs and larvae in the four groups. This showed that there was no difference in the Super males and males from parental or old established strains on fecundity and reproductive success of both sexes.

# Chapter 7

## General discussion

The current work highlights the challenges that the major malaria vector, *An. gambiae* s.s., face under laboratory conditions. The effects of environmental and genetic factors were assessed on the mosquito's reproductive success. Nutritional requirements and adult body size were used as environmental factors (Chapter 3); Impact of colonization and genetic modification were used as genetic factors (Chapter 4). One indirect strategy to combat malaria is the release of sterile male mosquitoes that compete with wild males and transfer sterile components (sterile sperm) to females, as a result of which the field population declines (Knipling *et al*, 1986; Lofgren *et al*, 1974). For example, *Aedes albopictus* (Skuse) pupae males were exposed to gamma rays then adults released in the field immediately to estimate adult population density in a SIT development program. Results showed that a significant sterility level at a range of 70-80% in the wild population was induced after releasing males at the rate of 896-1,590 males per hectare per week. Also, egg density declined in ovitraps (Bellini *et al*, 2013).

Generating a better male is a principal factor for any SIT program (Perez-Staples *et al*, 2013). Unfortunately, it is unknown what the best male qualities needed for SIT programs are. There is even less knowledge of what male qualities wild females prefer when selecting a mate (Perez-Staples et al, 2013). Nutritional manipulation of the larvae & adult environment may improve sterile male mating success under field conditions. In addition, SIT candidate strains of *Anopheles* spp. have been kept under laboratory conditions for many years, so the question raised by Benedict *et al* (2009) "Would these males mate with wild females and at what rate relative to wild males? " is a pertinent one. Furthermore, food availability during the larval stage could play a role in male mating success. Based on previous studies that found that changing the food quantity during larval

development produced adults of different phenotypic quality (Nayar, 1969; Reisen, 1975; Naksathit and Scott, 1998; Okanda *et al.*, 2002; Nghabi *et al.*, 2008; Aboagye-Antwi and Tripet, 2010), two different feeding regimes were used, the poor regime produced considerably smaller adults (based on wing length) and the good regime created larger adults across both sexes. The impact of water availability on adult mating success was also estimated as earlier studies showed that *An. gambiae* s.s. is found in moist areas under field conditions (Tsy *et al.*, 2003, Moreno *et al.*, 2004). It was found there was no impact of female phenotypic quality on female survival under hydric stress treatments. This indicated that small and large body size female mosquitoes could survive equally well even when subject to limited water conditions. The effect of environmental stress on mosquito reproductive success was indicative of an effect of female phenotypic quality on almost all studied reproductive success parameters. On the other hand, male phenotypic quality was found to have a significant effect on the number of eggs produced by mated females but only under limited water availability. Further studies are required to study the effect of environmental stress conditions on sperm quality and quantity to clearly decipher their exact role in determining overall egg numbers. Hydric stress and food availability studies established the importance of growth conditions on adult physiology and consequent resistance to dehydration (Aboagye-Antwi and Tripet, 2010). Environmental factors could play a principle role in controlling malaria risk. For instance, numbers of malaria cases were increased in the presence of water for irrigation surrounding the residential areas in northern coastal Peru (Guthmann *et al*, 2002). Aboagye-Antwi *et al* (2010) reported that when wild-caught *Anopheles gambiae* females were reared under extreme desiccation stress, females with oocystes displayed significantly reduced survival. However, females with sporozoites were not affected. The authors concluded that female's mosquitoes under

natural-like conditions might suffer fitness costs. All previous studies confirmed the important of environmental factors in reducing malaria risk.

Male mating behavior in Anophelines is poorly documented in comparison to female mosquitoes (Ferguson *et al*, 2005). It should thus be studied with other ecological factors that could affect male traits such as energetic resources, dispersal, longevity, risk of predation and habitat. (Ferguson *et al*, 2005). Understanding male reproductive physiology and biology in *Anopheles* species is required to make good on the promise of genetically modified mosquitoes for malaria control (Ferguson *et al*, 2005). Also, further knowledge about male fitness is needed to support genetic control (Ferguson *et al*, 2005). The impact of colonization and genetic modification on sperm quality, male's accessory glands and testes size in *An. gambiae* s.s. has not been documented. To address this, we set out to investigate the impact of colonization and genetic engineering on potential correlates of reproductive success in *An. gambiae* males were assessed by comparing sperm quality, testes size and accessory glands size in the progeny of wild mosquitoes versus transgenic and non-transgenic mosquito colonies (Chapter 4). Little is known about sperm length in *Anopheles* mosquitoes (Klowden and Chamber, 2004; Voordouw *et al*, 2008). Large variations are observed in sperm length in male *An. gambiae* s.s. across all studied groups. The sperm variation between groups that we reported here might have a fitness consequence on male mosquitoes. Whether this is a positive or negative effect is, at present, unclear and further studies are needed. The results in colonized and genetic individuals showed that sperm length is significantly reduced in older colonized strains compared to the progeny of field-collected females or newer and/or refreshed strains. Resion (2003) reported that mosquito colonized strains became very inbred within a few generations. In the current work, there was a link between sperm length and inbreeding degree as measured by age of colony. However, to estimate inbreeding degree directly

molecular techniques assessing heterozygosity and allelic richness would be needed. Additional studies are required to study whether sperm at different length are adapted for functions other than fertilization. Furthermore, it is unknown how sperm qualities affect future male mating rate (Reinhardt *et al*, 2011). A study by Thailayil *et al* (2011) suggested that sperm could be used as a good tool to induce female fertility reduction under field conditions.

Theoretical programmes utilising genetically modified mosquitoes- to control vector-borne diseases are currently comprised of one of two paradigms: population replacement, where a wild vector population is replaced by outcrossing to mass reared and released transgenic insects that are refractory to pathogens. And population suppression using for instance, transgenic alternatives to traditional chemo- or radiosterilisation-based SIT (Alphey, 2009). To study the effects of genetic modifications on sperm quality, we compared sperm size in KIL (the original non-modified strain), in EE (a KIL-based strain genetically-modified into a docking strain) and in Vida (EE loaded with an antimicrobial peptide) (Chapter 4). The transgenic strain EE did not differ significantly from the original KIL strain, but sperm size was reduced in the Vida strain, possibly because of a further genetic bottleneck (Futuyma, 2009), or due to insertional mutagenesis in the chromosome and the cost of transgene expression (Catteruccia *et al*, 2003; Li *et al*, 2008). Further studies are necessary to study each possibility on the impact of sperm quality in transgenic lines to produce a satisfactory quality of genetic modified mosquitoes.

Released males for SIT must create under laboratory conditions before being tested in the field to determine their use for control (Benedict *et al*, 2009). The success of any given SIT programme relies on sterile males performance and estimating male fitness components in the laboratory is the first step before their release (Massonnet-Bruneel *et al*,

2013). There are many questions yet to be answered regarding mosquito biology relevant to colonization and large-scale insect production. These will be answered both through laboratory and field trials and will hopefully lead to more optimized cage designs and standardizing rearing techniques to produce good quality males for less cost (Benedict *et al*, 2009).

There are already several success stories relating to releasing GM male mosquitoes in the field. For example, genetically modified mosquitoes *Ae. aegypti* (strain OX513A) were recently released in the Cayman Islands across ten hectares over a period of one month. It was observed that GM mosquitoes had successfully mated with wild females and induced them to lay sterile egg batches. The authors suggested that dengue disease could reduce by using genetic technology in target field populations (Harris *et al*, 2011). Another study by Lacroix *et al* (2012) demonstrated that releasing genetic modified OX513A *Ae. aegypti* mosquitoes in Malaysia was both a successful and safe approach. There was no significant difference in male's longevity between engineered strain and wild-type. Lee *et al* (2013) examined genetically sterile RIDL male mosquitoes (OX513A strain) mating competitiveness under semi-field condition. It was observed when transgenic male mosquitoes mixed with the wild under semi-field conditions, more than 45% of the matings were with RIDL males. This data confirmed that *A. aegypti* OX513A is competitive in this setting (Lee *et al*, 2013). Semi-field facilities which are used to assess transgenic insect technologies and effector genes should have special features such as: heightened biosecurity to prevent accidental release of altered strains, a location within and endemic area to more accurately simulate field conditions and must be in an isolated environment, close to all principle environmental conditions for the vector such as humidity, wind, solar energy, ovipostion sites, food availability for larvae and adult stages, swarming and mating sites. Also, semi-field environments should have enough space to

generate and maintain vector populations over several generations and fine tune methods to estimate changes in population (such as, to measure a decline or rise in the frequency of genotypes and/or phenotype over time). Semi-field conditions with excellent facilities are essential for GM mosquitos' research (Facchinelli *et al*, 2011).

Another interesting result demonstrated that all colonized and transgenic strains' accessory glands were significantly smaller than in the progeny from field-captured females. In contrast, testes sizes were larger in colonized and transgenic strains than in the progeny of field individuals. These patterns are thought to result from adaptations to laboratory conditions (Chapter 4). We then experimentally tested for the potential effects of inbreeding depression on reproductive traits by creating hybrid males from inbred colonized strains. Comparing sperm quality, testes and accessory gland sizes, and estimating the quantity of Plugin and Transglutaminase proteins in their accessory glands assessed the quality of these 'Super males' (Chapter 5). In the current study, the assumption was made that hybrid males were better than colonized lines. This seems to be true for sperm length because hybrid males had a long sperm length as did the field samples. However, the levels of Transglutaminase in the colonized lines were higher than in the hybrids. This suggests that not all the fitness qualities were affected by inbreeding. For example, the quantity of Plugin showed no difference between hybrids, colonized lines and field individuals. Accessory gland proteins (Acps) play a key role for the male in successful reproduction (Tram and Wolfner, 1999; Lung and Wolfner, 2001; Lung *et al.*, 2001; Wolfner, 2002). Rogers *et al.* (2009) identified twenty-seven MAGs proteins in *An. gambiae* by using mass spectrometry analysis and RT-PCR methods. Therefore, additional studies are necessary to estimate other MAGs proteins amounts and their role in male reproductive success.

From the observations reported here, there was no evidence of an impact of male or female genotypes across the four studied groups on the number of eggs and larvae (Chapter 6), as there was no significant difference in the number of eggs and larvae across the four experimental groups. This showed that there was no difference in the Super males and males from parental or older established strains in terms of fecundity and reproductive success in either sex. This may be because the Super males had similar genetic components inherited from their parents. Additional studies are needed to understand the molecular basis of fertility and reproductive success in *An. gambiae* s.s.in term of successful control malaria risk by using genetically methods.

Generally, the present study suggests that hybrid individuals (Super males) could represent a way to improve males' performance under laboratory conditions. There are still many open questions in terms of researching the reproductive biology and physiology of *An. gambiae* s.s. males that must be addressed before producing and releasing genetically-modified insects into the field. Determining the role of male accessory glands substances and sperm competition to progeny fitness, male survival, dispersal, mating success, and the factors affecting mate choice is of paramount importance to the future of sustainable, viable genetic control of mosquitoes. Thus it is important that, going forward, studies considering male mosquitoes should include experiments designed to investigate the ecological and evolutionary aspects of reproductive fitness (Ferguson *et al*, 2005). Carrying out these studies is essential to improve the quality of sterile Anopheline males produced in the laboratory, and is relevant to understanding of reproductive biology in the male of this species, in order to control malaria.

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# Appendix

**Appendix 1: Mosquito feeding solution**

1-Dissolve 0.5g PABA (Aminobenzoic acid) and 50g Glucose in 1litre distilled water (this gives 5% sugar solution).

2- Keep at 4°C for direct use or store at - 20°C.

**Appendix 2: 1xPBS**

10x PBS diluted with distilled water as one part in 10 times

**Appendix 3: Proteinase inhibitors (PI) + PBS**

One tablet of PI dissolves in 10ml of 1xPBS

**Appendix 4: Washing solution**

Prepare PBS+ Tween by adding 50ml of 10x PBS to the cylinder then complete with distilled water until 500ml. Cut the tip of the pipette with a sharp scissors and take 250µl of the tween and add it to 500ml PBS. Keep the bottle in fridge at 4C°.

**Appendix 5: Blocking solution**

Add 2.5gram of dried skimmed milk powder (5% Marvel) to 50ml of PBS +Tween 0.05%-vortex the tube

**Appendix 6: Primary antibody with blocking solution**

Mix 6ml of blocking solution with 6µl of antibody I° in tube for 96wells. The solution should prepare fresh and used immediately. The remain solution is discard.

**Appendix 7: Secondary antibody with blocking solution**

Prepare the 2nd antibody (Goat anti rabbit HRP 1:2000 in block) by adding 2µl of 2nd° antibody in 4ml of PBS-T+ Marvel (blocking solution)-vortex the tube.