MATING BIOLOGY OF MALE *Anopheles arabiensis* Patton (Diptera: Culicidae): IMPLICATIONS IN THE USE OF STERILE INSECT TECHNIQUE IN VECTOR CONTROL

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Dedication

To

My parents…
My brother…
My sisters…
My uncle Hyder…
And to the soul of Ali Khalid.
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# ACRONYMS AND ABBREVIATIONS

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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>Ae.</td>
<td><em>Aedes</em></td>
</tr>
<tr>
<td>An.</td>
<td><em>Anopheles</em></td>
</tr>
<tr>
<td>AS</td>
<td>Aerial Spraying</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control and Prevention</td>
</tr>
<tr>
<td>CI</td>
<td>Cytoplasmic Incompatibility</td>
</tr>
<tr>
<td>Cx.</td>
<td><em>Culex</em></td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro Diphenyl Trichloroethane</td>
</tr>
<tr>
<td>DONG</td>
<td>A colony of <em>An. arabiensis</em> originated from Dongola area at Sudan.</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
</tr>
<tr>
<td>FMOH</td>
<td>Federal Ministry Of Health</td>
</tr>
<tr>
<td>GM</td>
<td>Genetically Modified</td>
</tr>
<tr>
<td>IAEA</td>
<td>International Atomic Energy Agency</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITN</td>
<td>Insecticide Treated Net</td>
</tr>
<tr>
<td>KGB</td>
<td>A colony of <em>An. arabiensis</em> originated from Zimbabwe.</td>
</tr>
<tr>
<td>MAGS</td>
<td>Male Accessory Gland Secretions</td>
</tr>
<tr>
<td>Medfly</td>
<td>Mediterranean fruit fly: <em>Ceratitis capitata</em></td>
</tr>
<tr>
<td>MRR</td>
<td>Mark Release Recapture</td>
</tr>
<tr>
<td>NMCP</td>
<td>National Malaria Control Programme</td>
</tr>
<tr>
<td>s.l</td>
<td><em>Sensu lato</em></td>
</tr>
<tr>
<td>s.s.</td>
<td><em>Sensu stricto</em></td>
</tr>
<tr>
<td>SIT</td>
<td>Sterile Insect Technique</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations Children’s Fund</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Chapter One:

INTRODUCTION AND LITERATURE REVIEW

Hopelessly, developing countries are entrapped in a vicious cycle of disease, poverty and war. Vector borne diseases, which are transmitted by some arthropods, reside at the core of this cycle. Arthropods of medical importance, such as vespids, ticks, mosquitoes, mites, flies, and fleas, are major causes of morbidity and mortality throughout the world. Thus, arthropods-transmitted diseases such as malaria, Dengue fever, sleeping sickness, leishmaniasis, Chagas disease, and a number of arboviral hemorrhagic fevers remain important threats in much of the world. However, by far, the deadliest member among the animal kingdom is the one responsible for the largest loss of human life through transmitting malaria, i.e \textit{Anopheles} mosquito.

Overall, severity of vector-borne diseases is mainly attributed to their complex mode of transmission, requiring passage from man to man or animal to man through an arthropod vector. This method of transmission implies the simple principle that removal of the vector will lead to eradication of the disease.

1.1 Vector control methods:

Current efforts on vector control are concentrated on inducing death in mosquito population; thus, the control measures target already birthed life forms of the organism. The approach of death control is operationally translated into two methods, these are:

(1) Methods to control adult stage: these target mosquito females, including aerial spraying of insecticide (AS) to suppress females during the flight, indoor residual spraying of insecticides (IRS) to bait females during their resting time inside rooms, and
Insecticide-Treated bed Nets (ITNs) to chase them during times of blood-seeking behaviour.

(2) Methods to control aquatic stages: These target the immature forms of the vector, in particular the larval stage, regardless of the sex. The methods include simple mechanical engineering techniques such as drainage to eliminate mosquito-larval habitats, use of bioinsecticides, use of insect-growth regulators, and biological control that uses a predator or other organisms to attack larvae and pupae (Bates and Curtis, 2005).

Although these two methods are employed to decrease the disease burden by interrupting transmission, they do not entirely eradicate the pathogen leaving the vectors to thrive in their natural habitats. It has proven increasingly difficult to eradicate an arthropod vector, and where successful control has been shown, this was often the result of temporary interruption of transmission to clear the human reservoir from the pathogen, as has been the case of malaria. For instance, in many countries around the Mediterranean and in the continental USA, a phenomenon of ‘anophelism without malaria’ is known. However, in India and Sri Lanka, such temporary successes had led to adverse results (Bruce-Chwatt and De Zulueta, 1980 cited in Takken and Boëte, 2003).

1.1.1 Insecticides and Insecticide Resistance:

Extreme Successful stories on the control of malaria in Western Europe (Curtis, 2002), the Soviet Union (Bruce-Chwatt, 1959) and India (Saxena et al., 1992), and the control of dengue fever in the Americas (Gubler, 1987), had all relied-to a large extent- on vector control using insecticides.
Insecticides are classified, according to their chemical nature and origins, into four groups: Organochlorines, Organophosphates, Carbamates, and Pyrethroids (Cusida and Quistad, 1998). These insecticides used for malaria control all target the nervous system of the insect (Bloomquist, 1996).

However, great expectations on synthetic insecticides (such as DDT and Dieldrin), which were introduced during the early years of the twentieth century, are phased out (Najera, 1989; Takken and Böete, 2003). The main reasons for this failure were: (1) the emergence of insecticide resistance in disease vectors, (2) the negative response of the community against continuous house spraying, (3) and the lack of political will at the local level as well as the global one to offer adequate funds for control (Greenwood and Mutabingwa, 2002).

Insecticide resistance is defined as the ability of an organism to tolerate doses of a toxicant that proves lethal to a majority of individuals of a normal population of the same species (WHO, 1992).

Generally, four types of resistance mechanisms have been reported for mosquitoes:

1. **Vigour tolerance**; this involves tolerance of insecticide due to a thicker cuticle, feeding status, or environmental conditions (Hodjati and Curtis, 1998).

2. **Behavioural resistance**; this includes insecticide avoidance or changing of resting behaviour (Roberts and Andre, 1994).

3. **Target site insensitivity**; which is associated with point mutation(s) in a structural gene, such as the *para*-gene that confers cross-resistance to DDT and Pyrethroids (ffrench-Constantm *et al*., 1998).
4. Metabolic resistance; that is based on the overproduction of detoxifying enzymes.

Three classes of enzymes are responsible of metabolic resistance: Monooxygenases that confer Pyrethroid, Carbamate and Organophosphate resistance (Hemingway and Karunaratne, 1998); Glutathione-S-transferases that are reported for DDT resistance and in rare cases Pyrethroid resistance (Parpanthadara et al., 1996); Esterases, which are associated with Organophosphate and Carbamate resistance (Berge et al., 1998).

1.2. Genetic Control:

Differentially, the second approach of vector control is the birth control, whereby a measure targets the mosquito before it birthed. Indeed, the birth control approach is not operationally implemented, and the only manifestation of this approach is genetic control. Genetic control aims either to reduce density of the vector population towards elimination, or to replace competent vectors with genetically modified counterparts (GMO) that have been made refractory to parasite infection or development and no longer can transmit target pathogens or parasites. In 1964, World Health Organization (WHO) had defined Genetic control as “the use of any condition or treatment that can reduce the reproductive potential of noxious forms by altering or replacing the hereditary material". In the years of nineteen-sixties and nineteen-seventies, genetic control was studied using hybridization and sterile-insect technology.

1.2.1 Hybridization:

Hybridization result in production of sterile hybrids by cross mating two sibling species, was well observed between members of Anopheles gambiae (s.l.). Formerly regarded as a
single species with ecological salt-water variants, *An. gambiae s.l.* was shown later to be comprised of at least five freshwater and two salt-water species (Hunt et al., 1998).

The most important advance in the biology of *An. gambiae s.l.* stemmed from the work of Davidson (1962) and Davidson and Jackson (1962) who showed that certain freshwater strains of *An. gambiae (s.l.)* from different parts of West Africa produced sterile male progeny when crossed together. Further works reported by Paterson (1963) and Davidson (1964a, 1964b) revealed that the strains of *An. gambiae (s.l.)* studied up to that time fell into three mating groups, between which various degrees of genetic incompatibility existed. The reproductive isolation of these forms was established by Davidson (1964b) and Paterson (1964), and after that time they are regarded as good species.

However, until that time, no satisfactory morphological characters had been discovered in the adults, and the cross mating was the only method of identification. Cross mating of any two of the six known species of the *An. gambiae* complex results in both hybrid male sterility and -in some of the crosses- distorted sex ratios (Gillies and De Meillon, 1968).

Thus, an examination of the hybrid male reproductive system and estimation of the sex ratio from crosses-between unknown material and individuals of known species enabled identification of the unknown. Thirty possible crosses between the six sibling species of *An. gambiae* complex were easily conducted in laboratory mating cages (Davidson, 1964; Davidson and Hunt, 1973).

This dilemma on identification of members of the *gambiae* complex was solved after using cytogenetic (Coluzzi et al., 1979), biochemical isozymes (Miles, 1979) and molecular DNA polymorphism methods (Scott et al., 1993). Nonetheless, some
limitations were separately realized on the practicability of these methods (Krzywinski and Besansky, 2003).

The rarity of natural hybridization, where two or more species of the complex are sympatric, was demonstrated by a number of investigators (Paterson, 1964; Ramsdale and Leport, 1967). Indeed, there have been some reports of natural hybridization between *An. melas* and *An. gambiae* and between *An. gambiae* and *An. arabiensis* (Coz and Hamon, 1964; White, 1971; Shidrawi, 1972; Coz, 1973; Coluzzi et al., 1975). Nevertheless, extensive cytogenetic surveys have revealed an overall frequency of hybridization in nature lower than 0.2%, which points to the existence of some efficient pre-copulatory isolating mechanisms and this provides a basis for regarding members of the complex as distinct species (Paterson, 1963, 1964; Ramsdale and Leport, 1967; White, 1967, 1971; Bryan, 1979).

Disappointingly, field-failures in the use of sterile hybrid males as a tool to eradicate malaria were reported (Davidson et al., 1970). These are mainly attributed to the existence of mating barriers between sterile males and wild females (Davidson et al., 1970). Assortative mating might be accentuated by using a hybrid of two species to control a third one. Although mating behaviour may be disturbed in the experimental cages, conduction of laboratory studies on the members of the *gambiae* complex as well as their hybrids is an important tool to further knowledge on mating-segregation mechanisms of such phylogenic group (Okereke, 1980).

**1.2.2 Sterile Insect Technique (SIT):**

Sterile Insect Technique (SIT), also known as the sterile insect release method, is a species-specific and environmentally non-polluting method of insect control. SIT relies
on the mass rearing, sterilization and release of large numbers of insects (Knipling, 1955, 1979, 1998; Krafsur, 1998; Alphey et al., 2002). Mating of released sterile males with native females leads to a decrease in their reproductive potentiality; and ultimately, if males are released in sufficient numbers over a sufficient period of time, may result in eradication of the local pest population (Alphey, 2002; Benedict and Robinson, 2003). Central to the application of SIT is the area-wide concept in which the total population of the pest in an area, or region, has to be managed. SIT is also not a stand-alone technology and should be integrated in a package together with other pest control methods. However, SIT has a unique attribute of increasing efficiency with decreasing target population density; and as a result can lead to eventual eradication if applied systematically and on an area-wide basis over many generations. SIT is also the most environment-friendly pest management method as it is completely “species specific”: the sterility is induced exclusively in the target species, thereby affecting only the population of insect (Dyck et al., 2005).

Highly successful area-wide SIT programs have been conducted against the New World screwworm, Cochliomyia hominivorax, in the USA, Mexico and Central America (Wyss, 2000) and also in Libya, where SIT was used in the successful control of a serious outbreak in 1989 (Lindquist et al., 1992,). Other targets of area-wide SIT programmes include the Mediterranean fruit fly (Medfly), Ceratitis capitata, in various parts of Latin America (Hendrichs et al., 1995) and the codling moth, Cydia pomonella, in Canada (IAEA, 2001). These programs have been conducted on a massive scale; for instance, the El Pino facility in Guatemala alone aims to produce around one billion (10^9) sterile males
of Medfly per week, primarily for use in California and Guatemala (Hendrichs et al., 2002).

1.2.3 History of Mosquito SIT Projects:

The main objective of a SIT project is to reduce wild targeted populations by mating with virgin females, which lay only sterile eggs. In a model mosquito SIT project, a mass-rearing factory is employed to produce and separate millions of males, these males will be sterilized before releasing them into the field. Large imbalance ratios between sterile males and wild ones can be secured by timing the releases to the suppression season of wild populations i.e. “bottle neck” period. Since 1959, about 28 trials on mosquito SIT had been implemented in different parts of the world. The objective of most of these trials was not to suppress the targeted populations but to answer a specific research question. However, although a few suppression and/or eradication projects have been challenged, these were not of sufficient scale to be effective in non-isolated areas. In 1967, the first major success had been achieved at Myanmar against *Cx. quinquefasciatus*, which is infected with *Wolbachia*. Sterilization of the released males was achieved using cytoplasmic incompatibility (Cl)- (Laven, 1967).

Generally, several methods are available to obtain full sterile males such as chromosome aberrations, CI, irradiation, chemo-sterilization, or sex-ratio distortion due to meiotic drive. Equally, partial sterilization can be achieved using male-linked translocations. The latter method was applied to control a local population of *Cx. pipiens* near Montpellier, France (Laven et al., 1971 cited in Benedict and Robinson, 2003). On the other hand, *Cx. quinquefasciatus* was successfully eradicated using chemo-sterilized males on an island off Florida in the USA (Patterson. et al., 1970).
The first successful project against an anopheline mosquito was the eradication of an isolated population of *An. albimanus* using chemo-sterilized males released in a 15-km² area in El Salvador (Lofgren et al., 1974). When a larger scale attempt against the same vector was performed on the Pacific coast of El Salvador, population suppression was achieved only after the release area was contracted to 20 km² below that originally planned, and a sex separation strain based on a Y-linked translocations was introduced. Immigration was also reduced the effectiveness of the project. However, owing to a civil war [1980-1992] the project was terminated (Alphey and Andreasen, 2002).

Although in the last few years several studies reported successful transformation of mosquitoes (Jasinskiene et al., 1998; Catteruccia et al., 2000; Ito et al., 2002), controlling disease with transgenic mosquitoes is in the very early phase of development. A number of studies and reviews during the last three years reinforced the necessity to incorporate research on behavioural and physiological traits to ascertain that the transformed insects can compete with the wild populations which they are meant to replace (Takken and Böete, 2003; Catteruccia et al., 2003).

Despite its environmental benefits, SIT has been used against only a rather modest number of target species. Prominently, it is now well-realized that various technical causes had contributed to the failure of mosquito releases, among these are: production below planned levels due to absence of sexing methods or delays in production; immigration into release areas, and loss of fitness due to the less knowledge on male mating biology (Benedict and Robinson, 2003).

The released insects are required to compete for mates with wild insects. The production process, however, in particular sterilization of the insects by irradiation, causes a
dramatic loss of their mating competitiveness relative to wild type. Irradiated insects are less competitive and have reduced life spans. The combination of these two factors resulted in an estimated reduction of 4–10-fold in fitness of the Medfly (Shelly et al., 1994; Lance et al., 2000; Alphey, 2002). Therefore, the main question of “How transgenic insects will fare in a natural environment?” remains to be answered.

Surprisingly, a relatively little attention has been paid to this critical question on fitness of released transgenic mosquitoes compared to wild siblings that they are meant to replace and how parasites will respond to barriers of infection in their arthropod hosts (Clarke 2002; Boëte and Koella 2003). In 2003, Catteruccia et al., had reported a significantly reduced fitness of genetically transformed An. stephensi compared to non-transformed ones of the same laboratory stock.

As a whole, only seven releases of sterile anopheline males were carried out until now. These were carried out on An. quadrimaculatus at USA (Weidhaas et al., 1962; Dame et al., 1964); on An. gambiae at Burkina Faso (Davidson et al., 1970); on An. albimanus at El Salvador (Breeland et al., 1974; Weidhaas, 1974 and Dame et al., 1981); and on An. culicifacies at Pakistan (Baker et al., 1980; Reisen et al., 1981).

Past efforts of mass release of sterilized male mosquito had been less successful than expected partially because of low degree of competitiveness between sterile and wild males (Lounibos, 2003; Reisen, 2003). Release of 240,000 males over 9 weeks was unsuccessful in the reduction of a population of An. gambiae at Burkina Faso. Poor mating competitiveness was thought to be the reason (Davidson et al., 1970; Benedict and Robinson, 2003). For the same reason, release of 7500 sterile males in 1980 over a week at Pakistan was also failed (Reisen et al., 1981). However, in another release of
3100 males at Pakistan, these sterile males were competitive and no assortative mating was observed (Baker et al., 1980). In another trial, population reduction by release of 433,600 sterile males over 48 weeks at USA failed and this was attributed to a little semi-sterility of males (Weidhaas et al., 1962). Although some observations on mating between sterile males and wild females of 50,000 released at USA were demonstrated, it was not a concrete evidence for fitness of the males (Dame et al., 1964).

1.3 Age of Male and Mating Success:

 Evolutionarily, fitness of an individual is a function of both survival and mating success. In most cases, however, these two fitness components cannot be maximized simultaneously (Reznick et al., 2000). Overall, two contradicting hypotheses were proposed to predict which age category (i.e. young and intermediate males vs. old ones) of males would be preferred by the females in order to mate:

(1) The Viability indicator model of age-related mate choice: this predicts preference of females to older males since they have proven survival ability or because their signals are potentially more revealing (Kokko and Lindström, 1996; Kokko, 1997; Proulx et al, 2002). Such model argues that females use age as a reliable signal of male quality and that, by mating with older ones, they gain indirect benefits through the production of higher-quality offspring. This model has gained empirical support in a wide spectrum of animal species (Brooks and Kemp, 2001).

(2) The Direct trade-offs model: in this a trade-off between fitness components and age-specific differences in survival may reduce the fertility of older males, and instead promote the evolution of female preference for young and intermediate-age males
For instance, trade-offs model supports mating competency of young males of antler flies Protopiophila litigate, versus intermediate and old ones. Here, a high mating rate was associated with reduced longevity, because increased (short-term) mortality risk or accelerated ageing in traits affects viability of the individual (Bonduriansky and Brassil, 2005). In hiding beetle Dermestes maculates, intermediate-age males gained significantly higher mating success than younger or older males in a competitive mating environment (Jones and Elgar, 2004).

In Drosophila spp., age affects several aspects of sexual behaviour, such as virility (Kosuda, 1985), female receptivity, and female mating preferences (Obin et al., 1988).

In 2002, Olet and others investigated sexual receptivity and age in sleeping sickness fly Glossina pallidipes and showed that older males copulated more frequently than younger ones. Although Abila et al. (2003) had shown that 8 and 13-day old males of G. palpalis palpalis are more competent to mate than the 1 and 5-day old males; they revealed absence of age effect on the ability of G. f. fuscipes to inseminate.

Effect of age on mating competence had been investigated in laboratory-colonies of An. culicifacies, (Reisen et al., 1979); on An. stephensi (Mahmood and Reisen, 1982), and on An. gambiae s.l. (Charlwood and Jones 1979; Verhoek and Takken, 1994). However, comparative studies between populations of the same species, originating from different ecological habitats, may be important and reflect the genetic makeup of these populations.

In mosquito SIT project, the age and reproductive history of males of the targeted population(s) should be elucidated in order to select an optimal age of release for the
sterilized males (Reisen, 2003; Ferguson et al., 2005). Releasing sterile males of an optimal age would improve the efficiency of sterile males to out-compete the wild ones. This will contribute to the management of SIT project and will reduce its costs. It is therefore vital to investigate the most successful age for copulation and sperm transfer in male mosquito, with a view of choosing an optimal age of release.

1.4 Swarming behaviour of Mosquitoes:

As in most dipterans, anopheline mosquitoes mate during the flight. Aggregation is an important first phase in the mating behaviour of most mosquitoes (Clements, 1999). Swarming, “a nuptial flight assembly of males at a distinctive station”, is a distinctive phenomenon in the mating behaviour of mosquito. Many culicines swarm and mate near the vertebrate host; males of Mansonia spp. being attracted to host odours (Mclver et al., 1980) are able to locate females in search of a blood meal. In the genus Anopheles, mass swarms at (or near) aquatic emergence sites are the most common mating system (Nielsen and Haeger, 1960; Downes, 1969; Clements, 1999). The swarm may consist of a single individual, tens or thousands, related to a discrete swarm marker (Downes, 1969). Most anophelines mate in crepuscular swarms (Yuval, 2006). These include Old World vectors of malaria such as members of the An. gambiae complex (Charlwood and Jones, 1980; Marchand, 1984; Charlwood et al., 2002a), An. funestus (Charlwood et al., 2003), An. culicifacies (Reisen et al., 1982), An. maculipennis (Bates, 1949), An. stephensi, and An. subpictus (Panicker and Rajagopalan, 1984). In addition, some New World anophelines such as An. franciscanus (Belkin, 1951), An. pseudopunctipennis (Bates, 1949), and An. freeborni (Yuval et al., 1993), were shown to form crepuscular swarms.
However, species of the neotropical subgenus *Anopheles* (Nyssorhynchus) have not been observed in the field to mate in swarms (Lounibos *et al*., 1998) and apparently copulate at emergence sites, resting sites, or possibly near the blood meal host (Wilton and Fetzer, 1972; Lounibos *et al*., 1998). In most of the anopheline species, males are incapable of responding to host cues (Mclver *et al*., 1980).

In all swarming anophelines (whether at inanimate markers or near the host), females that enter the swarms are recognized by their wing beat frequency. There is no discernible courtship, and mating on the wing lasts between 10 and 30 seconds. Swarming males’ exhibit erect antennal fibrillae, which, in addition to the highly sensitive Johnston's organ, allow them to recognize the frequency of the female's wing beats and pounce on her as she approaches or enters the swarm (Yuval, 2006). The specificity of these frequencies is enough to distinguish between conspecific males and females and between females of closely related species (Brogdon, 1994). However, there is an extensive overlap between harmonic amplitudes of the wing beat of members of the *An. gambiae* group (Tripet *et al*., 2004). Thus other mechanisms—such as volatile (Takken and Knols, 1999) or contact pheromones (Nijhout and Craig, 1971; Polerstock *et al*., 2002)—must be invoked as final recognition cues before copulation.

In two non-anopheline genera: *Opifex* and *Deinocerites*, copulation— in the laboratory—is a lengthy affair (40 to 50) minutes, resulting in the filling of the three spermathecae of the female (Provost and Haeger, 1967). In *Culiseta inornata*, a contact pheromone and possibly a volatile pheromone aid recognition (Downes, 1969). Interestingly, a similar reduction of the swarming habit is shown in the closely related (but not blood feeding)
ground-dwelling crane flies (*Tipulidae*), in which females broadcast a volatile pheromone that alerts the patrolling males to their location (Adler and Adler, 1991).

Remarkably, members of the Sabethine tribe (*Sabethes, Malaya*, and *Wyeomyia spp.*), exhibit a completely different mating pattern. In this group, diurnal substrate-based sexual encounters are the rule. Males patrol resting sites, locate females on vegetation, and perform elaborate courtship displays before copulation (Hancock *et al.*, 1990). Copulation is significantly longer than in the swarming species, lasting (2-3) minutes (Hancock *et al.*, 1990; Philips *et al.*, 1996).

One of the most critical issues in mosquito mating is the lack of understanding of mate finding. Such swarms are often found in discernible sites, presumably guided by a visual marker (Marchand, 1984; Yuval *et al.*, 1993; Yuval and Bouskila, 1993; Charlwood *et al.*, 2002b; Charlwood *et al.*, 2003; Yuval, 2006). However, no distinctive attributes were distinguished neither on the selection of swarming station, nor on the rule of swarm formation; in other words, there is no information on how males approximate the swarming stations, how they aggregate and how females locate the sexual assembly. Nonetheless, two recent hypotheses suggested a role of volatile organic chemicals (Takken and Knols, 1999 ; Takken *et al.*, 2004), and/or stationary flocking model (Charlwood *et al.*, 2002b).

Observations on swarming behaviour of male *An. arabiensis* at both the laboratory and field (where a pilot SIT project will be implemented in Dongola) are needed to identify suitable station (s) to release sterile males and to obtain information on the general characteristics of swarming time, place and composition.
1.5 Feeding Source and Mating success:

One of the factors that may affect mating propensity of the male is the feeding source. Plant sugar is an essential part of the mosquito diet. In hematophagous insects, a female utilizes protein of blood meals to develop eggs. In addition, sugar provides the female with a ready source of flight energy (Nayar and Van Handel 1971) and can, in some cases, improve fecundity (Foster et al. 1989). The belief that sugar-feeding rarely occurs is based largely on collections of indoor resting and biting females that either contained no fluid in the esophageal diverticulum (Gillies, 1968) or mostly tested negative for fructose (Beier, 1996).

On the other hand, the mosquito male depends solely on carbohydrates of plant sugar to obtain the necessary energy to survive, disperse, swarm and mate (Van Handel, 1984; Foster, 1995). In nature, nectar is assumed to be the prevalent source of sugars, but probably all sorts of sugars in different concentrations are available (Foster, 1995). The availability of sugar solutions is not confined to nectar, but a wide variety of carbohydrates may be accessible, with honeydew probably the most important (Foster, 1995). *Ae. taeniorhynchus* is quite tolerant in accepting most sugars, while *Ae. aegypti* was reported to be more selective (Nayar and Sauerman, 1971).

Despite great efforts by several entomologists in the field, observations on mosquito feeding on flowers are relatively rare (Haeger, 1960; McCrae et al., 1969; McCrae 1989). Adult mosquitoes feed on nectar (Bidlingmayer et al., 1973; Magnarelli, 1983), cane sugar (De Meillon, 1967), extrafloral nectarines, and honeydew (Haeger, 1955) in the field. How mosquitoes locate natural sources of sugar remains unknown. Some flowers have ultraviolet-reflecting nectar guides, and many insects utilize these to locate nectar.
(Silberglied, 1979). Many Diptera are known to have UV receptors that are sensitive in
the 340-360 nm range (Stark and Tan, 1982). It is possible that diurnally active
mosquitoes use visual patterns and UV reflectance in particular, to locate flowers in the
field. Owing to the low light levels at dawn and dusk, crepuscular species are less likely
to use such cues (Allan et al., 1987).

In fact, foraging for plant sugar is costly of time and effort and makes necessary the
frequent nectar-collecting visits to flowers, which is the other main occupation of a male
mosquito. Foraging habit is also a dangerous activity, and it would scarcely persist where
it is not an integral part to the way of life (Downes, 1969).

In any mating event, success hinges on attending the encounter site, and for swarming
mosquitoes, this ultimately depends on sugar feeding. A comparison of the energetic
reserves of resting and swarming male An. freeborni revealed that males fed on nectar
during the night once swarming has concluded and that a swarming bout of 30 minutes
consumes more than 50% of their caloric reserves (Yuval et al., 1974). Thus, foraging
successfully is a prerequisite for participation in swarms.

In most sugar feeding studies, study parameters were confined to the survivorship of
adults (Gary and Foster, 2004; Impoinvil et al., 2004). However, it is unknown to which
extent a carbohydrate feeding meal can affect the propensity and competitiveness of a
mosquito male either in its flight dynamics to seek a mating site or in its ability to
inseminate a female. Accordingly, the limited knowledge of male feeding source in the
field urges research on behaviour and ecology of feeding of male. Usually, in most
anopheline laboratories, males are maintained on sucrose solutions with variable
concentrations ranging from 5%-10%.
1.6 The Genitalia of Male *Anopheles*:

In certain species, male genitalia are highly characteristic and provide useful confirmatory characters for species separation. However, they are subject to considerable variation and, in contrast, to the Culicinae; it is often found that they are of little value in the separation of sibling species of *An. gambiae* complex (Gillies and De Meillon, 1968). Morphologically, copulation between the female and male involves “terminalial touching”. Terminalial touching, which had been well illustrated in *Ae. aegypti* (Jones, 1974) and in *An. gambiae s.s.* (Charlwood and Jones, 1979), implies that male’s genitalia touches those of the female.

The abdomen of *Anopheles* spp. consists of eight normally visible segments together with two highly modified retractile terminal segments. The ninth segment in the male includes the clasping organs, while the latter and tenth together form the terminalia. The abdominal tergites are usually clothed in hairs only, but in many species, the last two sternites are ornamented with a few scales. In a certain number of species, mostly belonging to the series *Neomyzomyia* and *Cellia*, the tergites and sternites are densely clothed with broad scales, which may form laterally projecting tufts (Gillies and De Meillon, 1968).

The male terminalia are illustrated in Plate I (Evans, 1938). It consists of: coxite (= the composite basal unit of a paired segmental appendage of a genital segment.); harpago (= a variably shaped lobe of the interbasal fold arising free at the base of the gonocoxite.); phallosome (= all the structures surrounding or enclosing the opening of the male genital duct, i.e., the aedeagus, paramere(s), and parameral apodeme(s).); leaflets (= the one or more pairs of elongate leaflet-like structures usually occurring at the apex of the
aedeagus); style (= The movable appendage attached at or near the apex of a coxite); and the eighth, ninth and tenth sternites (dorsal plates of segments).

Plate I: Male terminalia (An. gambiae). a- Whole structure in sternal aspect (i.e. from above). c. coxite, h. harpago, l. leaflets, st. style, st. VIII eighth sternite, st.IX ninth sternite, X tenth or anal segment. b- Segments IX and X and coxites, tergal aspect. c. outline of coxite, t.VIII-t.X eighth to tenth tergites. e- Structures at base of terminalia to larger scale. a.s. accessory spine, d.l.p. dorsolateral plate, har. Harpago isolated, p. phallosome, p.sp. parabasal spines. d- Harpago isolatt. c. club, ap.b. apical bristle or hair. e- phallosome isolated. f - Tip of phallosome to show leaflets of one side. g- Largest leaflet seen in flattest aspect. (After Evans, 1938).
1.7 Insemination as an Indicator of Mating Success:

In most mosquito genera, during the copulation of mating, an ejaculate of the male is deposited into the bursa copulatrix of the female, from which sperms are transported to the spermathecae (Spielman et al., 1974). Uniquely, male Anopheles ejaculates directly into the single spermatheca (Yuval, 2006). The male deposits sperms, using his aedeagus, in the vagina of the female. Approximately 1000 sperms, suspended in male accessory gland secretions (MAGS), are retained viable for the reproductive life period of the females (Klowden, 1999). When eggs reach the opening of the spermathecal duct, the sperms leave the spermatheca and enter the eggs through the micropyle (Borror et al., 1981). Presence of sperms in the spermatheca of the female is known as insemination (Charlwood and Jones, 1979); and in most of mating studies, insemination was taken as a proof of mating success (Charlwood and Jones, 1980; Yuval, 2006).

In the crabhole-mosquito Deinocerites spp., an effective copulation fills all three spermathecae and females do not copulate again (Provost and Haeger, 1967). Furthermore, in this genus the temporal window of receptivity to copulation is narrow. Virgin females denied copulation for a week are no longer receptive when allowed access to males (Provost and Haeger, 1967). In aedines and culicines, spermathecal filling may provide short-term inhibition, until peptides produced in the accessory glands find their target in the female nervous system and effectively curtail female sexual receptivity (Craig, 1967; Young and Downe, 1987; Klowden, 1999). Craig (1967) demonstrated that female Ae. aegypti (L.) are rendered refractory to subsequent insemination because of the action of the male accessory gland Secretions (MAGS), which are received during an initial mating. Although Craig (1967) reported that the inhibition of insemination lasted
for the life of the mated female, several circumstances may promote re-mating in mosquitoes. Among these circumstances is an interrupted initial copulation or copulation with a sperm depleted male (Gwadz and Craig, 1970). Furthermore, physiological changes that occur with several gonotrophic cycles may result in remating (Williams and Berger, 1980; Young and Downe, 1982). Although the effects of gonotrophic aging on remating had not been completely supported, the pattern of lifetime refractoriness in *Ae. aegypti* appears to be the rule (Dickinson and Klowden, 1997).

In anophelines, a gelatinous mating plug is produced by the accessory glands and deposited into the vagina after sperm have been transferred (Giglioli and Mason, 1966). This plug often protrudes from the female genital opening, providing a short-term barrier (lasting 24 to 48 hours) against further copulations. Nonetheless, the storage in the spermathecae of a substantial ejaculate accounts for the long-term inhibition of female receptivity (Klowden, 2001). Previous studies of mating patterns of anopheline mosquitoes were behavioural observations either made on laboratory colonies or focused on field-collected material. Inferences made from laboratory observations have some limitations because mosquitoes are kept at high densities in small enclosures thus creating artificial environmental conditions that may result in a higher rate of multiple inseminations. On the other hand, field studies usually utilize genetic markers to detect if more than one male sired the progeny of wild caught females. In laboratory-mating studies on *Anopheles spp.*, reduced insemination rates of the insectary-reared colonies, are not expected to occur under natural conditions (Reisen *et al.*, 1979; Verhoek and Takken, 1994; Chambers and Klowden, 2001). Possible reasons may include not only the
effect of captivity on mating behaviour but also the rapid accumulation of recessive
behavioural genes in the colony.

1.8 Marking Methods of Insects:

Tracking the movement of insects in their natural habitat is essential for understanding
their basic biology, demography, and ethology. Generally, animal marking dates back to
218 B.C., when ornithologists distinguished ownership of birds by banding (Fisher and
Peterson, 1964). A wide variety of materials and methods have been used to mark
animals for biological research. Vertebrate biologists often mark their test subjects with
bands, brands, tattoos, tags, notches, paints and radiolabels (Stock, 1979 cited in Hagler
and Jackson, 2001; Basavaraju et al., 1998). Unfortunately, most vertebrate-marking
techniques are not practical for marking insects because they are cumbersome, heavy,
and/or costly (Southwood, 1978 cited in Hagler and Jackson, 2001). As a result,
entomologists are often challenged to develop unique methods for marking insects.
Particularly, insect marking for scientific studies began around 1920, when researchers
used paints, dyes, and stains in studying population dynamics (Dudley and Searles, 1923;
Irvine, 2000). Hundreds if not thousands of studies that required some way to label
insects have been conducted, but the search for a universal marker has proven to be
challenging (Hagler and Jackson, 2001). A wide variety of markers has been used to
assess insect population dynamics, dispersal, territoriality, feeding behaviour, trophic-
level interactions, and other ecological interactions (Hagler and Jackson, 2001).
Marking has been extensively utilized in insect dispersal studies known as Mark-Release-
Recapture (MRR); a researcher collects insects either from laboratory colonies or from
the field. The collected insects are then marked, released into the field, and recaptured at
given time and distance intervals after their release. The recaptured insects are then
checked for the presence of the marker to distinguish them from unmarked insects.

In many of the marking studies, an ideal marker for one insect species is a useless marker
for other insects (Su et al., 1991). An ideal marking material should be durable,
inexpensive, non-toxic (to the insect and the environment), easily applied, and clearly
identifiable. The method of choice for applying markers depends on the insect being
marked, the environment that the insect will encounter, and the nature of the experiment.

Insects can be marked individually or in large groups. Mass marking - usually in the form
of an application of dust, paint or dye- permits the identification of a group of insects
within a larger population. Dusts (also known as “powders”) have been used to mark
insects for >80 years (Darling, 1925). To date, they are probably the most commonly
used materials for externally marking a variety of insects (Service, 1993). Various kinds
of dusts have been used to label insects (Taft and Agee, 1962; Stern and Mueller, 1968;
Service, 1993; Southwood, 1978 cited in Hagler and Jackson, 2001). An invisible green
fluorescent dust used in crime detection was among the first dusts used to mark insects
(Taft and Agee, 1962). This dust is invisible under normal light, but it is easily detected
under UV light. The most common commercial dust used to mark insects is Day-Glo
(Day-Glo Colour Corp., Cleveland, OH), an affordable fluorescent dust that is available
in a wide variety of bright colours. Dusts are most frequently used for marking insects for
conventional MRR studies. Field-collected or laboratory-reared insects are dusted in
mass and released into the field for dispersal studies (Stern and Mueller, 1968; Schroeder
et al., 1981 cited in Hagler and Jackson, 2001). However, dusts have also been used to
mark insects for mark-capture studies by direct application in the field (Byrne et al., 1996; Prasifka et al., 1999). Moreover, different colours of fluorescent dusts can be used to mark different cohorts of individuals.

There are many aspects to the use of fluorescent markers in SIT programs. They are utilized to assess mating competitiveness of sterile males before they are released. Fluorescent markers could differentiate between captured couples of sterile and wild males in the laboratory and field-cages, but not in the wild (Shelly et al., 2002). In addition, they are useful to assess the ratio of releases to the wild populations (Batra et al., 1976). Moreover, spatial collection of marked males is a valuable method to investigate dispersal of the releases in the targeted area (Davidson et al., 1970; Milby et al., 1980; Reisen et al., 1981).

Hence, in a mosquito SIT project, there is an insisting demand to monitor the project in terms of ecology, dispersal and mating behaviour.

1.9 Malaria in Sudan and SIT Pilot Project:

Malaria is the major health problem in Sudan, with an estimated 7.5 million morbidity cases occurring annually and more than 35,000 deaths, most of these are children and pregnant women. Plasmodium falciparum is responsible for about 90% of malaria cases, in addition to P. malariae and P. vivax (UNICEF/WHO, 2003).

Despite a long history of malaria control and eradication (Elgadal et al., 1985), Sudan’s cases of malaria constitute half the number of all East Mediterranean Region countries (Malik et al., 2003). This tragic situation is due mainly to environmental degradation, climatic changes and the war.
Three malaria vectors are incriminated for the disease transmission. These are: *An. arabiensis* that actively transmits malaria all over the country from the dry north to the humid south; *An. gambiae (s.s.)* and *An. funestus*, both of them dominate the humid south region and some focal points in the west and blue Nile area (Lewis, 1956; White, 1974; Zahar, 1985).

The spread of drug and insecticide resistance had made the National Malaria Control Programs (NMCP), in the developing countries, to switch to more costly alternatives. Moreover, up till now, malaria vaccines have not proved sufficiently protective to warrant use in malaria control. Consequently, these limitations of current interventions pushed IAEA to renew its interest in the use of SIT for the suppression of malaria-transmitting mosquitoes in suitable areas.

Although no accurate statistical indicators are available, epidemics of other mosquito-borne diseases such as filariasis, dengue and yellow fevers occur and cause many deaths in Sudan (FMOH, personnel communication).

It is accepted that SIT would be used under specific conditions as an adjunct to more orthodox technologies, conforming to the principle of Roll Back Malaria (RBM) strategy to rely on multiple approaches of disease control. In 2000, the IAEA accepted a proposal from the Federal Ministry of Health of Sudan (FMOH) to envisage potentiality of introducing SIT in malaria vector control. Since that time, collaboration between the Sudanese counterpart represented in the Ministry of Science and Technology, and FMOH has been in progress. A mission in June 2000 to the Northern State of Sudan had assessed the suitability of the area and identified a potential site by the Nile River in the provinces of Dongola and Merowe. Two subsequent missions in August 2002 and
January/February 2003 confirmed the suitability of the area and a plan of research activities was developed for the period (2003-2006).

Although mass release is a final step in SIT project, it is a collective net of steps of rearing, factory selection, marking, handling, radiation and release (Benedict and Robinson, 2003). The main objective of a quality control on SIT project is to maximize competitiveness of laboratory-reared males to the wild ones. Upon each step of mass release, procedures of quality control should be sought to obtain well competent males.

In this study, some essential issues that are pertinent to the fitness of mass-releases of males of An. arabiensis were explored under laboratory conditions at FAO/IAEA laboratories-Austria. In addition, fieldwork was accomplished in the field at Dongola area-Sudan.

1.10 Objectives of the study:

Main objective:

To explore some issues on mating biology of male *Anopheles arabiensis* that are pertinent to the fitness of mass-releases of males in a SIT project.

Specific objectives:

1. To identify an optimal age (s) of mass-releases of sterile males.
2. To investigate the effect of adding-sex ratio on mating success.
3. To investigate the effect of mating-duration on mating success.
4. To investigate the effect of meal type of male on mating success.
5. To observe swarming and mating behaviour in the field and laboratory.
6. To identify swarming stations of *An. arabiensis* in the field.
7. To test susceptibility of both sexes of *An. arabiensis* to insecticides.
Chapter Two:

MATERIALS AND METHODS

2.1 Mosquito Rearing

2.1.1 At the IAEA mosquito laboratory in Siebersdorf- Austria:

Two colonies of An. arabiensis were used in the current study; DONG colony originated from Dongola city- Sudan where extreme conditions of high temperature and low humidity limit the northern geographical distribution of this malaria vector in the African continent; and KGB colony originating from Zimbabwe where optimal conditions of low temperature and high humidity prevail. The two colonies were reared in IAEA mosquito laboratory at Siebersdorf- Austria. Larvae were maintained in larvae room of the insectary on Tetramin™ food in plastic pans. These pans were placed on thermo-regulated racks of 29-31 ºC suitable for the development of larvae. Adults were transferred to the plastic cages, provided with sugar feeding (10% sucrose) and moved quickly to the adults’ room where a crepuscular light system of sunset-sunrise is utilized; temperature and humidity were adjusted in the adults’ room. Hobbo LCD logger, i.e. sensor, was utilized to record the time stamped relative humidity (RH) and temperature measurements. Hobbo logger was set from a host computer using running Boxcar Pro software to 1 minute intervals. Data of 24 hours was offloaded to a computer.

2.1.2 In the field laboratory in Dongola-Sudan:

Larval collections were made from the original breeding site of DONG colony using larval nets and scoops. Anopheline larvae were isolated from Culicines using pipettes. All larval pans were maintained in a wide room where the solar light entered from the north-sided windows during daytime (7:30- 19:00). Rice powder was provided to the larval
aluminium pans as the main source of feeding. Pupae were harvested each day and transferred in small trays to the adults’ cages. The latter were maintained in a small isolated room where solar light only entered through a closed glass-window. A feeding solution of 10% Sucrose was provided to each adult cage. Temperature and humidity were recorded in the morning 8:00; at midday 2:00; and at night 10:00.

2.2 Sexing Methods of Experimental Materials:

*Anopheles* species differ from other mosquitoes in that there is often no discernable difference in the female and male larval or pupal size and general appearance. Two methods were applied to ensure that no prior mating of females and males had taken place:

1. Sexing of pupae using a dissecting microscope to distinguish male’s claspers.

Using a pipette, 12 pupae were individually transferred to a white depression plate of 12 wells. Excess water was removed from the depression well so that the pupa is lying on its side. Under a dissecting microscope, the prominent genitalia of the male were distinguished from the females, particularly claspers (Plate II). A small brush was used gently to lift the paddles and show the male’s claspers.

2. The second method is the immediate separation of emerged females from the males within the first 12 hours after emergence using a sucking tube. Prominent apparent antennae of males were used to discriminate and isolate males from the females.
Plate II: Pupae-sexing of females and males of *Anopheles* spp. can be achieved under the microscope using male’s claspers (*Photo courtesy: M. Benedict*).

2.3 Experiments on the Effect of Age of Male:

Six series of experiments were conducted to determine the effect of male’s age on mating propensity. The pupae sexing method was employed in all these series of experiments. 50 ♀s + 50 ♂s were combined together in a mating cage for a night mating period of sunset-sunrise. Sugar feeding container of 10% sucrose was inserted in the mating cage. While the age of females was fixed in all experiments to 5 days old, six age categories of male were tested, these were: <One day old ,One day old, and 2,3,4 and 5 days old. Three replicates were run to investigate each age category. DONG and KGB colonies were separately investigated for age effect.
2.4 Experiments on the Effect of Male-biased Sex Ratio:

Sex ratio of (2:1) and (3:1) males to females were investigated in two series of experiments. 50 virgin females of 5 days old were merged in a mating cage with 100, 150 unmated males of the same age. All experiments were carried out for a one night mating period of sunset-sunrise. Three replicates were run for each series.

2.5 Experiments on the Effect of lengthening Mating Period

Experiments:

In these series of experiments, mating period was extended to two consecutive days, three consecutive days and four consecutive days. Starting with an age of 5 days old for each, 50 males were mixed together with 50 females in a mating cage and provided sugar feeding of sucrose10%. Three replicates were run for each series. Experiments were run separately for DONG and KGB colonies.

2.6 Experiments on the Effect of Meal type:

Four series of experiments were conducted on two age categories of males: one day and five days old. In the first series of experiments, emerged males were provided a water meal. In the second series of experiments, no meal was provided to the emerged males either in the males’ cage or in the mating cage. While a meal of 10% sucrose + 2% methyl parbene was provided in the third series of experiments, a juice of 10% date palm was the meal in the 4th series of experiments.
Females of 5 days old were used in all experiments and maintained on 10% sucrose after their emergence. Twenty-five females of five days old were mixed together with 25 males of one day, or five days old, in a mating cage and for one night mating period of sunset-sunrise. In the control group of experiments, males were provided sugar feeding of 10% sucrose either in the One day old or 5 days old groups.

2.7 Experiments on the Effect of Combined Factors (Mating Period + Sex Ratio + Meal Type):

The three factors of mating period, sex ratio and meal type were combined together in two groups of experiments. While males were provided a meal of 10% Sucrose in the first group, 10% Sucrose + 2% methyl parbene was offered to the second group. Three replicates were run for the first group but only one replicate was run for 10% Sucrose + 2% Methyl parbene.

Starting with 50 males of 5 days old, a mating experiment was conducted for four successive mating periods each period extended for three days. During the start of each mating period 25 virgin females were added to the mating cage. These females were removed before the start of next mating period, and so on. The Number of dead males was calculated after each mating period in each replicate.

2.8 The New Approach of Male Marking:

Marking dusts are usually applied by putting the insects in a container with a given amount of dust and shaking the container. It is also common to use an insufflator to blow
air inside the container in order to ensure marking of the mosquitoes. Often the container is as simple as a paper or plastic bag (Stern and Mueller, 1968; Narisu et al., 1999). In this new approach, males were topically treated on their last abdominal segments including claspers. Pink and green fluorescent powder dyes were used to mark the males. Males were anesthetized using carbon dioxide before 2 hours of the dark period in the insectary. Pipe cleaners were used to apply the dye under a dissecting microscope; gloves were worn each time of application of the dyes to exclude contamination. All females were not marked with either one of the dyes. After leaving the mating cage for the night mating period, females were individually separated using small plastic tubes. A fluorescent microscope was utilized to investigate the presence of fluorescent spots on the last abdominal segment of the female. Specimens were photographed under the fluorescent microscope using attached camera and the appropriate software “Analysis B”. Spermathecae of coded females were further dissected and investigated for insemination. Correlations were made between the results of fluorescent microscope and the binocular one.

2.8.1 Experiments on the Effect of Fluorescent Dyes on Mating Success:

In this regard, two series were conducted. In the first series, three treatments were run: a non-marked control group of 10 females + 10 males were contrasted against a green and red marked groups of the same numbers of females and males. In the second experiment, a group of 10 green marked males and 10 red marked ones were added to the mating cage of 10 virgin females for 3 consecutive nights.
2.8.2 Experiments on Age Competitiveness using the Fluorescent Dyes:

Two series of experiments were conducted to study mating competitiveness between different age groups. In the first series males of 5 days, 3 days and One day old were investigated. While Males of 5 days, 8 days and 1One days were subjected to the experiments in the second series. Three mating groups were run during the same time of the experiment. Combination rules of the three age categories of males were followed; these are:

1. Age categories were subjected to the three marking colours (green, pink and none).
2. In each mating cage, equal numbers of males of the three ages were put together.
3. In each mating cage duplications had been avoided neither in dust colour nor in age category.

2.9 Observations on Mating and Swarming Behaviour in the Laboratory:

Observations were extended from 16:30 hour before the dusk (light intensity = 5.3 lux) and until 19:00 hour after 90 minutes from sunset (light intensity = 0.2 lux) in the insectary (see Figure (1)). All Observations were done during the period (July –August) 2005. A mating cage made of transparent Plexiglas (60X30X30 cm) was employed for these observations. A lamp of infrared light was switched on before the insectary’s light was turned off, as the eyes of Anopheles mosquito could not perceive the intensity level of infrared. Objective of the observations was to characterize the swarm formation: start time of the swarm, number of swarming males at start, maximum number of swarming males, time of the maximum number, and time of the end of swarming.
2.10 Experiments on the Effect of Swarm Marker:

In this series of experiments, effect of presence of a vertical swarm marker inside the mating cage was examined. Two plastic cups were pasted together on their bases using a glue to form a stand of 14 cm. White papers were used to cover the surfaces of the stand. On the same way, a black marker was made using black papers. Three mating cages, each one containing 25 females + 25 males of 5 days old, were employed in this series. The white swarm marker was inserted in the 1st cage; the black marker was placed in the 2nd cage; and no swarm marker was added to the last one. Observations on swarming behaviour were made during the artificial sunset period. All mating cages were left for a night period of sunset-sunrise. Females were removed on the next day and dissected for insemination. Three replicates were run for DONG colony.

2.11 Observations on Mating and Swarming Behaviour in the Field:

Exploration of possible swarming stations was made at Dongola and its neighbouring villages during January 2006. A mining process on the relevant literature on the swarming behaviour of *An. gambiae s.l.*, had revealed a number of hints utilized to discover swarming stations, these were:

1. To look near the breeding sites in a circle of a diameter of 3 meters;
2. To look for prominent object contrasting in its features with the landscape;
3. To look up on the sky; at an altitude level of (1-4) meters;

The searching started at evening 17:30 hour before one hour from the dusk. Time at the starting of male swarming, number of starting males in the formation of the swarm, time at a maximum number of swarming males, maximum number of the swarming males,
time at the end of the swarm, number of the males at the end, were all recorded. Observations were also made on the occurrence of couples, number of couples, and duration of coupling pairs. Sweeping net (opening diameter= 25 cm, hand=1.5 meters) was used to catch the swarming males and forming couples.

2.12 Insecticides Susceptibility Tests:

Insecticide susceptibility tests were conducted by exposing sugar-fed adult males/females to filter papers impregnated with diagnostic concentration of the proposed insecticide according to the standard of WHO procedures (1998).

Susceptibility tests were carried out in the field on reared males collected from the field as larvae, eggs and pupae. Females and males were tested separately for 1 hour by aspirating 25 mosquitoes in each exposure and control tubes. Four replicates of the above number were used for each insecticide. During exposure time the numbers of mosquitoes that are knocked down were recorded after 10, 15, 20, 30, 40, 50 and 60 minutes of exposure. Exposure time was one hour for all the four insecticides tested. Temperature and relative humidity were measured during the performance of the test using thermometer and a hydrometer, respectively. Specifications of tested impregnated papers are shown in Table (1).

Table 1: Specifications of the impregnated papers used in Susceptibility tests

<table>
<thead>
<tr>
<th>Insecticide class</th>
<th>Tested Insecticide</th>
<th>Discriminating doses</th>
<th>Manufacturing date</th>
<th>Expiry date</th>
</tr>
</thead>
<tbody>
<tr>
<td>OrganoChlorines (OC)</td>
<td>DDT</td>
<td>4%</td>
<td>Sep 2005</td>
<td>Sep 2010</td>
</tr>
<tr>
<td>OrganoPhosphates (OP)</td>
<td>Malathion</td>
<td>5%</td>
<td>Jan 2004</td>
<td>Jan 2007</td>
</tr>
<tr>
<td>Carbamates (C)</td>
<td>Bendiocarb</td>
<td>0.1%</td>
<td>Sep 2005</td>
<td>Sep 2008</td>
</tr>
<tr>
<td>Pyrethroids</td>
<td>Permethrin</td>
<td>0.75%</td>
<td>Sep 2005</td>
<td>Sep 2006</td>
</tr>
</tbody>
</table>
2.13 Dissection of Spermathecae:

On the next day morning, after the mating period, females were anesthetized using carbon dioxide, chloroform or by shocking. Females were dissected using fine dissecting needles under a stereomicroscope in a physiological saline solution to extract spermatheca. The spermathecae were removed, and mounted on a microscope slide with a cover slip. Under a binocular microscope and using powers of 10X and 40X, each spermatheca was investigated for the presence of sperms.

The formula of physiological saline solution was NaCl = 7.5 g; KCl = 0.35g; CaCl₂ = 0.21g; added to H₂O to make a total volume of 1 litre (Ephrussi and Beadle, 1936).

2.14 Calculations and Statistical Analyses:

Data entry was made for each series of experiments using Microsoft Excel sheets. Data analyses were done using Excel and SPSS software.

The insemination rate was calculated for each replicate using the formula: %Insemination rate = No. of inseminated females/No. of dissected females x 100%. Mean and standard deviation (SD) of the insemination rate were calculated for each group of replicates.

F test was used to compare DONG and KGB colonies on the effect of age experiments. The F test was also used in male age experiments to compare the one-day-old males group with other age groups > One day.

Assuming equal variance, t-test was used to compare means of sex ratio groups. ANOVA test was used to compare means of mating periods either in DONG or KGB.
ANOVA test was also used to compare means of different meal types either for the 5 days males or for the One day old males. A Post hoc test of Tukey HSD was further used to determine significant differences on means between the meal types.

Multiple regression analyses were performed on the data of combined effects experiments by defining insemination rate as the Y function, and both the numbers of mating period, and the dead males as $X_1$ and $X_2$.

For insecticide resistance mortality of tested specimens in each replicate was recorded 24 hours after exposure time. If mortality of the control was equal to or higher than 20%, the test will be repeated. If the control mortality was between 5-20%, the mortality percentage would be corrected by Abbott’s formula as follows:

$$\text{Corrected } \% \text{ killed} = \frac{\left( \% \text{ alive control} - \% \text{ alive treated} \right) \times 100\%}{\% \text{ alive control}}$$

Interpretation of susceptibility results was made according to WHO recommendations (WHO, 1998), that mortality of exposed mosquitoes should be interpreted as following:

- If mortality is 100%-95%, susceptibility is confirmed.
- If mortality is <95% and >80, resistance is suspected and need more tests.
- If mortality is <80%, resistance is confirmed.
Chapter Three:

RESULTS

3.1 The Environmental Conditions at the Insectary:

In Figure (1), temperature was finely adjusted inside the insectary to a range of 26.5-26.9 °C. Relative humidity had fluctuated from 79.1% to 83.6%. Line of the light intensity shows that the dawn starts at 6:30 inside the insectary till it gets full bright at 7:45, a consistent daylight continues till 18:30. After which, a dusk period continues till it gets full dark at 19:00. Variation in light intensity was recorded during the daylight.

3.2 Age of the Male and Mating Propensity:

Results of females dissection for DONG and KGB are detailed in Tables 2 and 3, respectively. The mating experiments on six different age categories of males had shown no mating propensity by the males of <One day old neither in DONG nor in KGB colony- Figure ( 2).

Males were able to mate after they spent the first 24 hours after their emergence. While an average insemination rate of 12.08% ± 0.14 was recorded in KGB for the males of One day old, a less percentage was obtained for the same age category in DONG (insemination rate = 7.33% ± 1.533). In addition, as in Figure (1), KGB had shown a higher insemination rate than in DONG but not significant (F =2.26; p=0.144).

Interestingly, there is a significant difference on insemination rate between the males of One day old and above age groups either in DONG (F=5.225; p=0.016) or in KGB (F=4.173; p=0.03). However, mating success of all age replicates for one night was less than 60% and 70% in DONG and KGB, respectively.
Figure 1: time-scaled records of the physical conditions inside the adults' room of the insectary
Table 2: Results of mating experiments in five age groups of males of DONG colony

<table>
<thead>
<tr>
<th>No. Rep.</th>
<th>Males' age particulars</th>
<th>&lt; one day</th>
<th>One day</th>
<th>2 day</th>
<th>3 day</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>No. Dissected females</td>
<td>40</td>
<td>50</td>
<td>40</td>
<td>39</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>6</td>
<td>21</td>
<td>8</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>12</td>
<td>52.5</td>
<td>20.51</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. Dissected females</td>
<td>43</td>
<td>50</td>
<td>43</td>
<td>50</td>
<td>46</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>6</td>
<td>20</td>
<td>29</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>12</td>
<td>46.51</td>
<td>58</td>
<td>58.7</td>
<td>48.39</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. Dissected females</td>
<td>50</td>
<td>49</td>
<td>47</td>
<td>50</td>
<td>39</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>6</td>
<td>18</td>
<td>31</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>12.25</td>
<td>38.3</td>
<td>62</td>
<td>48.72</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 3: Results of mating experiments in five age groups of males of KGB colony

<table>
<thead>
<tr>
<th>No. Rep.</th>
<th>Males' age particulars</th>
<th>&lt; one day</th>
<th>One day</th>
<th>2 day</th>
<th>3 day</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>No. Dissected females</td>
<td>45</td>
<td>45</td>
<td>50</td>
<td>48</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>3</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>6.67</td>
<td>44</td>
<td>45.83</td>
<td>44</td>
<td>24</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. Dissected females</td>
<td>49</td>
<td>44</td>
<td>50</td>
<td>38</td>
<td>47</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>4</td>
<td>23</td>
<td>9</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>9.09</td>
<td>46</td>
<td>23.68</td>
<td>44.68</td>
<td>59.09</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. Dissected females</td>
<td>50</td>
<td>48</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>No. Inseminated females</td>
<td>0</td>
<td>3</td>
<td>21</td>
<td>17</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>%insemination rate</td>
<td>0</td>
<td>6.25</td>
<td>42</td>
<td>34</td>
<td>58</td>
<td>31.11</td>
</tr>
</tbody>
</table>
Figure 2: Effect of male age on mating success of An. arabiensis (DONG and KGB colonies):

The effect of males’ age on mating success

- Insem rate DONG
- Insem rate KGB
3.3 Results of the Male-biased Sex Ratio:

Results of females dissections for groups of 2:1 and 3:1 are detailed in tables 4 and 5, respectively. The effect of sex ratio on 5 days old males and females of DONG and KGB is shown in Figure (3).

Although a non-significant difference was shown in DONG colony on mating success between the group of 2:1 sex ratio (insemination rate=68.7% ± 23.5; t=-1.77; p=0.151) and the group of 1:1, a significant increase was recorded between the last group and the group of 3:1 (insemination rate=78.5% ± 11.87; t=-3.18; p=0.03).

Similarly, a significant increase was obtained in KGB colony for the available data on the group of 3:1 sex ratio (insemination rate= 80.57% ± 2.18; t=-22.69; p=0.00002) in a comparison to the group of 1:1. However, only one replicate was conducted on the 2:1 group of KGB (insemination rate= 65%).

Table 4: Results of mating experiments of male-biased sex ratio 50 females + 100 males

<table>
<thead>
<tr>
<th>No. Replicate</th>
<th>Colony</th>
<th>DONG</th>
<th>KGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>No. of dissected females</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>60.98</td>
<td>65</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. of dissected females</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. of dissected females</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>95.12</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5: Results of mating experiments on male-biased sex ratio 50 females + 150 males

<table>
<thead>
<tr>
<th>No. Replicate</th>
<th>Colony</th>
<th>DONG</th>
<th>KGB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Replicate 1</strong></td>
<td>No. of dissected females</td>
<td>42</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>83.33</td>
<td>78.72</td>
</tr>
<tr>
<td><strong>Replicate 2</strong></td>
<td>No. of dissected females</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>65</td>
<td>82.98</td>
</tr>
<tr>
<td><strong>Replicate 3</strong></td>
<td>No. of dissected females</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>87.23</td>
<td>80</td>
</tr>
</tbody>
</table>
Figure 3: Effect of sex ratio on mating success of 5 days old males of *An. arabiensis* (DONG and KGB colonies):
3.4 Results of Lengthening Mating Period:

Results of females’ dissections for time groups are detailed in Tables (6.1), (6.2) and (6.3). Effect of lengthening mating period is shown in Figure (3).

Elongating mating period to more than one night had significantly augmented the mating success in DONG colony up to 90.52%±4.36 in the group of 2 consecutive nights period, up to 91.04%±4.34 in the 3 nights group, and up to 92.37%± 4.43 in the 4 nights group (F=21.73, p<0.001). Correspondingly, a significant increase on insemination rate (F=34.67, p<0.001) was shown in KGB colony over the 1 night group (insemination rate=49.46 % ± 0.82), for the 2 nights (79.7% ± 4.3), 3 nights (78.14 % ± 8.33), and 4 nights group (86.25% + 3.88).

Table 6: Results of mating experiments on lengthening mating period for:

6.1 Two consecutive days of mating:

<table>
<thead>
<tr>
<th>No. Replicate</th>
<th>Colony</th>
<th>DONG</th>
<th>KGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>No. of dissected females</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>44</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>88</td>
<td>82.5</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. of dissected females</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>95.56</td>
<td>76.6</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. of dissected females</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>No. of Inseminated females</td>
<td>44</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>88</td>
<td>80</td>
</tr>
</tbody>
</table>
6.2 Three consecutive days of mating:

<table>
<thead>
<tr>
<th>No. Replicate</th>
<th>Colony</th>
<th>DONG</th>
<th>KGB</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. of dissected females</td>
<td>30</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated females</td>
<td>26</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Insemination Rate %</td>
<td>86.67</td>
<td>68.57</td>
<td></td>
</tr>
<tr>
<td>Replicate 2</td>
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<td></td>
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<tr>
<td>No. of dissected females</td>
<td>43</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated</td>
<td>41</td>
<td>37</td>
<td></td>
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<tr>
<td>Insemination Rate %</td>
<td>95.35</td>
<td>83.78</td>
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<td>Replicate 3</td>
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<tr>
<td>No. of dissected females</td>
<td>45</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated females</td>
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<td>32</td>
<td></td>
</tr>
<tr>
<td>Insemination Rate %</td>
<td>91.11</td>
<td>82.05</td>
<td></td>
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</tbody>
</table>

6.3 Four consecutive days of mating:

<table>
<thead>
<tr>
<th>No. Replicate</th>
<th>Colony</th>
<th>DONG</th>
<th>KGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of dissected females</td>
<td>50</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated females</td>
<td>44</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Insemination Rate %</td>
<td>88</td>
<td>90.48</td>
<td></td>
</tr>
<tr>
<td>Replicate 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of dissected females</td>
<td>39</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated</td>
<td>38</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Insemination Rate %</td>
<td>97.44</td>
<td>85.42</td>
<td></td>
</tr>
<tr>
<td>Replicate 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of dissected females</td>
<td>48</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>No. of Inseminated females</td>
<td>44</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Insemination Rate %</td>
<td>91.67</td>
<td>82.86</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4: Effect of mating period on mating success of *An. arabiensis* (DONG and KGB colonies):

![Graph showing effect of time duration on mating success](image)

- DONG colony
- KGB colony

<table>
<thead>
<tr>
<th>Time Duration</th>
<th>Insemination Rate (Average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day of mating</td>
<td>38.07 % ± 49.46</td>
</tr>
<tr>
<td>2 days of mating</td>
<td>90.52 % ± 79.70</td>
</tr>
<tr>
<td>3 days of mating</td>
<td>91.04 % ± 78.14</td>
</tr>
<tr>
<td>4 days of mating</td>
<td>92.37 % ± 86.25</td>
</tr>
</tbody>
</table>
3.5 Results of Male-Meal Type:

Results of mating experiments on meal type for one-day old and five- days old, males are
detailed in Tables (7) and (8), respectively.

In Figure (5), feeding of males for 5 consecutive days either on 10%Sucrose + 2%Methyl
parbene, water, or juice of 10% date palm had significantly reduced the mating success in
comparison to 10%Sucrose (F=24.43;p=0.0002). In the same way, feeding of One day
old males on 10%Sucrose gave a significant higher insemination rate than feeding on
10%Sucrose +2%Methyl parbene, water or 10%Date-palm juice (F=3008.3, p<0.001).

Multiple comparisons on the effect of meal types between 5 days old males and 1-day-old
males are shown in Tables 9 and 10, respectively.

In all meal experiments except 10%Sucrose + 2%Methyl parbene, males of 5 days old
showed a higher insemination rate over the males of One day old but was non-significant
difference  (F=0.66 ;p=0.247).

Table 7 : Results of mating experiments on meal type for 1-day-old males:

<table>
<thead>
<tr>
<th>No. Rep.</th>
<th>Meal type</th>
<th>10%Sucrose</th>
<th>10%Sucrose + 2%Methyl parbene</th>
<th>10%Date-palm juice</th>
<th>water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of dissected females</td>
<td>19</td>
<td>21</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Replicate 1</td>
<td>No. of Inseminated females</td>
<td>7</td>
<td>8</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>36.8</td>
<td>38.1</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>No. of dissected females</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. of Inseminated</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>35</td>
<td>30</td>
<td>0</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>No. of dissected females</td>
<td>24</td>
<td>22</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. of Inseminated females</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Insemination Rate %</td>
<td>32</td>
<td>27.27</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>
Table 8: Results of mating experiments on meal type for 5 days old males:

<table>
<thead>
<tr>
<th>No. Rep.</th>
<th>Meal type</th>
<th>10%Sucrose</th>
<th>10%Sucrose + 2%Methyl parbene</th>
<th>10%Date-palm juice</th>
<th>water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>No. of dissected females</td>
<td>22</td>
<td>22</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Replicate 1</td>
<td>No. of Inseminated females</td>
<td>11</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Replicate 1</td>
<td>Insemination Rate %</td>
<td>50</td>
<td>31.8</td>
<td>32</td>
<td>35</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. of dissected females</td>
<td>24</td>
<td>22</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>No. of Inseminated females</td>
<td>16</td>
<td>8</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>Insemination Rate %</td>
<td>66.7</td>
<td>36.4</td>
<td>30</td>
<td>36.4</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. of dissected females</td>
<td>24</td>
<td>23</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>No. of Inseminated females</td>
<td>14</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>Insemination Rate %</td>
<td>58.3</td>
<td>26.1</td>
<td>30.4</td>
<td>33.3</td>
</tr>
</tbody>
</table>

Table 9: Tukey’s HSD test for multiple comparisons between groups of meal type for 5 days old males

<table>
<thead>
<tr>
<th>(I) meal type</th>
<th>(J) meal type</th>
<th>Mean Difference (I-J)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Sucrose</td>
<td>+2%Methyl parbene</td>
<td>25.02***</td>
<td>13.40</td>
</tr>
<tr>
<td>10%Sucrose</td>
<td>10%Date-palm juice</td>
<td>27.22***</td>
<td>15.60</td>
</tr>
<tr>
<td>Water</td>
<td>+2%Methyl parbene</td>
<td>23.22***</td>
<td>11.60</td>
</tr>
<tr>
<td>Water</td>
<td>10%Date-palm juice</td>
<td>4.00</td>
<td>-7.62</td>
</tr>
</tbody>
</table>

*** Significant under the .001 level.
Table 10: Tukey’s HSD test for multiple comparisons between groups of meal type for 1-day-old males

<table>
<thead>
<tr>
<th>(I) meal type</th>
<th>(J) meal type</th>
<th>Mean Difference (I-J)</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%Sucrose</td>
<td>+2%Methyl parbene</td>
<td>3.47***</td>
<td>2.11 4.84</td>
</tr>
<tr>
<td></td>
<td>10%Date-palm juice</td>
<td>35.00***</td>
<td>33.64 36.36</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>22.67***</td>
<td>21.30 24.03</td>
</tr>
<tr>
<td>Water</td>
<td>+2%Methyl parbene</td>
<td>-19.19***</td>
<td>-20.56 -17.83</td>
</tr>
<tr>
<td></td>
<td>10%Date-palm juice</td>
<td>12.33***</td>
<td>10.97 13.7</td>
</tr>
</tbody>
</table>

*** Significant under the .001 level.
Figure 5: Effect of meal type on mating propensity of One day and 5 days old males of *An. arabiensis* (DONG colony):

**effect of meal type on mating propensity of males**

![Bar chart showing the effect of meal type on mating propensity of males. The chart includes data for 10% sucrose, 10% sucrose + 2% methylprbne, palm date juice, and water meal for both 5 days old and 1 day old males.](chart.png)
3.6 Results of Combined Factors (Period + Sex ratio + Meal Type):

A gradual steady decrease on insemination rate was observed in the 10%Sucrose+2%Methyl parbene group; from 92% in the 1st mating period, to 83.3%, 80.95%, and 61.9% in the 2nd, 3rd and 4th ones, respectively - Figure (6). On the other hand, in the same figure, there was an abrupt decrease on insemination rate from the 1st mating period (90.56%+9.6) to the 2nd one (72.59% ±6.7) in the group of 10%Sucrose. However, an increase on insemination rate was recovered in the 3rd mating period (80.95%±6.7) and in the 4th mating period (78.9%±6.51).

Numbers of dead males after each mating period were recorded for each replicate and are shown in Figure (7). Briefly, multiple regression analyses had shown a significant intercept for the Number of dead males and mating period on insemination rate in the methyl parbene replicate and in one replicate of the non-methyl parbene group- Table 11.

Table 11: regression statistics on the combined effects (No. of dead males+ mating period + meal type (DONG colony).

<table>
<thead>
<tr>
<th></th>
<th>DONG1 (+10%Sucrose)</th>
<th>DONG2 (+10%Sucrose)</th>
<th>DONG3 (+10%Sucrose)</th>
<th>DONG4 (+ methyl parbene2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R</td>
<td>0.93</td>
<td>0.56</td>
<td>0.75</td>
<td>0.95</td>
</tr>
<tr>
<td>R Square</td>
<td>0.86</td>
<td>0.31</td>
<td>0.57</td>
<td>0.90</td>
</tr>
<tr>
<td>Adjusted R Square</td>
<td>0.57</td>
<td>-1.07</td>
<td>-0.3</td>
<td>0.71</td>
</tr>
<tr>
<td>Intercept (t stat)</td>
<td>13.21*</td>
<td>3.98 a</td>
<td>7.34 b</td>
<td>12.32*</td>
</tr>
<tr>
<td>Mating period (t stat)</td>
<td>-2.23</td>
<td>0.21</td>
<td>-0.78</td>
<td>0.39</td>
</tr>
<tr>
<td>No. dead males (t stat)</td>
<td>1.92</td>
<td>-0.47</td>
<td>0.66</td>
<td>-2.23</td>
</tr>
</tbody>
</table>

* p<0.05; a. p=0.09; b. p=0.16.
Figure 6: Effect of combined factors (mating period + sex ratio + meal type) on mating success of male *An. arabiensis* (DONG colony)

<table>
<thead>
<tr>
<th>Mating Period</th>
<th>Dongola (D1,D2,D3)</th>
<th>DONG4 (+ methyl parbene)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>90.56</td>
<td>92.00</td>
</tr>
<tr>
<td>2nd</td>
<td>72.59</td>
<td>83.33</td>
</tr>
<tr>
<td>3rd</td>
<td>77.04</td>
<td>80.95</td>
</tr>
<tr>
<td>4th</td>
<td>78.90</td>
<td>61.90</td>
</tr>
</tbody>
</table>
Figure 7: Effect of combined factors (time + sex ratio + meal type) on mating success: No. dead males after each mating period.
3.7 Results of the Effect of Fluorescent dyes:

Similarly, successful mating attempts are recorded for the non-marked, and green and red marked females as shown in Table 12. On the other hand, unsuccessful mating attempts were dominant by the non-marked females.

Table 12: effect of fluorescent marker on mating success using three groups each contains 10 males + 10 females (all of 5 days old).

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>No. of ♀s inseminated</th>
<th>No. of ♀s not inseminated</th>
<th>Total No. ♀s dissected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Green</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Red</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>
Plate III: Fluorescently marked male:

(a) With the red dye.

(b) With the green dye:
Plate IV: Both fluorescently red and positively inseminated female

Plate V: Both fluorescently green and positively inseminated female
Plate VI: Fluorescently red but (negatively) not inseminated female

Plate VII: a three positive case: Red+ Green + inseminated female
Plate VIII: Contamination by the fluorescent powder; this is possible on other body parts but will not fake the case.
3.8 Results of Mating Competitiveness: Males of One day, 3 and 5 days old:

Males of One day old are uncompetitive to mate with the females in comparison to 3 and 5 days old males as shown in Table 13 (a). However, there is dominancy for the 5 days old males to unsuccessfully attempt to mate over the other two age groups - Table 13(b).

Table 13: mating competitiveness between 3 age groups of males (10 ♂s of One day, 10 ♂s of 3 days and 10 ♂s of 5 days old) on 10 ♀ of 5 days old in each replicate.

(a): Successful mating attempts:

<table>
<thead>
<tr>
<th>Colour + insemination</th>
<th>One day old</th>
<th>3 days old</th>
<th>5 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red coloured+ inseminated</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Green coloured + inseminated</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Non coloured + inseminated</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Total No.</td>
<td>0</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

(b) Unsuccessful mating attempts:

<table>
<thead>
<tr>
<th>Colour + insemination</th>
<th>One day old</th>
<th>3 days old</th>
<th>5 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red coloured + not inseminated</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Green coloured+ not inseminated</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total No.</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>
3.9 Results of Mating Competitiveness: Males of 5, 8 and 11 days old:

Males of 10 days old are uncompetitive to mate with the females in comparison to 5 and 8 days old males as shown in Table 14(a). However, numbers of unsuccessful attempts were less and equalled between the three age groups — Table 14(b).

<table>
<thead>
<tr>
<th>Colour + insemination</th>
<th>5 days old</th>
<th>8 days old</th>
<th>11 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red coloured+ inseminated</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Green coloured + inseminated</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Non coloured + inseminated</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total No.</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

(a): Successful mating attempts:

(b) Unsuccessful mating attempts:

<table>
<thead>
<tr>
<th>Colour + insemination</th>
<th>5 days old</th>
<th>8 days old</th>
<th>11 days old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red coloured + not inseminated</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Green coloured + not inseminated</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total No.</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
3.10 Observations on Mating and Swarming Behaviour in the Laboratory:

Swarms of *An. arabiensis* in the transparent mating cage have no definite shape. Complete darkness had set on at 18:02. However, before 20-30 minutes from the darkness the antennae of some males were erected. The swarming flight was started by 1-6 males in a chaotic form, moving every where in the space of the cage. The swarm was linked to a location inside the cage. However, when the maximum number of swarming males were flying (7-10 male), the swarm was fairly organized; back and forth movements of the males were routed through the swarming station. It was clear that not all the males are joining the swarm; some males had never erected their antennae and stayed resting on the cage walls. It was also common that some males depart from the swarm, rest for a while and then resume swarming.

Time-records of the swarming start; when 5 males joined the swarm and maximum number is in the swarm, and the ending of the swarm are shown in Table (15).

3.10.1 Observations on Coupling:

Over 22 days, 33 coupling pairs were watched. It was difficult to distinguish all detailed events of coupling behaviour which were described by Charlwood and Jones (1979). But the formation of the couple was a very rapid event (approximately 1-3 seconds), and the coupling pair moves out of the swarm. The narrowness of the cage space had left the coupling pairs with a very short time to last.
Table 15 : Time-records of swarming events observed in the laboratory during the period (June-August) 2005.

<table>
<thead>
<tr>
<th>Observation</th>
<th>Time Range of observation</th>
<th>No. observations(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of swarming</td>
<td>17:53-18:05</td>
<td>15</td>
</tr>
<tr>
<td>5 males swarming</td>
<td>17:53-18:07</td>
<td>22</td>
</tr>
<tr>
<td>Maximum No.</td>
<td>18:00-18:15</td>
<td>22</td>
</tr>
<tr>
<td>End of swarming</td>
<td>18:25-18:43</td>
<td>22</td>
</tr>
</tbody>
</table>

3.11 Results of Swarm Markers :

3.11.1 Observations:

Observations on swarming behaviour were extended from the hour 17:30 till the hour 18:30. Small numbers of males were seen in a flight of swarming in the three replicates; the number of swarming males was smaller in the cage of the dark swarm (N <6). There is no indicator in either group that swarm marker is discriminated by the flying males. Although some times they individually go and fly over the marker; but mainly the marker is in a site while the swarming males are flying in another site of the cage. Mating pairs were observed on two occasions: in the non-marker and in the white -marker cages.

3.11.2 Effect on Mating Success:

According to Table (16), presence of either black or white markers in the vicinity of the mating cage had made no difference on mating success of An. arabiensis in comparison to the non –marker group.
Table 16: effect of the swarm marker on mating success of *An. arabiensis*

<table>
<thead>
<tr>
<th>mating cage</th>
<th>No. of dissected females</th>
<th>No. of inseminated females</th>
<th>%Insemination rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-marker</td>
<td>24</td>
<td>13</td>
<td>54.2%</td>
</tr>
<tr>
<td>Black –marker</td>
<td>18</td>
<td>9</td>
<td>50.0%</td>
</tr>
<tr>
<td>White- marker</td>
<td>22</td>
<td>12</td>
<td>54.6%</td>
</tr>
</tbody>
</table>

### 3.12 Observations on Mating and Swarming Behaviour in the Field:

#### 3.12.1 Swarming Stations:

Surroundings of three breeding sites of *An. arabiensis* were explored for possible swarming stations. Two of the breeding sites have open surroundings; the first one was a large water pool splitting the riverbank while the second one was a broken water pipe lying on the east boundary of the main road. The third breeding site was also composed of a broken water-pipe, close to three inhabited house, which have no surroundings. Proliferation capacity of the latter one is huge compared to the first ones as this was estimated by the larval dipping method using trays. Interestingly, three swarming stations were only identified near the third breeding site. The first swarming station is 10-12 meters east of the breeding site. In this station, males were observed swarming at a height of 3-4 meters. This station is partly shaded by a palm tree lying 2 meters north of the station. There is no distinctive feature on the ground to be used by the swarming males as a marker i.e. “arena”, but on the south there is an irrigation canals.

The second swarming station lies 3-5 meters south of the breeding site; it was beyond the mud wall of a ruined building, on its northeast corner. Here the ground is elevated...
approximating the swarm in a less height of 2.5-3 meters. Regularly, this station was also used for swarming by the midges (Chironomids) earlier from 18:00-18:20 ± 3.3 (N=7).

Similarly, males were occasionally observed in the third swarming station. This station lies 3 meters west of the second station and 7 meters southwest from the first one. It is also beyond the mud wall over the adjacent west corner.

In addition to the mud fence, there is an assembly of rubbish on each corner of the building, which may be used as a swarming marker by the males.

Microscopic classification was made on random catches of swarming males, using sweeping net; this had shown that An. arabiensis was the only swarming species (N=57) in the three stations.

3.12.2 Observations: In most of the observations, a swarm was first formed at the 2nd station by one or two males, and then after 5-8 minutes 5-7 males swarm at the 1st station. The swarm in the latter is large in size (30-40 males), of a spherical shape and of more compact nature. Adversely, the swarm at the 2nd station is small in size (5-20) males, of a flattened shape and more illusive. During its occasional formation, the swarm at the 3rd station is similar in its features to the 2nd one. In fact, it always formed when the swarm of the 2nd arena exceeds its size (>20 males). Walking slowly beneath the 2nd swarm to the east or west direction had let the swarm to depart its station up to 2 meters, but after a while the swarm returned to its original place. It seems that relatively small numbers of females entered the swarm, and in one occasion a female was swept. Interestingly this female was found inseminated when it was dissected.

Time-records of swarming start; of 5 males, 10 males, of maximum number males joining the swarm, and ending of the swarm, over the three stations, are shown in Table 17. The
earliest time for males to start swarming was observed at 18:32 evening. The maximum number of swarming males was shown at 18:58:30 ± 00:04:30 at the first station; 19:02:30 ± 00:12:30 at the second station; and at 19:06:30 + 00:01:30 at the third station. However, complete darkness had set on at 19:05 preventing observation of the end of swarming.

Stunningly, five coupling pairs were watched at the first station during three days at 18:45, 18:47, 18:50, 19:00 and 19:05. The female enters the swarm; after 5-10 seconds the female is recognized by a male which grasped her and quickly both of them moved away of the swarm. The couple slipped down in a slow motion (up to 1 meter above the ground), then the couple stabilized and flew up and disappeared from the scene. In one observation, the couple had flown up before it had fallen down.

Table 17: Time-records of swarming events observed at the three stations (January 2006).

<table>
<thead>
<tr>
<th>Observation</th>
<th>Time Range of the observation (No.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1(^{st}) station</td>
</tr>
<tr>
<td>5 males swarming</td>
<td>18:40-18:49 (10)</td>
</tr>
<tr>
<td>maximum No.</td>
<td>18:54 -19:03 (10)</td>
</tr>
</tbody>
</table>

* Complete darkness had set on, preventing observation of the end after this time.
Plate IX: study site. Kaptood is a typical example of the villages of Dongola (70 km north), characterized by the date palm trees and the mud houses. *Anopheles arabiensis* breeds on either pools alongside the riverbanks or artificial sites such as breaks of water pipes, irrigation canals and water reservoirs of brick-making factories.
Plate X: Males of *An. arabiensis* swarms at a height of 3.5-4.5 meters above the ground in the first station (as shown by the tip of the sweeping net). The largest swarm was observed in this closed-aerial station; a maximum of 30-40 males had been shown swarming. The horizontal arrow points to the swarming site in the sky, while the vertical one indicates the breeding site.
Plate XI: The height of the swarm is shorter at the second station; 2.5-3.5 meters above the ground as shown by the tip of the sweeping net. However, altitude level was higher at this station compared to the first one. This station was also utilized by swarming chironomids 30-60 minutes before Anopheles swarms. The arrow points to the swarming site in the sky.
Plate XII: The third swarming station was near the second one; also associated with the mud fence. In the same picture, the angle of vision to watch the swarm is much easier by the upwind looking to the brightest point in the sky. At all the three stations, orientation of the swarm was always from west to east. The arrow points to the swarming site in the sky.
**Plate XIII:** catching of swarming males using sweeping net. Sucking tube (held on the left hand) is used to transfer live males to a paper cup (put on the wall). The swarm darts to the opposite direction when the sweeping net is smoothly moved through it. The swarming flight is disturbed after every capture, resulting in a lag time of (1-5) minutes to let the swarm resituate to the station. The arrow points to the swarming site in the sky.
3.13 Results of Susceptibility Tests:

Physical conditions recorded during the performance of the susceptibility tests are summarized below:

- Average Temperature at start of the tests = 24.4°C ± 1.5
- Average Temperature at end of the tests = 24.16°C ± 1.7
- Average Relative Humidity at start of the tests = 38.43% ± 2.8
- Average Relative Humidity at end of the tests = 35.22% ± 2.5

According to Tables 18 and 19, both females and males of *An. arabiensis* were confirmed susceptible to the four tested insecticides, respectively. There is no death in the control group observed in either test. Though, males are fully susceptible to DDT, Malathion and Permethrin than females (100% mortality rate). Bendiocarb is most effective to kill all the females of *An. arabiensis*.

**Table 18: Results of insecticide susceptibility tests on female *An. arabiensis***:

<table>
<thead>
<tr>
<th>Tested Insecticide</th>
<th>Control</th>
<th>Treated</th>
<th>Mortality Rate%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. replicates</td>
<td>No. ♀</td>
<td>No. ♀ dead</td>
</tr>
<tr>
<td>Bendiocarb</td>
<td>1</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>DDT</td>
<td>1</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Permethrin</td>
<td>2</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Malathion</td>
<td>1</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 19: Results of insecticide susceptibility tests on male *An. arabiensis*:

<table>
<thead>
<tr>
<th>Tested Insecticide</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. replicates</td>
<td>No. ♂ dead</td>
</tr>
<tr>
<td>Bendiocarb 0.1%</td>
<td>1 25 0 4 100</td>
<td>100 100 100.00%</td>
</tr>
<tr>
<td>DDT 4%</td>
<td>1 25 0 4 100</td>
<td>10 100 100.00%</td>
</tr>
<tr>
<td>Permethrin 0.75%</td>
<td>1 25 0 4 100</td>
<td>100 100 100.00%</td>
</tr>
<tr>
<td>Malathion 5%</td>
<td>1 25 0 4 100</td>
<td>100 100 100.00%</td>
</tr>
</tbody>
</table>

### 3.13.1 Knockdown Results:

Knockdown of 50% of the specimens for each insecticide had shown variation between males and females.

Equally, permethrin quickly knocked down 50% of both males and females during the first 15-20 minutes of exposure -Figure (8) and Figure (9). However, among the other three types of insecticides, 50% of the males were able to tolerate exposure to Malathion for a longer exposure period before knocked down (55 minutes for Malathion vs. 35 and 22 minutes for Bendiocarb and DDT, respectively). On the other hand, the females were able to tolerate DDT more than the other two insecticides (42 minutes for DDT vs. 35 and 22 minutes for Bendiocarb and Malathion, respectively).
Figure 8: Knockdown results of male *An. arabiensis* exposed to four types of insecticides versus exposure time intervals of 10 minutes (Mean ± S.D.).
Figure 9: Knockdown results of female *An. arabiensis* exposed to four types of insecticides versus exposure time intervals of 10 minutes (Mean ± S.D.).
Plate XIV: Susceptibility tests were conducted in the field laboratory of Dongola. Pupae were transferred to the adults-cage in order to emerge. Sexing of adults (separation of males and females) was done during the first 12 hours of their teneral life.
Chapter Four:

**DISCUSSION**

The success of mosquito SIT projects will significantly hinge on the fitness of sterilized males in the field, i.e. their ability to survive, locate a mating swarm, compete with other wild suitors, successfully couple with a female and finally inseminate her. It is argued that released sterile males should have at least a power of 70% of fitness relative to those wild ones it is meant to compete in order to sustain a success. Nevertheless, aspects governing male fitness are poorly understood and appear to be difficult to estimate, particularly in the field (Charlwood, 2003; Reisen, 2003).

The main objective of this study is to improve fitness of sterilized released males in terms of mating propensity and mating competitiveness. However, technologies of mass rearing, sterilization and release of the males might all have a negative impact on final field fitness, indicating that larger numbers of insects must be released than those predicted by simple models. Hence, over the past few years, there has been a growing realization that quality of the produced insects has to assume a much greater role in SIT implementation, and it will be a major concern for any mosquito release (Benedict and Robinson, 2003).

In the past and present, males are chosen for release, because release of blood-feeding females may increase disease risk by enhancing the rate of transmission. Though, there is a potential role of SIT to eliminate malaria burden in developing countries, this is much challenged by the less available knowledge on biology of male malaria mosquito, particularly mating biology (Benedict and Robinson, 2003, Knols and Scott, 2003; Ferguson et al., 2005).
The available literature on male biology reveals that most of the research work in this area was associated with the nineteen sixtieth and seventieth’ trials to use SIT as a tool of vector control. This situation implies that little funds have always been devoted to balance the gap of knowledge between the two sexes. Consequently, innovation of measuring tools and methods to study the biology of male mosquito was much limited. The denominator parameters used to measure mating success in the previous studies and this one is the calculation of insemination rate (after the dissection of spermathecae) and recording time of behavioural observations.

4.1 Age of Male and Mating Propensity:

An optimal age for the mass release of male malaria mosquito was identified in this study. After one day of emergence, all males are physiologically able to mate. These findings are in line with the fact that young males can be distinguished because their terminalia on emergence are un-rotated, a process which takes from 12 to 24 hours depending on temperature (Charlwood, 2003; Takken et al., 2004); thus no mating will be performed during this period by the male. However, it is unknown whether this unfitness on mating is only attributed to the morphological immaturity of copulatory organs of males or may be some mating traits are also to be acquired during this period (Takken et al., 2004). The above assumption should be considered since a significant difference between the one-day old males and other above age categories is shown in this study.

Another morphological attribute that limits mating ability of newly emerged males, was previously shown in *Ae. aegypti*. The antennal setae are folded along the shaft in a single
pencil. In this condition a mating response can be elicited by a loud sound of appropriate pitch (even though the insect is still incapable of mating, the terminalia not yet being fully rotated), but not by the female herself. If the flagellum is cemented to the pedicel the response is abolished (Roth, 1948).

These findings are in line with the previous studies, which found male insemination success varied with age (Reisen et al., 1979; Bock et al., 1983; Mahmood, 1986; Siddiqui et al., 1978; Verhoek and Takken, 1994). In 1978, Siddiqui et al. worked at laboratory settings on Clx. tritaenorrhyncus. Despite all males’ cohorts of ages (4-8) are 100% sexually mature, the maximum mating ability was recorded on the males of 5 days old. In 1979, Reisen and others had shown that mating activity of males of An. culicifacies was at the maximum on day 3 after emergence, followed by a rest interval, after which mating activity was again renewed on day 7. Under insectary conditions, Bock et al. (1983) had shown the greatest number of mating per day of males Clx. tarsalis, to occur between days 15 and 21 of adult life. In 1994, Verhoek and Takken showed in An. arabiensis and An. gambiae that males of 7 days old had higher insemination rates compared to other cohorts of tested ages.

From an evolutionary perspective, a female chooses a mate of high benefit to her fitness. In An. gambiae, Chambers and Klowden (2001) had shown that females have higher fecundity when mated with 2-day-old males than with older males.

Females, by contrast, are ready to mate almost as soon as they emerge from the pupal cases. Extraordinarily, in some species females are inseminated immediately following emergence by males which sit and wait next to the emergence site to pounce on any female as they are unfolding their wings, or they even grab the female pupa shortly
before emergence (Provost and Haeger, 1967). In most species, though, there is a 24–48-hour time lag between emergence and mating. Mating is not needed for egg development and maturation, but in most species, eggs can only be deposited when insemination has occurred (Clements, 1999).

4.2 Adding Ratios of Males and Mating Success:

The overall adult sex ratio is a key factor affecting sexual competition (Kvarnemo et al., 1995; Parker and Simmons, 1996). Current findings on male biased sex ratio are in line with the hypothesis that higher male density and more male-biased sex ratios should result in lower female resistance and thus increases both mating frequency and non-assortative mating (Arnqvist, 1992; Lauer et al., 1996).

On the other hand, the effective release numbers of sterilized males should not only be calculated in respect to the population size targeted by mosquito SIT but also in a respect to the mating ability of males. The lag on insemination rate between the sex ratio 3:1 over the sex ratios of 2:1 and the 1:1 ratio may raise a question of “why there are many males unable to mate even in the sex ratio of 2:1”; in other words, whether all males have the same innate propensity to mate under the same controlled conditions of the insectary.

Although mosquito male is physiologically able to mate with more than one female, (i.e. polyandrous), production of equal sex ratio of males and females at emergence (either from breeding sites in the field or from larval pans in the laboratory) practically results in monogamous females and monoandrous males.

In SIT project, male biased sex ratio is selected to overweigh wild males by sterile ones. Consequently, this male biased system causes a “tough” male to male competition;
awarding the female with a benefit of remating with fitter male. However, this assumption on the effect of sex ratio is controversial.

In the Medfly, Saul and McCombs (1993) had found substantial increase on remating rates both in male-skewed and female-skewed laboratory strains, compared with a standard strain. Gaskin et al. (2002) had shown that the number of male–male interactions in med flies was higher at more male-biased sex ratios, but whether this treatment augmented remating rate in the female was undetermined. Hendrichs et al. (1996) found no consistent differences in remating under different sex ratios (up to 10 males per female) in Medfly. Remating frequency in Drosophila melanogaster is affected by adult density in the field and laboratory, but the direction of this association is not consistent between strains (Gromko and Gerhart, 1984; Harshman et al., 1988; Ochando et al., 1996).

Typically, males devote less time and energy than females to offspring production and then have a higher potential rate of reproduction. Peculiarly, in only a few species where male parental input is relatively higher, females may compete for males if males vary in quality. As a result, the male makes mate choice; i.e. the courtship role is reversed. This abnormal trend was observed in thymine wasps (Alcock and Gwynne, 1987); in certain katydids Tettigoniidae spp. (Gwynne and Simmons, 1990; Gwynne, 1991) and in burying beetle Nicrophorus orbicollis (Robertson, 1993).

4.3 Experience, Remating and Mating Success:

Results of lengthening mating period imply that sexual conflict between females and males is a timely-paced phenomenon. A number of assumptions could be thought to
interpret this difference over time. It is possible that early mating experience inherited over the first night by males and/or females secured mating success instead of conflicting. Thus, experience of mating trials could modify the pre-copulatory behaviour of the male and female affecting "male's handling" for the female, or the female response to the "end-to-end flight". Otherwise, a pheromone may be emitted by the males and recognized by the female hampering her antagonistic response. In insects, early experience modifies the response to behavioural targets, affecting male's courtship and the female’s preference or response to specific visual targets or courtship, respectively (Hirsch and Tompkins, 1994).

Another possible assumption is that remating had occurred, implying that fitter males which are a subgroup of the tested males, had inseminated all the females. However, in remating incidents, the individual experience is most certainly involved (Pruzan, 1976; Markow, 1978; Ehrman, 1990; Kim et al., 1992, 1996). In *Drosophila melanogaster*, Markow (1978) found that females are able to distinguish between males that have never copulated and those that had a previous mating experience, showing preferences for virgin ones.

Certainly, the two assumptions of experience and remating may work together. In *Drosophila pseudoobscura*, Pruzan (1976) observed that copulatory experience modified frequency dependent selection according to genotypes of males. Thus, rare males enjoyed the greatest advantage with females previously mated with males of the same genotype as the rare one, indicating that females modify their behaviour as a result of previous experiences. This aspect of learning is suppressed when the female flies are fed a protein synthesis inhibitor (cyclohexamide) prior to observation (Pruzan et al., 1977). Variably,
Zawistowsky and Richmond (1985) proposed a very extraordinary postulation on abnormal competitive ability of mature and experienced males towards immature ones in *Drosophila melanogaster*, describing it as “a homosexual courtship activity”. This experience-dependent courtship modification seems to be closely associated with chemical contact stimuli emitted by immature males, i.e. pheromones (Vaias *et al.*, 1993).

**4.4 Differences on Mating Success between DONG and KGB Colonies:**

The higher insemination rates shewed on KGB colony in comparison to DONG colony should be discussed under the light of utilized F generations, these were from 51-55 in KGB, and from 21-25 in DONG. It is argued that within 3 generations of insectary rearing, field genotypes may be lost and assortative mating (i.e. a biased non-random mating with the sister females) occurs (Davidson *et al.*, 1970; Reisen, 2003); this phenomenon is not expected in wild mosquito and might minimize fitness of the released males compared to the wild ones. The present comparative studies on male mating biology using DONG and KGB colonies imply a very rapid evolution within the males. In 2004, Lima and others had shown during the colonization of *An. albitarsis* that the males not the females had evolved faster to adapt to the insectary conditions.

On the other hand, the present findings explicit how much the factory rearing technologies of controlled conditions might mask the behavioural traits of the males, appending another burden on SIT programs to use insectary- reared males in the release.
4.5 Sugar Meal Type and Mating Success:

Most of previous studies have focused on sugar feeding of females and how this affects their vectorial capacity (Okech et al., 2004; Spencer et al., 2005) with only limited interest in sugar feeding of males. In this study, four meal types were utilized separately as the sole source of food for males, providing them with the necessary energy to engage in mating activities. Five days and one-day-old males were compared in these experiments in order to investigate the effect of teneral reserves on mating ability of males.

According to the current experiments a meal of 10% Sucrose augmented mating success more than a meal of sucrose plus methyl parbene in either one of the two age groups. Recently, the insectaries at CDC and FAO/IAEA laboratories had adopted addition of 2% of methyl parbene to the 10% Sucrose in order to enhance life longevity of Anopheles in the insectary (Mark Benedict, personal communication). Thus, the above findings reassure the advantage of conventional use of 10% Sucrose in mosquito laboratories.

Markedly, feeding on water for five consecutive days did not restrain ability of males to mate; implying there is an effect of pre-eternal reserves on mating behaviour. However, this finding should be cautiously discussed as these experiments were run in the laboratory. In a study on Medfly, sterile males that were provided water and apples following four days of feeding on protein and/or sugar, were significantly more likely to copulate than their starved competitors who had access to water only (Yuval et al., 2002). It is important to emphasize that the four meal types were only selected in a purpose to prime the seeking of an optimal meal type, a meal that could result in the male fitness trading off its survivorship and mating competitiveness. Significantly, research on male
Anopheles meal is important for strategies in which males are released in the field with the aim to effectively compete with wild ones. Moreover, sugar feeding is routinely used for mosquito colony maintenance and the search for additional food sources that can be used in mass rearing is still in its infancy (Hood et al., 2006).

In Medfly, protein-fed males are more likely to copulate than sugar-fed or starved flies. Yuval et al. (2002) demonstrated that protein nutrition increases a male’s probability of emitting pheromone in a lek\(^1\) by the males. Proteins fed males were also found to engage in critical elements of close-range courtship.

In a mosquito SIT project, research on foraging ecology and behaviour of the male is an urgent demand; how sterile males locate food resources and how far these resources from swarming stations are just examples of research questions demanding answers.

### 4.6 Mating Success versus Survivorship:

Under the combining settings of meal, period and sex ratio, although, males fed on sucrose + methyl parbene had survived better than those on sucrose alone. Among all, the non-methyl groups were better mated than other groups through out the four mating periods. These findings confirm the results that methyl parbene is inadequate in terms of mating success; but on the other hand, it is adequate in terms of survivorship. The increase of mortality of males along the four mating period implies that there is an adverse interplay between survivorship and mating on the fitness of males. Therefore, further research-work is indispensable to obtain long-surviving and mating-competent males.

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\(^1\) Lek: a swedish derived term, used in animal behaviour to describe gathering of males of certain species of animal for the purposes of competitive mating display.
In 1982, Mahmood and Reisen dissected males of *An. stephensi* and estimated that between 40 and 60% of males had ejaculated the previous evening, which would imply a very low survival rate. In *Saltella sphondylli* (Diptera: Sepsidae), copulation *per se* was found to decrease the longevity of males but not females (Martin and Hosken, 2004).

### 4.7 The New Male-Marking Method:

Interestingly, a progress in the study of mating biology of male mosquito is now attained by using a new approach of male marking. While mosquito males were topically treated with fluorescent dyes on their abdominal segments close to the genital system, investigation for the marker was alternatively bounded to the females. In other words, to distinguish which male mated with the female, only the female was checked. This approach would be useful in studying mating competitiveness between the sterilized males and wild ones either in the field or in laboratory.

Monitoring the progress of SIT project is the hardest phase in the whole process. It is vital to monitor if the whole process of SIT is achieving its goals in terms of birth control. Moreover, SIT programs require a constant monitoring of wild matings versus sterile ones to adjust the release rate over time. Although a number of methods were utilized to evaluate whether or not SIT project is succeeding in eradication of targeted population, no observation had ever been recorded on the mating itself (sterile males with wild females). In fact, the routine monitoring methods of SIT programs extensively relied on sampling either of females from the field (which will be subject to the dissection of their genital systems), or sampling of fertile and sterile males (which will be investigated for the presence of a pre-release marker).
In the successful SIT eradication project of Tsetse fly at Zanzibar, young females (1-2 ovulations) were sampled. The presence of an egg in embryonic arrest at the uterus, or empty uterus (as a result of expulsion of a dead embryo), was taken as a proof for mating with sterile male (Vreysen et al., 2000). In the classical SIT eradication projects of screwworm Cochliomyia hominivorax in a number of countries around the world, the association of the fly with mammals had restricted its spatial mating. Profoundly, this characteristic had facilitated the monitoring process of the project (Myers et al., 1998).

In mosquito SIT project, monitoring of mating success is more complex, copulation lasts for a few seconds (not for minutes or hours like in Tsetse and med flies), and is accomplished at ambiguously less identifiable sites. Moreover, due to the poor understanding of male ecology, efficient male sampling techniques are lacked.

Literature on mating competitiveness of mosquito either in field-cages or laboratory, had shown a bias on results of captured couples (Ng’habi et al., 2005). It is impossible to distinguish whether retrieved data of non-inseminated females are attributed to the performance of investigators or these cases were of unsuccessful mating trials per se.

Short evaluation on the new marking method was carried out, encouraging dependence on this very applicable approach. Although not investigated here, a few drawbacks on using dusts had been reported in previous studies (Hagler and Jackson, 2001). For example, excessive amount of dust can kill the insect or produce adverse behavioural effects. In addition, dusts may inhibit normal dispersal behaviour (Chang, 1946) and decrease insect longevity (Sheppard et al., 1969; Reinecke, 1990; Messing et al., 1993). Moreover, persistency of fluorescent dusts in long-term studies is questionable (Hagler and Jackson, 2001). Another possible drawback on using the dusts is that their particles
can be transferred to unmarked insects in the field or in traps and sweep nets used for sampling (Miller, 1993). In this study, contamination by the fluorescent dusts, on other body parts such as thorax, was occurred in some females but did not fake the reading of unmated females. According to the above, there is a need to standardize the procedures of male marking; such as optimization of the amount of applied dust, selection of the tool of male marking and the handling method of males during the operation.

The specimens of green and yellow marked females, which were found non-inseminated, indicate unsuccessful mating attempts. This may be attributed to the effect of the marker, or the occurrence of antagonistic conflict between the two sexes after coupling.

The few cases of females marked with both markers (the green and red ones), and which were also inseminated, are very appealing findings. It is unclear if one of these cases is inseminated with the two types of males i.e. polyandrous; or she was inseminated with one male but unsuccessfully tried to mate with another one.

While no evidence of polyandry was found in An. dirus, An. maculatus (Baimai and Green, 1987), or An. messae (Novikov, 1981 cited in Clements, 1999). Low rates (1%-4%) of re-mating were found in females of both An. gambiae (Tripet et al., 2003) and An. freeborni (Yuval and Fritz, 1994). A higher rate of polyandry was exhibited by females of An. nuneztovari, of which 15% mated with at least two males (Scarpassa et al., 1992).

The frequency of insemination may be a key element in determining the success of vector control programs based on SIT. If a female mated with a sterile male is able to mate again with a wild one, this might threat the success of SIT in the elimination of the vector (Tripet et al., 2003).
A recent advance in SIT monitoring tools was reported by Hood et al. (2006); the stable isotope of carbon $^{13}$C. This isotope was evaluated in the laboratory as a potential chemical marker for *An. arabiensis*. Fixation of the isotopic label in adults was accomplished and detection of the label at an appropriate concentration was signaling up to 21 after the emergence. Integration of the latter and present approach in mosquito SIT projects may give the opportunity (for the first time) to monitor mating aspects.

4.8 Age of Male and Mating Competitiveness:

In the Medfly, sterile males are not reproductively mature when released (1-3 days old) and therefore need to survive several days in the field before they can mate and contribute to SIT programs (McInnis et al., 2002). In contrast and according to this study, sterile mosquito males are reproductively mature after one day of the emergence. Hence, in mosquito SIT project, the survival of released males will be essential to hurdle the elapsed time needed for acclimatization and finding of a mate. In addition, some experiments on mating competitiveness were conducted using the male marking approach. These experiments indicate that males in the range of (3-8) days old are more competitive to mate than the 1One days and 1-day-old males. A universal optimum age of mass release should consider among all fitness to couple and mate with the wild females. In a recent review on the necessity of research on male mosquito mating, the authors mentioned “*Were this to be found, releasing transgenic males of only this age class could ensure a greater reproductive return from each released individual, and thus minimize the number of releases required*” (Ferguson et al., 2005).
Certain males often enjoy a mating advantage over others. This differential mating success is modulated by two important mechanisms: male–male competition and female mate choice. It is believed that there is no direct female choice in aerial mating mosquitoes (Yuval, 2006). Although both mechanisms of sexual selection have been individually the focus of considerable research, a unified understanding of how this duet interacts remains elusive (Qvarnström and Forsgren, 1998).

Age competition among potential suitors is expected to facilitate female choice if this generates a mating outcome that is consonant with net fitness gains to the female, directly by increasing her fecundity and/or survival, as well as indirectly by awarding genetic benefits that will improve viability and/or attractiveness of the offspring (Wong and Candolin, 2005). However, it is argued that female’s mate choice may not work together with the male-male competition, ending her with a less fitting male. Thus, a further work would be important to investigate the interplay of the two mechanisms of sexual selection; in other words, “whether an intensive competition between young and old males affects the mating success rate”.

4.9 Mating and Swarming Behaviours of *An. arabiensis*:

Swarms at the three stations in Dongola were exclusively composed of *An. arabiensis*, these are in line with the fact that it is the predominant anopheline mosquito species at Dongola area (Dukeen and Omer, 1986). *An. pharoensis* had been scarcely reported to coexist, but not in this study (Lewis, 1956). Although larvae of *Culex* spp. were present in the breeding site adjacent to the swarming stations, no culcines were captured from these swarms. Most investigators have found swarms to be composed of males of a single
species, even when several species were swarming in the same area (Clements, 1999). However, mixed swarms have occasionally been reported in literature. In Tanzania, *An. funestus* swarmed at sites only marginally different from those used by sympatric populations of *An. gambiae* and *An. arabiensis* and overlapped with them to such an extent that mixed swarms were common (Marchand, 1984). A few females of *Cx. quinquefasciatus* were occasionally recorded in swarms of *An. gambiae* at Burkina Faso (Diabate et al., 2003). Mosquito species that have the same characteristics of swarming may form mixed swarms in sympatric areas but this does not mean that they will copulate. In view of the fact that males and females must come close before mating, a pre-copulatory mechanism (s) may work to prevent cross-mating.

4.10 Characteristics of the Swarms of *An. arabiensis* in the Field:

In 1940, Cambourance and Hill wrote on *An. atroparvus*: “the swarms usually form in the same place each evening but we have not been able to discover any factors influencing the choices of positions except that of proximity to the light day-time shelters and protection from the wind”. Amazingly, this dilemma is persisting as “the black box” in *Anopheles* biology -as Takken et al. (2004) had phrased. Unfortunately, at this part of the study we came to the same “stuck”: “what are the characteristics that enabled males to maintain these swarming stations”. Many authors were charmingly able to numerate many (rather in fact a lot of many) characteristics that could be utilized by the males to find swarming stations (Downes, 1969). Their conclusions were always indefinite, mixing these characteristics of some certainty with those of none at all. So, these characteristics of some certainty will only be considered in the current discussion.
Firstly, the swarming stations were near the breeding site, but which in turn near the inhabited houses (3-5 meters away from the swarming stations). On the counterpart, the other two-screened-breeding sites were far from the houses, and no swarms were observed there. Similarly, the centre of swarms in Tanzania, were observed close to the female feeding site rather than the breeding site (Marchand, 1984). Hence, swarms of *An. arabiensis*, in this area, should be sought not only near the breeding sites but also adjacent to sources of blood feeding. Although the anopheline male is a non-hematophagous (i.e. blood feeding) gender; it was shown –by accident-that males of *An. arabiensis* tend to rest indoors, (Charlwood *et al.*., 2001). While resting outdoor may involve a risk of death to the males through desiccation, indoor resting especially in man-made constructions may minimize such a probability (Gillies, 1988). Nonetheless, a further work is needed to find shelters where males rest during the daytime.

In Burkina Faso, swarms were observed about (5-10) minutes after sunset. A few males started dancing in a flight and other males gradually joined the swarm until the number reached several hundreds (Diabate *et al.*, 2003). The aggregations progressively dispersed as the light faded, 20-30 minutes after formation. Swarms were not formed over sites with obviously similar characteristics but most of them were localized close to cowherds. However, cows may not be essential determinants for swarm formation. In Tanzania, Marchand (1984) observed no discernible markers associated with swarms of *An. gambiae* and *An. arabiensis* which were found above flat, open ground within an area of scattered huts and trees. However, these mosquitoes use horizon markers for orientation and require a minimum angle of view of the sky. In The Gambia, two factors appeared to be important in determining the swarming area of anophelines: first, its openness and
second, features associated with the surrounding skyline (Charlwood and Jones, 1980). In Mozambique, swarms of male *An. funestus* were observed within the sandy clearings surrounding houses (Charlwood *et al.*, 2003). However, there was no obvious pattern than locations of the swarms, which where at the edge or in the middle of the clearing. These swarms occurred 2–4 m off the ground, occupied a similar volume, and appeared to consist of a similar number of insects. At some sites two or more swarms occurred within a few meters of each other. In this study, restitution of the swarms after the disturbance (by a sweeping net at all the stations or by a body walking beneath at the 2nd one), indicates that visual cues were the only ones involved in swarm maintenance at Dongola.

Secondly, swarming continued for (15-40) minutes in the field (*Dongola*) compared to (20-50) minutes in the laboratory. Although the difference on swarming duration between the field and laboratory may be a counterfeit (due to the difficulty of vision after sunset), swarming *per se* is a lengthy affair. In general, the genitalia of male mosquitoes are remarkably adapted for a rapid coitus and efficient insemination (Spielman *et al.*, 1974) rather than for species recognition or copulatory courtship (Yuval, 2006). Thus, most of swarming time is devoted to “advertise” mating location. On the other hand, swarming for this lengthy time requires foraging successfully during the previous night. In *An. freeborni*, a swarming bout of 30 minutes was found to cost the male more than 50% of its caloric reserves (Yuval *et al.*, 1974).
Thirdly, no vertical swarm marker was easily distinguished on the swarming ground. Males of *An. arabiensis* might use the surroundings of palm trees and/or mud walls as horizontal markers to find swarming sites. This notice is in line with that observed in Tanzania by Marchand (1984). There, the author wrote on the mixed swarms of *An. gambiae* and *An. arabiensis*; “Only rarely were swarms seen over low vegetation such as grass” and that “there was no indication of ground markers for orientation”. In contrast, in São Tomé, Charlwood and other colleagues (2002b) reported that all swarms of *An. gambiae s.s.* formed over markers, many of which were on low vegetation. In addition, swarming at the second station was started earlier than those ones at the other two stations indicating the males may approximate the two stations by the presence of the swarming males at the first one. Unfortunately, this assumption on “remote control” of “satellite” swarming stations by a master one was not investigated here, demanding a further attention at the future.

On the same trend, no effect was detected in the laboratory between the black and white artificial markers. It appears from the literature that swarms of *An. gambiae s.l.* may not form over a marker in eastern Africa, while they do in western Africa (Charlwood *et al.*, 2003). One possible explanation was suggested by the latter authors that the member of the complex observed in São Tomé (which was the M form of *An. gambiae*) is not found in Eastern Africa. Cytogenetically, populations of *An. arabiensis* in Sudan, are thought to be panmictic (i.e. unstructured, random-mating populations), demonstrating more similarities with the populations of westwards than those of eastwards from the Great Rift Valley (Petrarca *et al.*, 2001).
Another characteristic of the swarms in Dongola was the high elevation of (2-4) meters above the ground. Similarly, in São Tomé, males of *An. gambiae* (s.s.) and *An. funestus* (s.s.) had swarmed at least 2.5 meters, and sometimes 4 meters or more, off the ground (Charlwood *et al.*, 2002b). Swarming at different heights was thought to prevent inter-specific contact of males of *Cx. quinquefasciatus* and males of *An. gambiae* with the other partners. On the contrary, swarms of *An. gambiae* in Tanzania had occurred typically about 1 m above the ground (Marchand, 1984).

On the other hand, swarming of DONG colony in the laboratory was shown in very confined space (small cages of 50 cm dimensions). With no doubt, both findings on swarming elevation in the field and laboratory, may complicate the situation to release “stenogamic” sterile males (in which mating takes place in large swarms of 2 meters or above the ground) to compete eurygamic wild ones (in which mating takes place in a small cage).

### 4.11 Importance of Swarming Time in the Field and Laboratory:

Conventionally, a dimmer (automatic lighting machine) is utilized to artificially simulate dusk and dawn photoperiods inside the laboratory. A lag on the dusk time between the latter and field had resulted in an earliness of swarming in the laboratory (30-50 minutes before that in the field). Correct photoperiod entrainment in the mass-rearing factory is critical to SIT programs. In one release of *An. culicifacies* males failed to both egress from shelters and swarm at the correct time of the day, because they had been entrained on an aberrant midsummer insectary photoperiod (Baker *et al.*, 1980). Similarly, the dimmer should be reprogrammed to simulate the natural photoperiod of Dongola area.
Considerations should also be given to the seasonal variations of sunset and sunrise during the year. Although previous studies minimized the function of swarming of *An. gambiae* during dawn time (Gillies and De Meillon, 1968; Charlwood *et al.*, 2002b), a further work is required to explore the presence and magnitude of the dawn swarms at Dongola.

On the other hand, the erection of antennal fibrillae of males that were collected after the setting of darkness indicates swarming had lasted further time than expected. It was proved that eyes of anophelines are extremely sensitive to low light levels (Land *et al.* 1997).

### 4.12 Formation of the swarm :

It was rarely possible to see the first male at the swarm site; rather, several insects started together swarming at the same time. Thereafter, number in the swarm built up rapidly and within five minutes of the start, it had reached maximum size and density. Though, it was impossible to determine precisely the number of males in swarms or the average number of days a specific male would spend swarming. Since it is difficult to compare between the observed number of copulations and the number expected to occur, extrapolating the proportion of matings in the swarms was impossible in this study.

Although, small numbers of males were observed in these swarms (relative to previous observations on *An. arabiensis*) and that only four couples had been observed, the evolutionarily argument on the function of swarming as a mate finding mechanism (Nielsen and Haeger, 1960; Downes, 1969) remains. In other words, whether the swarming behaviour is a pre-request or a vestige to the mating success of *An. arabiensis*?
Obviously, the answer will much affect the success and monitoring of SIT mosquito project.

4.13 Coupling as a Subset of Mating Behaviour:

Although, many couples were observed in the laboratory compared to the field; observations on coupling event *per se* was more absorbing in the field than in the laboratory. Coupling had taken a shorter time in the latter implying a rapid ejaculation of sperms by the males inside the genitalia of female in the small cages. Further work should clarify if this difference between the field and laboratory strains causes assortative mating, and hence affecting the success of SIT project.

In 1979, Charlwood and Jones had video-filmed the coupling event of *An. gambiae s.l.*, they analyzed coupling into four stages. Unfortunately, no film was recorded in this study, but it is worth to quote their sketched diagram (Plate XV). They wrote describing the diagram: “The sequence is as follows, (a) A male approaches flying female; (b) The tarsal claw of the male’s forelegs hooks onto of the female’s legs, usually also foreleg; (c) The male swings under the female, quickly circles his abdomen up and his genitalia locate those of the female; (d) The male releases his hold of the female’s legs and adopts the end-to-end position. After 13-14 s, he vibrates vigorously from side to side; then, after further 2-3 s, the claspers open and he flies away. Copulation therefore lasts approximately 17s (mean 16.8 s, SD 2.6 s). During the initial process, flight is inhibited by contact, but in the end-to-end position, both insects resume flight”. However, their observation on duration of coupling may be particular to the settings under which they worked.
Plate XV: Diagrammatic representation of coupling sequence in *An. gambiae* (after Charlwood and Jones, 1979)
4.14 Importance of the Susceptibility Results in SIT Project:

SIT campaigns are usually initiated with a pre-release phase that involves population suppression methods, and then followed by sequential mass release of factory-reared and sterilized males (Dyck et al., 2005). Classically, insecticides are the main choice of population suppression in SIT programs. Hence, the main purpose of testing susceptibility of local population of *An. arabiensis* to insecticides is to explore which choice (s) of insecticide will be in hand to SIT suppression phase. In Unguja (Zanzibar), suppression of the Tsetse population was initiated in 1988 by both applying residual Pyrethroids as a pour-on formulation to livestock, and deploying insecticide-impregnated screens in some of the forested areas. This was followed by sequential releases of gamma-sterilized male flies by light aircraft (Vreysen et al., 2000).

According to the current results of susceptibility tests, either one of the tested insecticides (Bendiocarb, Malathion, Permethrin and DDT) could be utilized to suppress the malaria vector *An. arabiensis* at Dongola. However, the 100% effectiveness of Bendiocarb in killing both females and males deposits this insecticide as a first choice for the suppression phase of SIT.

For such complete suppression, one would have to use an efficient insecticide, which has no history of resistance in the proposed area. In fact, the history of insecticide resistance in Sudan dates back to 1970s when intensive use of Malathion at Gezira province had resulted in selection of resistance by *An. arabiensis* (Hemingway, 1983); *An. arabiensis* had also selected resistance against DDT and Dieldrin (Haridi, 1974), and Pyrethroids in
a number of country regions (NMCP, *Personal Communication*). On the other hand, the malaria control programme had never implemented Carbamates insecticides (such as Bendiocarb) in vector control policy at the Northern State (NMCP, *personal communication*).

Alternatively, the integration of SIT in vector control at Dongola area should be accomplished under the light of these findings. The rationalized use of insecticide at this area must be conserved in order to grant SIT project with a “multi-choice answer”.

A good scenario to improve mating success of sterile males could be imagined as following: Resistance to Bendiocarb would be artificially induced in the males by exposing reared colony to a high pressure of low concentrations of Bendiocarb (less than 0.1%). Inevitably, the insecticide pressure will induce genetic resistance in the offspring after a number of exposed generations. Thereafter, suppression of local population could be continued during the release phase, as the sterile males would be in advantage compared to the wild ones.

A computerized model developed by Vale and Torr (2005) predicts Tsetse control by SIT is inappropriate measure in terms of cost and effect. They assumed integrating SIT with insecticide use after suppression would be more effective than insecticide alone. However, one would require great confidence in the effectiveness of the suppression strategy to persuade malaria control programme to release resistant males at the project area; thus preventing effective use of the compound that is commonly employed in chemical control.

Another prospect to use the results of susceptibility tests in SIT project will be in genetic sexing. Genetic sexing allows propagation of males rather than females under one set of
selective conditions. While males of a few insect species can be separated by physical methods such as sexual dimorphism-based technique (Sharma et al., 1976; Alphey and Andreasen, 2002), these methods are inapplicable to anopheline mosquitoes, and rarely could give 100% sex separation (Alphey and Andreasen, 2002). Conversely, an effective method for anophelines involves selection of insecticide resistance in order to induce translocation and link the resistant gene to the Y-chromosome of the males. This genetic sexing method was employed in An. gambiae and An. albimanus (Curtis et al., 1976; Seawright et al., 1978). This system was successfully employed to produce a million sterile males per day of An. albimanus in Salvador (Dame et al., 1981).

In another perspective and regardless of variations on chemical and physical properties of tested insecticides, results of 50% knockdown would be useful to step further in inducing resistance to Malathion in the males of An. arabiensis.

4.15 Perspective: Community Concerns on the Use of SIT at Sudan

During 1970s, WHO and Indian Council of Medical Research (ICMR) initiated SIT project to eradicate three diseases vectors: Cx. quinquefasciatus, Ae. aegypti and An. stephensi (Pal, 1974). Although the educational programme gained public perception, a disastrous relationship arose with a certain section of the Indian media and politicians accusing the project of carrying out research supporting biological warfare (Sehgal, 1974; Jayaraman, 1982). Eventually, the community resistance imposed the Indian government to abandon this ambitious project.
During the course of this study and before that during the IAEA experts’ missions to the Northern State, I had frequently listened to the scary question asked by the public there:

“are you going to disperse unsafe irradiated mosquitoes in the area?” Although it is easy to react to the latter query, the theme is broadening to include other two queries: *How can we successfully convince the Sudanese community that this goal is desirable, feasible and can be accomplished safely? What could happen if SIT failed?*

One of the big challenges to implement SIT in Sudan is the community perception. Although the success of the mosquito SIT project will largely depend on the accumulation of scientific findings and advancement in relevant technologies to SIT, the local community in the Northern State will much determine a failure of such ambitious work. The building of public awareness and confidence is essential to develop implementation strategy that involves the end-user community at Northern State. The local community leaders should be involved such as to raise their awareness and build confidence about the benefits and risks of the use of SIT. The public should be equipped with the necessary means to be sufficiently knowledgeable in order to make informed decisions about the merits of deploying the project. The local community should easily find these means to include their inputs into the implementation phase of SIT.

On the other hand, assessment of risk factors should not only be confined to the health impact (on malaria transmission), but also to ethical, legal and social aspects of the use of SIT. The mechanisms to obtain individual and group consent specifically should be promoted for SIT as a public health intervention. Also, there is a need to translate risk-assessment procedures into local language(s) of the community (may be into *Nubian* language in case of Northern State). Moreover, local community in the targeted area
should have the choice to select releasing sites and plans for deployment, and in promoting and understanding real measures of success for the project (Macer, 2002; Toure et al., 2003).

For the second concern, a failure of SIT project would not be accepted at all for several reasons. Firstly, the tragic situation of malaria in Sudan urges a very prompt solution (shamefully 35,000 deaths occurs annually). This situation had imposed on NMCP to adopt effective strategies of prevention and treatment to combat malaria. ITNs strategy is among these preventive measures adopted by NMCP but hardly accepted by the community over the last decade. Therefore, suffering community may aggressively respond to a false hope of using SIT, as a lasting solution to malaria problem. Unless the risk of using SIT in malaria control is excluded, NMCP should not adopt uncertain hopes that may expose the Sudanese community to a worse situation.

Secondly, the targeted zone of SIT at Sudan overlaps with the Gambia project area that is a successful mosquito eradication co-project between Egypt and Sudan to eradicate malaria using vector control measures at a restricted bordering area. In this border zone, IRS has been successfully implemented to conserve the south of Egypt and north of Sudan free of malaria. However, the current technologies of genetic sexing and sterilization might not demand fertile females to be unreleased. The release of fertile females in this red zone may threaten the Gambiae project. Therefore, authorities of the two countries will never tolerate or accept probability of such a threat.

Thirdly, Sudanese scientific community is moving closer to conduct operational research servicing the public. Thus, a success of SIT project will ultimately result in further reliance on science in solving public problems. The Sudanese politicians are getting day
after day to realize that many troubles of this poor country are of scientific origin. The Sudanese scientists should play a central role in the development and future of our country.

**IMPLICATIONS OF THE STUDY:**

Important implications of this study on mosquito SIT project in terms of resources management and quality assurance may be summarized as follows:

1. **Mass rearing:**
   Behavioural traits, in particular mating traits, should be conserved –as much as possible - during the colonization of *An. arabiensis*. In a mass rearing settings, old generations must be substituted on a periodical basis with younger colonies derived recently from the field. Designing of adequate mating cages, isolation of production lines of different ages of mating and simulation of wild conditions of humidity, light and temperature are ways to minimize cost of acclimatization to the factory. Adequate source of sugar feeding should be sought not only with respect to mosquito survival but also with regards to mating capability of the males.

2. **Sexing, Sterilization, Packaging and transport:**
   Designs of the above methods and tools should be of least damaging effects on the males either in terms of morphological characteristics or in terms of behavioural traits. Regardless of employed methods, it is important to confirm mating fitness of the manipulated males. Further mating studies will be needed to compare sexed,
sterilized, packaged and transported males to manually separated, fertile, unpackaged and un-transported ones.

3. **Release:**

Selection of release time should be earlier than usual to ensure acclimatization of sterile males before they swarm. In this respect, the release should be during the most optimal conditions of humidity, light, temperature and wind velocity to the males. On the other hand, releasing sites should be selected in relation to the characteristics of swarming stations of *An. arabiensis* (near potential breeding sites and adjacent to human dwellings); and in accord to community choice. From a perspective of resources management, it is not a mandatory to release a fixed age; the age of the release could be pooled to a number of consecutive optimal days (e.g. 3-7 days).

4. **Mating Competitiveness:**

Improving mating competitiveness of sterile males could be attained only during the pre-release phase by combining factors affecting mating ability. “Fittest” sterile males would be of an optimum mating age, fed on ideal meal, and had a sort of experience to mate with the females (Is it sound to expose sterile males to mate with females before they are released?).

5. **Suppression :**

Suppression of local population of *An. arabiensis* before the release could be implemented using one of four tested insecticides. However, application of IRS of
insecticide is not ideal because sterile males may rest indoors. On the other hand, suppression after the release of sterile males could be accomplished using Insecticide Treated Nets (ITNs), which only target the blood-seeking females.

6. Monitoring:
The new male marking method would be a useful tool to monitor the progress of SIT project in terms of mating behaviour. Further work is needed to prove applicability of this method in the field (for how long the marker will be retained on the female body?). Furthermore, observing the swarming stations after the release, collecting swarming males and couples using sweeping net would be useful in the monitoring phase.

7. Addition:
Frequency of addition and addition rate at each release site should be defined in terms of mating competitiveness, survival in the field and dispersal from the release site. In addition to mating propensity of the sterile males, calculation of operational sex ratio must consider the addition rate of wild females.

8. Database:
Scarcity of literature on male mosquito biology urges a need to construct a database on this issue. Inclusion of comparative literature on other models of insects and animals in this database would be an invaluable procedure.
SUMMARY AND CONCLUSION:

1) Age of the male has an effect on mating propensity in *An. arabiensis*. Males were able to mate after they spent the first 24 hours after their emergence. However, there is a significant increase in insemination rate for the males of age above of one day old either in DONG colony or in KGB colony.

2) There is an effect of lengthening mating period on mating success. Elongating mating period to two, three and four nights had significantly augmented the mating success in DONG and KGB colonies compared to one night.

3) Male’s biased sex ratio has an effect on mating success. While a non-significant difference was shown in DONG colony when the number of males was doubled; a significant increase was recorded in both DONG and KGB colonies when the number of males was tripled, compared to the equal sex ratio group.

4) Meal type has an effect on the mating ability of the males. Feeding males of five days old from emergence on 10%Sucrose+2%Methyl parbene, water, or 10%Date palm juice had significantly reduced the mating success in comparison to 10%Sucrose. Feeding one-day-old males on 10%Sucrose resulted in a significant higher insemination rate over the other feeding meals.

5) Combining the factors of mating period, male’s biased sex ratio and meal type had influenced mating success. A decrease on mating success was observed in the
10% Sucrose + 2% Methyl parbene group; from 92% in the 1st mating period, to 83.3%, 80.95%, and 61.9% in the 2nd, 3rd and 4th ones, respectively.

6) A successful male marking method was described in this study. Fluorescent dyes were used to mark the males on their terminali segments. Females were checked for the fluorescent dye as a proof of a mating trial. Marked females were dissected to investigate whether mating trial succeeded or failed.

7) Using the new male marking method, experiments of mating competitiveness showed differences between different age groups of males. Males of One day old are uncompetitive to mate with the females in comparison to 3 and 5 days old males. Males of One days old are uncompetitive to mate with the females in comparison to 5 and 8 days old males.

8) There is no effect of swarm marker colours on swarming or mating success obtained in the laboratory.

9) Three swarming stations were identified in a village at Dongola near a breeding site. However, no ground marker was distinguished on any one of the three swarming stations at Dongola.

10) The earliest time for males to start swarming was observed at 18:32 hour evening. The maximum number of swarming males was shown at 18:58.30 ± 00:04:30 at the first station; 19:02:30 ± 00:12:30 at the second station; and at 19:06:30 + 00:01:30 at the third station.

11) Both sexes of males and females of *An. arabiensis* originated from the breeding site near the swarming stations are susceptible to 0.1%Bendiocarb, 4%DDT, 5%Malathion and 0.75%Permethrin.
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