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Behavioural, ecological and genetic determinants of mating and gene flow in African malaria mosquitoes

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Thesis

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> Abstract

Malaria is still a leading threat to the survival of young children and pregnant women, especially in the African region. The ongoing battle against malaria has been hampered by the emergence of drug and insecticide resistance amongst parasites and vectors, respectively. The Sterile Insect Technique (SIT) and genetically modified mosquitoes (GM) are new proposed vector control approaches. Successful implementation of these approaches requires a better understanding of male mating biology of target mosquito species. This thesis explored the potential behavioural, ecological and genetic determinants of mating and gene flow in the major African malaria vector *Anopheles gambiae s. l.*

This thesis specifically investigated (i) the effect of larval density and nutrition on the mating competitiveness of adult male *Anopheles gambiae s.s* mosquitoes (ii) compared the physiological fitness of male *Anopheles gambiae* mosquitoes between laboratory and field populations, (iii) the potential for establishing a self-replicating *Anopheles arabiensis* population in an enclosed semi-natural environment and observing its genetic variation over time, (iv) the development of a PCR-based method for assessing male mating success among inseminated female *An. gambiae* and (v) the population genetic structure of *An. gambiae s. l.* along the Kilombero valley (southern Tanzania).

Results indicated that environmental factors experienced during larval development, affect adult male mating behaviour. These factors can be experimentally manipulated during mass-rearing to enhance male mating success. Laboratory reared males are physiologically different from wild males. Field males have more lipids than laboratory-reared males. This thesis also reports the first ever establishment of an *Anopheles* population in an enclosed semi-natural environment. Conditions within the system were suitable for maintaining the genetic variation which is frequently lost under traditional laboratory cage conditions. The newly developed PCR-based method to assess insemination success will help to understand the mating biology and ecology of *An. gambiae* mosquitoes within the enclosed semi-field system. Results also show that the *An. arabiensis* populations of the Kilombero valley display a population substructure whereas *An. gambiae s. s.* showed no population substructure.

It is concluded that environmental factors, such as crowding and larval nutrition can be manipulated in the laboratory to enhance male mating success. Given the successful establishment of a semi-field mosquito population and the evidence that it does maintain more genetic variation than laboratory colonies, I suggest that studies aimed at exploring male mating biology /ecology and feasibility of SIT/GM can be executed in an enclosed semi-field environment. Information obtained in the semi-field system may be more representative of field mosquitoes than that from caged colony mosquitoes. The results of this thesis, therefore, provide useful information to strengthen current and future vector control approaches.

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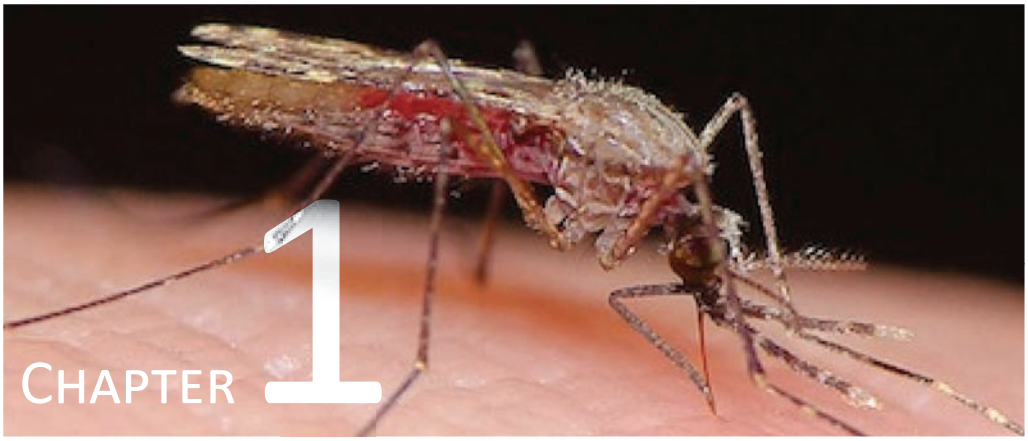


General introduction

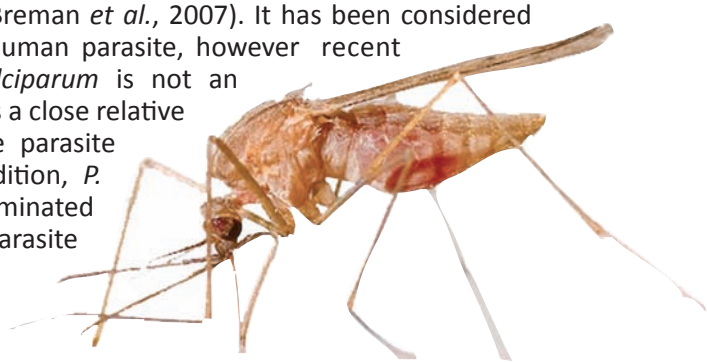
Ng’habi KR

The research reported in this thesis focused on the determinants of gene flow, and in particular the ecological, behavioural and genetic determinants of mating success, in African malaria mosquitoes. This introductory chapter aims to give an overview of the malaria burden, control efforts and the prospects for new vector control approaches. Then it gives an overview of ecological, behavioural and genetic determinants of mating success and gene flow in mosquito vectors.

Background

Malaria Burden

Despite intensified control efforts, malaria remains one of world's most burdensome diseases (Hay *et al.*, 2005; Hay *et al.*, 2004; Snow *et al.*, 2005). Malaria accounts for an estimated 240 million cases and 280, 000 deaths worldwide, with over 80% occurring in African region (WHO, 2009); the majority of these are children of under five years of age (WHO, 2008). Pregnant women are another vulnerable group as malaria causes serious adverse effects including abortion, low birth weight and maternal anaemia (Adam *et al.*, 2005; Newman *et al.*, 2003; Rogerson *et al.*, 2007). The economic toll of malaria has been high in Africa and has been linked with poverty (Sachs and Malaney, 2002). Malaria is caused by a protozoan belonging to the genus *Plasmodium*. There are five malaria parasite species of this genus that can infect humans. The first two includes the *P. vivax*, and *P. malariae.* For *P. ovale* recent evidence indicates that it may be consisting of two species (Duval *et al.*, 2009) and *P. falciparum* is the fifth malaria parasite*. P. falciparum* is the most deadly species responsible for most of the deaths occurring in Africa (Breman *et al.*, 2007). It has been considered that *P. falciparum* is a strictly human parasite, however recent studies have found that *P. falciparum* is not an exclusively human parasite and is a close relative of *P. reichenowi*, a chimpanzee parasite (Prugnolle *et al.*, 2010). In addition, *P. knowlesi* recently been incriminated as the sixth human malaria parasite (Prugnolle *et al.*, 2010; White, 2008a).



Malaria vectors

Only some seventy *Anopheles* species can vector malaria (Service, 1993). Of the 422 known *Anopheles* species, members of the *An. gambiae s.l.* Giles complex are responsible for malaria transmission in Africa (White, 1974). The *An. gambiae* complex consists of seven sibling species (Hunt *et al.*, 1998). Two members of the complex, *An. gambiae s.s.* Giles and *An. arabiensis* Patton are considered to be the major vectors of malaria in Africa. Although they are mostly found in sympatry, the two species differ greatly in their behaviour and physiology. *Anopheles arabiensis* is more zoophilic and exophilic (Gillies, 1955), and able to tolerate higher temperatures and lower humidities than *An. gambiae s.s.* (Kirby and Lindsay, 2004) whereas *An. gambiae s.s.* is more anthropophilic and endophilic (Gillies, 1955). As a result *An. arabiensis* is widely distributed in arid areas (Coetzee *et al.*, 2000) and more likely to change its behaviour in response to interventions such as residual insecticides than *An. gambiae s.s.* (Geissbühler *et al.*, 2007). *An. arabiensis* is therefore believed to replace *An. gambiae s.s.* (Lindblade *et al.*, 2006) in some areas and to be present as the sole vector of malaria in other places. Cytogenetic evidence has suggested that *An. arabiensis* is monomorphic for the 2La chromosomal arrangement whereas *An. gambiae s.s.* is a complex of five chromosomal forms, referred to as Savanna, Forest, Mopti, Bamako and Bissau (della Torre *et al.*, 2002; Touré *et al.*, 1998). These chromosomal forms are expected

to present a major challenge in a genetic control approach due to the existence of partial or complete reproductive isolation between them, e.g. for the Mopti, Bamako and Savanna chromosomal forms (Lanzaro *et al.*, 1998; Tripet *et al.*, 2003). While the three other members of the complex, *An. merus*, *An. melas* and *An. bwambae* have restricted ranges, the other two members, *An. quadriannulatus* subspecies A and B are not considered to be vectors of malaria as they are largely zoophilic. However, anthropophilic behaviour of *An. quadriannulatus* subspecies B was recently reported in Ethiopia (Pates *et al.*, 2006). In addition to *An. gambiae s.s.* and *An. arabiensis*, the *An. funestus* complex is another major vector in Africa and plays a significant role in malaria transmission (Cohuet *et al.*, 2004). *An. nili* and *An. moucheti* are the major vectors of malaria in West and Central Africa (Fontenille and Carnevale, 2006).

Malaria control

Malaria control advocates two major approaches, i.e. the control of parasites through the use of antimalarial chemotherapies (parasite control) and reducing human-vector contact (vector control). Based on these approaches, the WHO laid down a global malaria control strategy with the main goals to reduce world's burden of malaria to 50% by 2010, 75% by 2015 and also to have an effective vaccine by 2025. To achieve these goals, the WHO in partnership with the Roll Back Malaria (RBM) programme has developed the Global Malaria Action Plan (GMAP) which outlines a global framework for action to coordinate partnership's efforts with the vision for a substantial and sustained reduction of malaria burden in near, mid-term and total eradication in a longer-term. The GMAP outlines targets in order to reach this vision which includes; achieving universal coverage of control approaches, to reduce malaria cases by 50% in 2010 and 75% 2015, to reduce malaria deaths by 50% in 2010 and near zero in 2015, malaria elimination in some countries by 2015 and total eradication in long-term through progressive elimination in countries. The RBM has outlined three-part global strategies to achieve these targets; (i) to control malaria to reduce the current burden and sustain as long as possible (ii) to eliminate malaria over time country by country and (iii) researching new tools and approaches to support the ongoing control and elimination efforts (RBM, 2008).

Parasite control

Malaria treatment is aimed at parasite clearance besides alleviating illness, pains, and discomfort in infected individuals as malaria symptoms disappear quickly after malaria parasites are killed. Malaria is diagnosed by clinical symptoms, microscopic examination of blood smears as well as the use of rapid diagnostic tests (RDT) (Msellem *et al.*, 2009). Early diagnosis and prompt treatment is an appropriate approach in treating malaria (WHO, 2001) and is one of the four basic technical elements of the global malaria control strategy of the World Health Organisation (WHO, 1993). The challenge facing malaria efforts in Africa is the emergence of drug resistance among the deadly malaria parasite, *P. falciparum,* which has become resistant to chloroquine, the cheapest and most widely available antimalarial (Hastings *et al.*, 2002). Furthermore, resistance to sulphadoxine-pyrimethamine (SP), the cheapest alternative to chloroquine has emerged and spread through most African countries (Marks *et al.*, 2005; Mockenhaupt *et al.*, 2005). As a result, the WHO has recommended countries to change their treatment policies to advocate and distribute combination therapies which combine several drugs in an effort to slow down the emergence of drug resistant parasite strains

(Mutabingwa, 2005; Sutherland *et al.*, 2005). The use of Artemisinin Combination Therapy (ACT) has so far resulted in recommendable reductions in malaria morbidity and mortality (Barnes, 2005; Bhattarai, 2007). However, it is more expensive than the conventional monotherapies (Mutabingwa, 2005). ACT is now the recommended first-line treatment for falciparum malaria throughout the world. However, ACT-resistant *P. falciparum* has recently been reported in Cambodia and was suggested to be a result of the long-term use of artesunate monotherapies (Maude *et al.*, 2009; White, 2008b). Despite significant research progress, malaria vaccine development has also been slow, despite WHO's aim to have an effective vaccine ready by 2025 (WHO, 2006).

Conventional vector control

Malaria can radically be reduced or eradicated from an area by reducing mosquito populations, making vector control the best approach in malaria control (Okogun, 2005). Malaria transmission intensity (as indexed by the entomological inoculation rate (EIR)) is defined as the number of infective bites a person receives per given unit of time. Malaria models have shown that the EIR can be reduced through vector control (adult or larval control) and that this can reduce malaria transmission in a range of endemic areas (Killeen *et al.*, 2002). The current ongoing large-scale campaigns such as the Roll Back Malaria programme and the President's Malaria Initiative (PMI), promote the effective implementation of existing vector control methods such as insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) (Barnes, 2005; Bhattarai, 2007; Fegan *et al.*, 2007; Noor *et al.*, 2009). Other vector-control approaches focus on larval control using larvicides such as *Bacillus thuringiensis israelensis (Bti)*, a bacterium that produces toxins that are effective in killing mosquito larvae and which is used as a biological control agent (Fillinger *et al.*, 2003), albeit at a much smaller scale.

The use of ITNs is now a top priority in vector control in many African countries, advocating the use of long-lasting impregnated bednets (LLINs) as they have a long life span and do not need re-impregnation with insecticides. In order to maximise ITN coverage, some countries have adopted a voucher scheme to increase uptake in some risk groups including children under five years of age and pregnant women (Magesa *et al.*, 2005). Despite challenges such as distribution and socio-economic factors associated with their use, ITNs have resulted in substantial reductions in malaria mortality and morbidity (Fegan *et al.*, 2007; Noor *et al.*, 2009). However, the major challenge to this approach has been the emergence of vector strains that are resistant to the only class of insecticides approved for net impregnation (pyrethroids) (Greenwood *et al.*, 2008; Ringwald, 2007).

Larval control (larviciding) is another vector control approach (Figure 1). Larvicides control mosquitoes before they reach the adult stage, thus preventing disease transmission (Killeen *et al.*, 2002). In addition, larval control is effective at reducing malaria vectors compared to adult control because larvae have a lower mobility than adults which compromises their ability to avoid interventions such as excito-repellent insecticides (Darriet *et al.*, 2005). Although some studies have shown promising results (Fillinger and Lindsay, 2006; Fillinger *et al.*, 2009), the large-scale application of larvicides in Africa has been a challenge given the heterogeneity and extensive number of breeding habitats, which makes repeated treatments (e.g. after every 14 days) difficult and expensive to undertake (Killeen *et al.*, 2002; Knols, 2010; Majambere *et al.*, 2010). Dichloro-diphenyl-trichloroethane (DDT) has historically been the most widespread

Figure 1. Mosquito larva (Photo: Tibor Bukovinszky).

indoor residual insecticide for reducing mosquito populations, given its high effectiveness, durability and low costs (Mandavilli, 2006). Although the use of DDT was banned in the 1970's, the WHO announced the re-application of DDT in 2006 on a limited and controlled scale to maximize impact on malaria vectors (Mandavilli, 2006; Sadasivaiah *et al.*, 2007). The limited use of DDT in areas where it was approved for re-use, has shown tremendous reduction in mosquito densities (Curtis, 2002), even in areas of high pyrethroid resistance (Maharaj, 2005). However the possible impact of DDT on the environment and human health remains a major concern. Therefore the use of DDT for malaria control needs to be limited to avoid misuse and the development of resistance in major malaria vectors (Hargreaves *et al.*, 2003).

The current intensified malaria-control efforts have suggested the delivery of multi-intervention packages for vector control, which aims at controlling transmission. The WHO has therefore called for the promotion of integrated vector management (IVM), to combat neglected tropical diseases (WHO, 2008). IVM aims at the integration of different sustainable vector control methods that reduce the use of pesticides to the lowest level possible. Despite success in some areas (Chanda *et al.*, 2008), IVM is facing a number of difficulties including a lack of stable funding for mosquito control operations and the absence of well-coordinated malaria entomological information. This hampers the establishment of a centralised, high quality, information database and reference for monitoring vector control interventions. Although the IVM global strategic framework is designed to overcome some of these problems, in practice this still proves to be difficult (Beier *et al.*, 2008). However, IVM is a promising approach for an effective, environmentally benign and long-lasting malaria control. In addition to the existing IVM components, approaches that need to be included as part of IVM include the use of larvivorous fish to control mosquito vectors (Fletcher *et al.*, 1993; Marti *et al.*, 2006). Other new components that may be incorporated are being developed and are presented below.

Other vector control approaches

These includes the recent successful development of soil-borne entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana,* to infect and kill adult mosquitoes (Blanford *et al.*, 2005). Although the application of these fungi has proven to be successful in reducing the lifespan of adult wild mosquitoes (Scholte *et al.*, 2005), it is still not available for large-scale application and studies to optimise methods for production and application are underway to ensure effectiveness at the time of application (Farenhorst *et al.*, 2008; Mnyone *et al.*, 2009).

Genetic vector control

The emergence of vector and parasite strains that are resistant to insecticides and locally available cheap antimalarials, respectively, have increased the interest to develop new control approaches to supplement the existing ones in order to reach the millennium development goals with respect to malaria (Águas *et al.*, 2008; Feachem and Sabot, 2008; Greenwood *et al.*, 2008). Two genetic control approaches have been proposed. An established method is the sterile insect technique (SIT) (Dyck *et al.*, 2005). This method relies on the sterilisation and release of male mosquitoes into the field to mate with wild females causing a population crash (Dyck *et al.*, 2005). Male sterility is induced by ionising radiation or chemosterilisation, hybridisation or by chromosomal rearrangement (Knipling *et al.*, 1968). SIT is not a new technology and a number of SIT programmes have been successful in the past including the eradication of the tsetse fly, *Glosinna austensi* Newstead, in Zanzibar (Vreysen *et al.*, 2000) and the New World Screwworm *Cochliomyia hominivorax* Coquerel in Central and North America (Snow, 1988) and Libya (Lindquist *et al.*, 1992). Other ongoing SIT programmes include those to control the herbivorous Medfly *Ceratitis capitata* Wiedemann in America, Europe and Australia (Robinson, 2002). In mosquitoes, a SIT programme against *Anopheles* was undertaken in El Salvador in the early 1970's. The results were successful and promising. In this programme against *An. albimanus* Wiedemann, population suppression was achieved especially when the release area was limited (Benedict and Robinson, 2003). However, a number of SIT trials against mosquitoes were not successful and achieved no satisfactory results. For example the SIT release trial in Bukina Faso to reduce the *An. gambiae* population in 1968-1969 was not successful due to poor male mating competitiveness (Davidson *et al.*, 1970). Also the failure of the *Culex tarsalis* Coquillett SIT control trial in California (1981) was a result of negative assortative mating between released and wild strains (Reisen, 1982). Failure of most genetic control trials was associated with incomplete understanding of the mating biology of the target mosquito species (Benedict and Robinson, 2003; Ferguson *et al.*, 2005). Thus, comprehensive knowledge of the mating biology of the target mosquito species is required for successful genetic control (Benedict *et al.*, 2009; Ferguson *et al.*, 2005). Another promising genetic control method is the possibility of rendering wild vector populations refractory to parasite infection by releasing genetically-modified mosquitoes (GM). The creation of GM that are refractory to infection by malaria parasites (Catteruccia, 2007; Ito *et al.*, 2002) and dengue virus (Franz *et al.*, 2006) has fuelled the optimism that these diseases can be controlled by releasing a sufficiently large number of transgenic males into the wild that can drive the refractoriness genes into natural populations. It is suggested that gene-drive systems (e.g. transposable genetic elements) may allow relatively small releases of such transgenic laboratory-

reared mosquitoes to drive *Plasmodium*-refractory genes to fixation in wild vector populations, even if these genes confer substantially reduced fitness. This suggestion is still based on laboratory-based findings. The challenges to move GM technology into field application are discussed in this thesis.

SIT and GM area-wide genetic control methods are advantageous over the current approaches because they are based on the principles of area-wide integrated pest management (AW-IPM), non-reactive, and not insecticide-based (Hendrichs *et al.*, 2007). However, crucial to the success of these approaches is the ability of laboratory-reared transgenic and /or sterile males to compete successfully for female mates in the presence of their wild counterparts (Ferguson *et al.*, 2005; Kiszewski and Spielman, 1998; Scott, 2002). The African malaria vectors *An. gambiae* and *An. arabiensis* studied in this thesis are the principal vectors of malaria in sub-Saharan Africa (White, 1974) and the primary candidates for new genetic-based control programmes (Andreasen and Curtis, 2005; Ito *et al.*, 2002; Moreira *et al.*, 2004; Tabachnick, 2003).

There are five major steps to be considered in genetic control approaches (Knols and Louis, 2006); (i) laboratory colonisation of field-collected insect vectors, (ii) sex separation (iii) genetic manipulation, (iv) up-scaling (mass-rearing) of genetically manipulated insect vectors and sterilization (for SIT) (v) release of genetically manipulated or sterile males into target wild populations. Each of these steps poses its own challenges in terms of sustaining genetic diversity, reducing fitness and competitiveness due to colonisation, genetic alteration, sterilisation (for SIT), and mass-rearing. For example, laboratory colonisation of field-collected specimens has been shown to reduce genetic variability that imposes higher inbreeding depressions (Mukhopadhyay *et al.*, 1997; Norris *et al.*, 2001), reduces their competitive ability for female mates (Lounibos, 2007) and survivorship (Swindell and Bouzat, 2006), as well as adaptation to a changing environment (Reed and Frankham, 2003). This has also been shown during previous SIT trials where laboratory-colonised insects had low levels of mating competitiveness (Lounibos, 2003; Reisen, 2003). With regard to genetic manipulation and /or sterilisation a variety of mechanisms such as chemo-induced sterility (Lofgren *et al.*, 1974), cytoplasmic incompatibility (Laven, 1967), chromosomal translocations (Curtis, 1968) or meiotic drive (Hickey and Craig, 1966) have been tried with the post-release biology of released vectors as the major cause for variable successes (Takken and Scott, 2003). Mass rearing of insect vectors has been blamed to affect life-history components such as mating behaviour of emerging adults (Howell and Knols, 2009). This indicates that factors that affect adult behaviour which are experienced during the early developmental (aquatic) stages subject individuals to competition which in turn affects their mating competitiveness as adults. Since females can still transmit diseases even when sterile, only males are required for the release in a mosquito genetic control programme. Thus an efficient sex separation mechanism is required to achieve high effectiveness in an operational SIT/GM programme. A number of sex separation methods have been employed in mosquitoes including mechanical sex separation (Lowe *et al.*, 1980) and a genetic sexing strain (GSS) (Dame *et al.*, 1981), for example the W-chromosome linked dominant conditional lethal mutation (DCLM) which kills only females in Lepidoptera (Marec *et al.*, 2005). The most recent and promising technique is the separation of males expressing enhanced green fluorescent proteins using both manual and automated sorting machines (Catteruccia *et al.*, 2005).

Given the side effects imposed by male irradiation, other methods have been proposed as alternatives to irradiation including the release of insects carrying a dominant lethal gene (RIDL) (Thomas *et al.*, 2000). For example, when males carrying a tetracycline-repressible female-specific lethal gene are released to mate with wild females, all resulting female progeny dies. This is due to the fact that males and females carrying this gene can survive well under tetracycline supplemented medium but only males survive under normal medium (Heinrich and Scott, 2000). Although promising, it requires that the permissive condition (e.g. tetracycline) used for rearing the strain prior to their release, should not be present in the field for it to be effective (Alphey and Andreasen, 2002).

Prior to field release, the feasibility of genetic control approaches needs to be well evaluated in semi-field environments. Studies to understand the fate of genetic modification on male reproductive fitness need to be undertaken in enclosed semi-field systems to avoid unforeseen side effects that are irreversible after open field releases. Unless the reproductive quality of released males is maximised, it is likely that attempts to reduce malaria and other diseases through the release of genetically-modified and /or sterile males will not be successful.

Also, it is imperative that the genetic structure of target field populations be well characterised in advance before such releases are to commence. Sub-divided field populations will greatly hinder the introduction of parasite-refractoriness or sterility in field populations. Understanding of the population genetic structure will help to develop an appropriate implementation method for these strategies, e.g. multiple genetic modifications for sub-species that appear not to freely interbreed in nature such as mosquito chromosomal forms (Tripet *et al.*, 2005).

Therefore this thesis focuses on the study of behavioural, ecological and genetic factors that mediate sexual behaviour and ultimately drive male mating success. The reported studies focused on *An. gambiae* and *An. arabiensis*, under laboratory, semi-field and field conditions, with the aim to improve our fundamental understanding of what governs mating success in anophelines and how this can be manipulated. These insights will find practical application in the design and execution of genetic control programmes.

Determinants of mating success and gene flow

The most fascinating and important part of the evolutionary process in sexually reproducing animals is the way genetic information is transferred between individuals and from one population to another population of the same species. In this process genetic traits are transferred between individuals and passed between generations and then among populations. There are many factors that influence mating of insects. Factors such as size, age, physiological status and chemical cues influence mating behaviour, either singly or in a complex fashion. Many insects are readily receptive to cues that govern mating behaviour, resulting in copulation. These behavioural cues can also serve as pre-copulatory reproductive isolation barriers.

In contemporary genetic control programmes to control vector-borne diseases, the ultimate and most convenient mechanism to deliver the desired traits or genes into a target field population is through mating (Catteruccia, 2007). Mating is therefore the

only and ultimate approach for successful transfer of sterile or transgenic sperm from males to females - even if target genes are linked to efficient transposable elements (Tabachnick, 2003). Failure of such programmes will likely be attributed to insufficient understanding of behavioural and ecological processes that underpin male reproductive success (Clements, 1992; Reisen, 2003; Scott, 2002). The need to understand pre-copulatory isolation barriers is not only important because of their influence upon the genetic structure of wild malaria vector populations (Donnelly *et al.*, 2002) but will also will determine the potential future success of endeavours to prevent these mosquitoes from spreading malaria (Kiszewski and Spielman, 1998).

Behavioural determinants of mating success

Swarming behaviour is a fascinating mating strategy, deployed by many mosquito species. It has evolved repeatedly in various groups of insects (Downes, 1969; Sullivan, 1981), including old-world anopheline malaria mosquitoes, and is linked to mating behaviour that delivers reproductive benefits (Parker, 1978; Sullivan, 1981). Unlike communal courtship grounds (leks) where groups of males congregate on a fixed habitat feature or area and compete for a territory within it (Kruijt and Hogan, 1967), swarming occurs in a three-dimensional arena whose physical structure is determined by the flight activity and abundance of males (Downes, 1969; Yuval, 2006). A wide variety of insects, including dipterans, trichopterans, lepidopterans, hymenopterans, hemipterans, ephemeropterans, and odonates mate in swarms, which are typically maintained in a fixed position above a swarm marker (Downes, 1969). Females are attracted to a swarm from a relatively long distance (Clements, 1992). The long-range attraction cue that draws females to swarms is not yet known, but it is plausible that visual or olfactory detection of the swarm is involved. Approaching females are quickly mated and leave the swarm (Charlwood and Jones, 1980; Reisen *et al.*, 1977). Anopheline females are short-lived, lay multiple clutches of eggs after insemination and mostly mate only once (Shuster and Wade, 2003; Tripet *et al.*, 2003; Yuval *et al.*, 1994). These female characteristics make mating choice and mating competitiveness important components of female fitness, especially in anopheline swarms where male-male competition is high (Charlwood and Jones, 1980). With only few males having a chance to win a female mate in a swarm, it is difficult to understand how differences in male quality are recognised by females in such a competitive environment. Studies have suggested that male mating success within swarms is random (Charlwood *et al.*, 2002).

The limited studies on mating swarms have suggested that male mating success increases with phenotypic traits such as body size (Alcock, 1996; Blanckenhorn *et al.*, 2004; Crean *et al.*, 2000; Patridge and Faquhar, 1983) and energetic reserves (Blanckenhorn *et al.*, 2004; Yuval *et al.*, 1994), which are often direct correlates of other fitness measures such as survival and resistance to starvation and desiccation (Jennions *et al.*, 2001). Although studies in mosquitoes have yielded conflicting results, with some studies showing that large body size yields a mating advantage (Yuval *et al.*, 1993), others that mating is random with respect to size (Charlwood *et al.*, 2002), studies in other insect mating swarms have suggested that being small is advantageous as it can increase speed and maneuverability (Fyodorova and Azovsky, 2003; Lebas and Ritchie, 2004; Marshall, 1988; Neems *et al.*, 1998; Vencl and Carlson, 1998). These inherent and innate characteristics of mating biology in mosquitoes pose major

challenges to current approaches designed to alter either phenotypic traits (such as engineered refractoriness to disease parasites) of populations through replacement strategies or induce sterility as a population reduction strategy. Thus systematic experimental investigation and identification of what, if any, traits are correlated with male mating success in a mating swarm is needed, besides determining if these traits are positively or negatively correlated with other measures of male 'quality' such as survival and resource accumulation. This thesis aimed to investigate the differences in physiological status between laboratory and wild male mosquitoes.

Ecological determinants of mating success

In nature, mosquito larvae hatch and grow in a range of aquatic habitats, from small, temporary man-made pools, tree holes, to large natural swamps (Aditya *et al.*, 2008; Fillinger, 2004; Gimnig *et al.*, 2001; Munga *et al.*, 2005; Sota *et al.*, 1994). The number of larvae and the amount of food available in a particular breeding habitat varies from one site to another and from one physical location to another (Blaustein and Kotler, 2008; Ye-Ebiyo *et al.*, 2003). In the absence of predators and pathogens the number of larvae in a particular habitat and the amount of food available to them determines the number of adults that will emerge from it (Gimnig *et al.*, 2001; Ye-Ebiyo *et al.*, 2003) as well as their survival (Hawley, 1985; Ye-Ebiyo *et al.*, 2003) and body size (Gimnig *et al.*, 2002; Koella and Packer, 1996; Lyimo *et al.*, 1992). Higher larval density (crowding) results in greater competition for food and space (Ye-Ebiyo *et al.*, 2003), higher levels of toxic waste accumulation, crowding chemicals and physical interference from other larvae (Moore and Whitacre, 1972; Reisen, 1975; Roberts, 1998). Crowding also extends larval developmental time and reduces larval survival and weight at emergence (Arnaldo, 2009). While low food quantity in larval habitats during larval development results in reduced fecundity and growth retardation in adult females (Dominic *et al.*, 2005; Waldbauer, 1968), excess food results in poor larval survival (Arrivillaga and Barrera, 2004). The impact of these ecological/environmental factors on adult reproductive success has been well investigated in female mosquitoes (Arrivillaga and Barrera, 2004; Lyimo *et al.*, 1992; Mercer, 1999; Merritt *et al.*, 1992), no doubt due to their direct role in disease transmission. In this thesis the impact of these factors on male mosquitoes are studied.

Genetic determinants of gene flow

The success of the transgenic mosquito control approach will depend on the spread and ultimate fixation of genes that inhibit parasite development in field populations. This necessitates the need to understand the population genetic structure and level of gene flow within and between target mosquito populations. Characterisation of genetic linkages between wild vector populations is a two-step process that will first require characterisation of the nature and magnitude of genetic complexity itself within poorly described populations using an array of currently available markers. This is followed by measurement of the level of gene flow between those populations, which is critical because vector control strategies could be highly compromised by subdivided populations (Lanzaro *et al.*, 1998; Lanzaro and Tripet, 2003). Chromosomal inversion is one of the factors that plays an important role in disrupting genetic exchange among wild vector populations (Bush *et al.*, 1977; Coluzzi *et al.*, 2002; Kirkpatrick and Barton, 2006; Schaeffer and Anderson, 2005). It is a chromosome rearrangement in which a segment of a chromosome is reversed end to end. It has been considered to be

an important barrier to gene flow in many groups of animals through its effect on recombination suppression in heterokaryotype individuals (Bush *et al.*, 1977; Coluzzi *et al.*, 2002; Kirkpatrick and Barton, 2006; Schaeffer and Anderson, 2005). Chromosomal inversions decrease recombination in heterozygous individuals thereby limiting gene flow from one lineage to another which promotes population differentiation that may lead to speciation (Navarro and Barton, 2003; Rieseberg, 2001). This phenomenon has been well documented for West African malaria mosquitoes. Chromosomal inversion also dictates insect morphological traits such as body size (Colombo, 2004; Colombo *et al.*, 2001) which is a strong correlate of male mating success (Brockett *et al.*, 1996; Butlin *et al.*, 1982). Although this phenomenon has been reported in other insects, investigation is needed to assess whether and how this influences sexual selection and mating success in anopheline mosquitoes.

Another factor affecting gene flow between individuals and within mosquito populations is the colonisation process. Laboratory-colonised mosquitoes have been reported to maintain low genetic variation and experience more inbreeding depressions, leading to loss of fitness (Matthews and Craig, 1987; Mukhopadhyay *et al.*, 1997; Norris *et al.*, 2001). Inbreeding depression is defined as reduced fitness of a population resulting from breeding closely related individuals (Swindell and Bouzat, 2006). Inbreeding is known to affect insect reproductive fitness, by reducing mating ability as well as reducing adult survivorship (Snoke and Promislow, 2003; Vermeulen and Bijlsma, 2004). The effects of inbreeding on survivorship are in line with the mutation accumulation theory of senescence which states that deleterious effects of 'aging genes' are limited to late age stage (Snoke and Promislow, 2003; Swindell and Bouzat, 2006). The consequence of inbreeding depression are much more severe in smaller laboratory maintained insect colonies where genetic exchange occurs among a limited number of individuals and the environmental conditions are homogeneous (Matthews and Craig, 1987; Mukhopadhyay *et al.*, 1997; Norris *et al.*, 2001). Contrary to the above, individuals in heterogeneous environmental conditions have the opportunity to occupy and adapt to a range of microhabitats which then determine the total genetic diversity of the local population (Gram and Sork, 2001; Nevo, 1978). Therefore, inbreeding depression can affect a population's ability to adapt to and survive in a changing environment. Understanding the impact of colonisation and inbreeding depressions is not only of interest to evolutionary biologists but also has direct practical implications for genetic control programmes. The development of new molecular approaches to render wild insect populations refractory to parasite infection alone (Blandin *et al.*, 2004; Ito *et al.*, 2002; Osta *et al.*, 2004; Shahabuddin *et al.*, 1998) does not guarantee the success of such campaigns. A comprehensive knowledge of genetic factors that affect mating competitiveness and a mechanism to avoid such genetic artifacts in laboratory colonies is required to improve protocols designed for mass rearing and release of genetically-engineered or sterile mosquitoes for disease control programmes.

Furthermore, mutations affect an individual's fitness. Natural selection therefore, tends to act on the available mutations among individuals by allowing advantageous mutations to survive and multiply and disadvantageous mutations to die out. Some of the mutations, in disease vectors and other pests, include those which make them resistant to insecticide (Aminetzach *et al.*, 2005; Berticat *et al.*, 2008; Tripet *et al.*, 2007). Recent studies have shown that insecticide resistance imposes a fitness

cost in mosquitoes by reducing male mating competitiveness (Berticat *et al.*, 2002) and fecundity (Bourguet *et al.*, 2004). Depending on the mechanism of resistance, other studies have reported the absence of fitness costs related to insecticide resistance (Bielza *et al.*, 2008; Okoye *et al.*, 2007) further studies are needed to understand the impact of insecticide resistance on male mosquito mating success. Furthermore, irradiation is another source of mutation that affects mosquito fitness. It has been shown that, irradiation to induce sterility in male mosquitoes may reduce their mating ability and survival (Andreasen and Curtis, 2005; Helinski *et al.*, 2008).

Research objectives and problem definition

The central focus of the research presented in this thesis was to study the reproductive biology of two key African malaria mosquito species that serve as candidates for new proposed genetic control approaches. The project aimed to investigate behavioural, ecological and genetic factors that are important components of African malaria mosquito mating biology.

In general, mating success is governed by biological parameters such as body size and energetic reserves that are dictated by either environmental factors such as resource availability and crowding effects experienced during the early larval stages or genetic forces operating during adult life. The impact of these parameters has been well investigated in female mosquitoes due to their role in disease transmission. How these factors affect male mosquito fitness, however, remains poorly investigated (Ferguson *et al.*, 2005). For example, little is known about the effect of larval density, larval nutrition as well as adult energetic reserves on male mating competitiveness. These aspects were studied in this thesis.

Male mating competitiveness in genetic control is considered as the ability of a male to win a female mate in mating competition. That is, the ability of a transgenic or sterile male to out-compete a wild male and inseminate a wild female mate. Mating behaviour of *Anopheles* mosquitoes is unique because they mate in aggregations (mating swarms) which are time (dusk) and space limited (specific markers, as point sources in a landscape) (Charlwood and Jones, 1980). The inherent difficulty in assessing the mating status of females in the field would also implicate difficulty in assessing insemination success during feasibility studies in contained semi-natural environments; thus, one of the objectives of this thesis was to develop a PCR method to assess male mating success among inseminated females. Apart from being robust, the PCR technique allows the screening of freshly collected and long-time collected (dry) specimens.

Even though laboratory-based studies can give valuable information needed for genetic control programmes, laboratory-maintained colonies have been reported not to be representative of field populations as they maintain low extant natural variation compared to their field counterparts and higher inbreeding depression (Matthews and Craig, 1987; Mukhopadhyay *et al.*, 1997; Norris *et al.*, 2001). Because most genetic control approaches are limited to laboratory findings, the best way to move these laboratory findings towards field application is to assess the feasibility of such approaches under semi-field conditions. One of the objectives in this thesis was to simulate field conditions in an enclosed semi-field system (Knols *et al.*, 2002; Knols *et al.*, 2003) and to establish a self-replicating *An. arabiensis* population. Additionally

the genetic variation of colonies maintained in cages and a semi-field environment was compared over time to that of the field population from which the former two groups originated.

Prior to any release the genetic structure of the target population should be well characterised, and it should be assessed whether there are genetic barriers that may prevent mating between released males and target wild females. Genetic factors such as chromosomal inversions and mutations are also responsible for reproductive isolation among field populations. This thesis characterised the genetic structure of *An. gambiae s.s.* and *An. arabiensis* populations in the Kilombero valley of Tanzania. This is an area with intense malaria transmission where, during the course of this project, a large semi-field system to study the behaviour, ecology, and genetics of malaria vectors was constructed (Ferguson *et al.*, 2008). This thesis therefore, will provide answers to the following questions; Do environmental conditions experienced during larval development impact male mating success? Do free-living males differ in their physiological fitness from those maintained in the laboratory? Can we establish an intermediate, self-sustaining study population in a confined near-natural environment? What is the nature and extent of genetic variation in *Anopheles* mosquitoes when maintained across a continuum of environmental conditions, i.e. from laboratory cages to their natural distribution? What is the nature of the genetic structure of *An. gambiae s.s*. and *An. arabiensis* populations along the Kilombero valley?

The specific objectives of the work presented here were to:

1. Determine the effect of larval density and nutrition on the mating competitiveness of adult male *Anopheles gambiae s.s* mosquitoes.
2. Compare the physiological status between male *Anopheles gambiae* mosquitoes from a laboratory and field population.
3. Establish a self-replicating *Anopheles arabiensis* population in an enclosed semi-natural environment and compare its genetic variation with that of its source field population.
4. Develop a PCR-based method for assessing male mating success among inseminated female *Anopheles gambiae*.
5. Characterise the population genetic structure of *Anopheles gambiae s. l.* along the Kilombero valley and assess the forces responsible for the genetic structuring of mosquito populations.

Thesis outline

Chapter 2 The density of larvae in natural breeding habitats is not homogeneous, such that some habitats are highly crowded and others less crowded. Crowding results in individuals with small body sizes. In this chapter the effect of larval density on the emerging adult energetic reserves, body size and mating competiveness of male *Anopheles gambiae s. s*. is reported.

Chapter 3 Besides density, the amount of food present in a breeding habitat determines the body size and energetic reserves of emerging adults. This chapter presents the effects of larval diet on the body size, energetic reserves, and mating competitiveness of male *Anopheles gambiae s.s.*

Chapter 4 Laboratory colonisation impacts the genetic variation of colonised individuals. This chapter compares the physiological status between free-living and laboratory colonised male *Anopheles gambiae s. l*.

Chapter 5 The fate of introduced genes prior to field application can be simulated in a self-replicating vector population established in a semi-natural environment. This chapter presents the successful establishment of an *Anopheles arabiensis* population in an enclosed semi-natural environment.

Chapter 6 Crucial to genetic control feasibility studies is the ability to assess mating success of released males. This chapter presents a new PCR-based method to determine male mating success among inseminated females of *Anopheles gambiae s. l.*

Chapter 7 Mosquito populations maintained under semi-field conditions are suggested to be more representative of field populations than those maintained under standardised laboratory conditions. This chapter presents the comparison of genetic variation between semi-field, field and laboratory-maintained populations of *Anopheles arabiensis.*

Chapter 8 Prior to release, the population structure of a target population needs to be well understood. In this chapter, the population genetic structure of sympatric *Anopheles gambiae s. l.* populations of the Kilombero valley was examined.

Chapter 9 Chromosomal inversion plays an important role in population isolation and speciation. This chapter investigated the application of a 2La molecular karyotyping method in the *Anopheles gambiae s. l.* mosquitoes of the Kilombero valley, Tanzania.

Chapter 10 General discussion: The results of all chapters are discussed, integrated and conclusions synthesised, specifically with regard to the future application of genetic control techniques.

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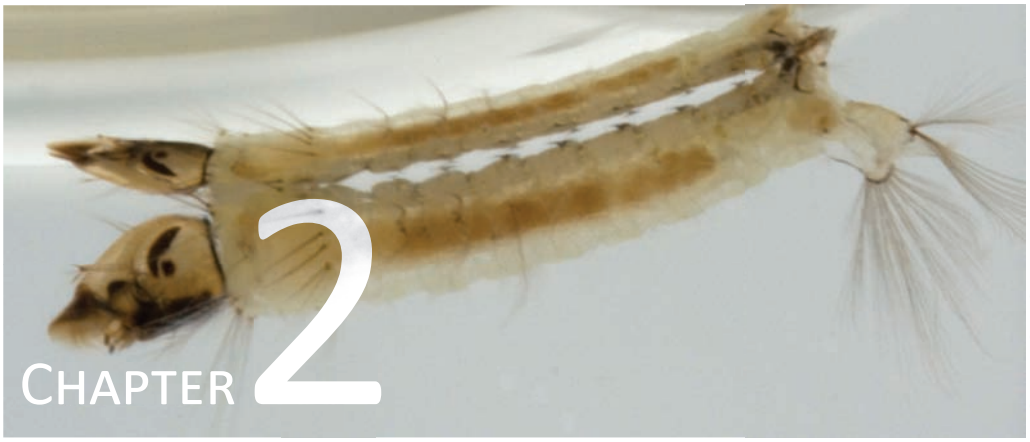

CHAPTER 2

TIBOR BUKOVINSZKY

The effect of larval density on the mating competitiveness of adult *Anopheles gambiae* mosquitoes

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> **Abstract**

The success of sterile or transgenic *Anopheles* for malaria control depends on their mating competitiveness within wild populations. Current evidence suggests that transgenic mosquitoes have reduced fitness. One means of compensating for this fitness deficit would be to identify environmental conditions that increase their mating competitiveness, and incorporate them into laboratory rearing regimes. *An. gambiae* larvae were allocated to three crowding treatments with the same food input per larva. Emerged males had to compete with one another for access to females, and their corresponding longevity and energetic reserves measured. Males from the low crowding treatment were much more likely to acquire the first mating. They won the first female approximately 11 times more often than those from the high crowding treatment (Odds ratio = 11.17), and four times more often than those from the medium crowding treatment (Odds ratio =3.51). However, there was no overall difference in the total number of matings acquired by males from different treatments (p= 0.08). The survival of males from the low crowding treatment was lower than those from other treatments. The body size and teneral reserves of adult males did not differ between crowding treatments, but larger males were more likely to acquire mates than small individuals. Larval crowding and body size have strong, independent effects on the mating competitiveness of adult male *An. gambiae*. Thus manipulation of larval crowding during mass rearing could provide a simple technique for boosting the competitiveness of sterile or transgenic male mosquitoes prior to release.

Introduction

Mosquitoes within the *Anopheles gambiae* species complex are the most important vectors of malaria in sub-Saharan Africa (Gillies and DeMeillon, 1968; White, 1974). The infective bite of these mosquitoes is in large part responsible for an estimated 240 million cases and 280, 000 deaths worldwide, with over 80% occurring in African region (WHO, 2009); the majority of these are children of under five years of age (WHO, 2009). Currently, the two most widely implemented vector control strategies are indoor residual insecticide spraying and insecticide-treated bednets (ITNs), both of which have proven effective in the reduction of malaria transmission in some areas (Alten *et al.*, 2003; Hawley *et al.*, 2003; ter Kuile *et al.*, 2003; Wiseman *et al.*, 2003). However, multiple insecticide resistance is emerging amongst the major malaria vectors *An. gambiae* (Yawson *et al.*, 2004) and *Anopheles funestus* (Brooke *et al.*, 2001), and there are complications associated with introduction, distribution and proper use of ITNs (Curtis and Maxwell, 2002; Lengeler, 2004) that indicate these strategies alone may not be sufficient to eliminate malaria transmission. New tools aimed at stopping malaria development in humans are promising, but the development of an efficacious antigen for vaccine production is slow, and parasite resistance to locally available drugs is increasing whilst new drugs that are effective are often unaffordable (WHO/UNICEF, 2003).

One promising new control prospect is the possibility of rendering wild vector populations less susceptible to infection by releasing mosquitoes that are genetically modified to resist infection (Benedict and Robinson, 2003), or sterile males that will mate with wild females and stop them from reproducing (Kiszewski and Spielman, 1998; Mshinda *et al.*, 2004; Tabachnick, 2003). In the case of a genetically modified mosquito (GMM) strategy, malaria could be reduced by fixing a resistance gene in vector populations, (Benedict and Robinson, 2003; Ferguson *et al.*, 2005), and in the case of sterile male release, malaria could be cut by a collapse in the vector population due to a high frequency of unviable matings. Any such release of sterile or GM mosquitoes should consist only of males (Knols *et al.*, 2002; Scott *et al.*, 2002) because this sex does not blood feed, and thus they will not increase the number or nature of mosquito bites per person at release sites. The success or failure of a GMM or sterile programme will depend largely on whether released males can successfully compete for mates against wild males (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Moreira *et al.*, 2004). Current evidence from laboratory experiments suggests that GMMs have reduced competitiveness and are generally out-competed in the presence of unmodified laboratory-reared males (Benedict and Robinson, 2003; Ferguson *et al.*, 2005). Operationally, the consequences of releasing males with poor competitiveness are dire. For example, the general failure of mosquito control programmes launched in the 1970s that aimed to reduce vector populations by releasing sterile males can be largely attributed to their poor mating competitiveness (Boëte and Koella, 2002), and to a lesser extent, the dispersal of fertile males into control areas. In the case of GMM, some argue that even if modified males have lower fitness than the wild type, refractory genes will still spread provided they are linked to an efficient genetic drive mechanism (Ferguson *et al.*, 2005). However, such a drive mechanism could only act if insemination occurs in the first place (Gould and Schliekelman, 2004; Riehle *et al.*, 2003; Tabachnick, 2003), which it may not if GMM competitiveness is very low.

Furthermore, no efficient genetic drive mechanism has yet been identified for *Anopheles* and even assuming one, there are doubts about whether it could be tightly linked to a potentially costly resistance gene (Ferguson *et al.*, 2005). The enhancement of male competitiveness thus remains crucial for successful gene introduction. Gaining an understanding of the ecological factors that govern *Anopheles* mating biology in general, and promote male competitiveness in particular, will increase the chances of success of future GMM and sterile male-based control efforts (Agnew *et al.*, 2002; Hawley, 1985; Koella and Lyimo, 1996; Lyimo *et al.*, 1992). One ecological factor known to have a great influence on the life-history of adult Anopheline, Culicines and *Aedes* mosquitoes is the density at which larvae develop (Fillinger, 2004). In nature, larvae of *An. gambiae* hatch and grow in a range of aquatic habitats (Gimnig *et al.*, 2002; Ye-Ebiyo *et al.*, 2000). In the absence of predators and pathogens, the number of larvae in a particular habitat and the amount of food available to them determines the number of adults that emerge from a habitat (Hawley, 1985; Ye-Ebiyo *et al.*, 2003), their survival (Koenraadt *et al.*, 2004; Lyimo *et al.*, 1992; Schneider *et al.*, 2000) and body size (Ye-Ebiyo *et al.*, 2003). Crowded larvae are thought to be at a disadvantage because they are faced with greater competition for food (Koenraadt *et al.*, 2004; Koenraadt and Takken, 2003), and are exposed to higher levels of toxic waste products, crowding chemicals and physical interference from other larvae (Koenraadt and Takken, 2003; Lyimo *et al.*, 1992; Mercer *et al.*, 2005).

Whereas the importance of larval density to female Anopheline and *Aedes* mosquitoes has been broadly investigated (Gomulski, 1985; Koenraadt and Takken, 2003; Schneider *et al.*, 2004), no doubt prioritized because of their direct role in disease transmission, little is known about its consequences for male mosquito vigour. Of the few known studies (in *Anopheles* and *Aedes sp*.) that have considered how larval density could influence male development (Briegel, 2003; Holliday-Hanson, 1997) their focus has been on the effect of food limitation, not that of chemical or physical interference.

Here the effect of larval crowding on the mating competitiveness of adult male *An. gambiae* was investigated. The focus was specifically on the effects of crowding in larval habitats, not on food limitation, which was controlled for by providing each larva with an equal amount of food per unit time. Crowding was prioritized for study because, space rather than food was believed to be the biggest limiting factor when mass-producing transgenic or sterile mosquitoes for field release. In addition to conducting mating assays, the teneral reserves of males from different crowding conditions was also quantified to test if any observed differences in competitiveness could be explained by energetic limitation. Energy reserves influence mosquito behavioural activities such as swarming and feeding (Scott, 2002), and may vary in response to larval crowding. In addition to testing the effect of larval crowding on mating competitiveness, it was also examined whether it influences male longevity, as this is another potential determinant of male lifetime reproductive fitness.

Materials and Methods

Rearing

Anopheles gambiae sensu stricto from a colony at the Ifakara Health Institute (IHI), Tanzania, were used in this study. This colony was established from a wild population located near Njage village in 1996. First instar *An. gambiae s.s.* larvae were obtained from colony cages and assigned randomly to density treatments of 100, 200 and 300 larvae per rearing tray (37 × 14 ×13 cm). Each tray was filled with 1L of water and supplied with fish food (Tetramin®). In each tray, 0.2 mg of Tetramin® was added for each larva, thus 20mg, 40mg or 60mg was added to the low, medium and high crowding treatment trays respectively, each day. Trays were inspected visually twice a day for the presence of pupae. Once detected, pupae were collected, counted and held individually in vials to allow for emergence. Batches of males from all three larval treatments that emerged on the same day were compared against one another in mating trials using females from the low-crowding treatment.

Marking

From the time of emergence, males were pooled according to crowding treatment and held in separate cages. On the second day after emergence, cohorts of adult males from two of the three rearing conditions were marked with green or pink fluorescent dusts respectively. One group was left unmarked. Marking treatments were alternated between crowding treatments across trials to ensure no systematic bias in performance due to dusting. Furthermore, pilot studies where males from the same crowding condition were marked with different colours revealed no effect of dust presence or colour on mating performance.

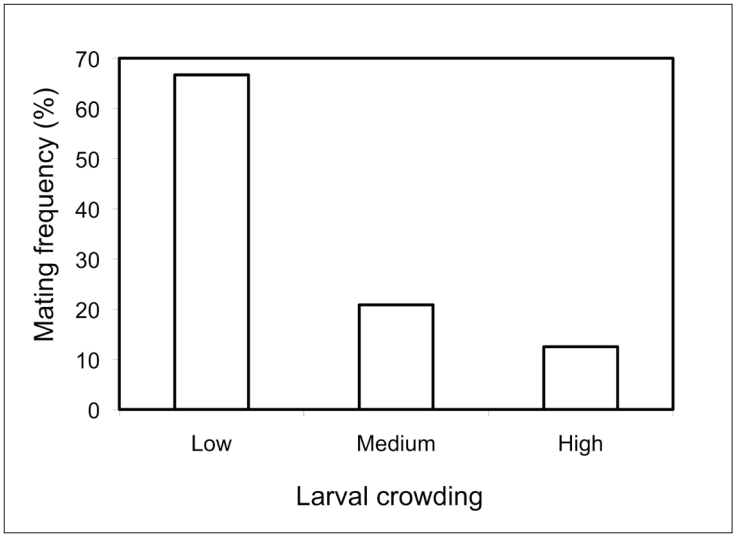

Figure 1. Frequency at which males from high, medium and low crowding conditions were the 'first-to-mate' in 28 nights of mating trials. The error bars represent the standard error as estimated from the binomial distribution.

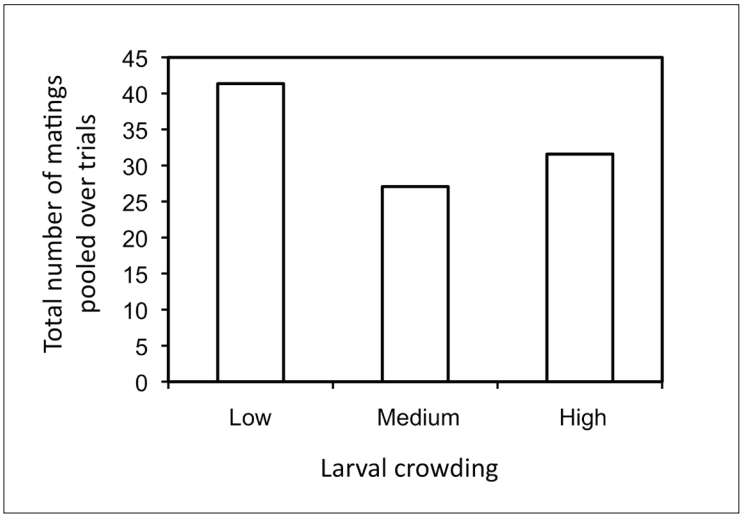

Figure 2. Proportion of total matings in 28 nights of trials going to males from low, medium and high larval crowding treatments. Error bars are the standard error as estimated from the binomial distribution (n= 133).

Mating experiments

On the third day after emergence, 30 males (10 males from each crowding treatment) were put together in one cage (15 × 15 × 10 cm). The cage was exposed to natural light a few hours before dusk. Observation of the cage began approximately 10 minutes prior to dusk. One or two males were observed to initiate the swarming process, just above a black disc (a swarm marker) that was placed on the bottom of the cage, with most of the remaining males joining the swarm after a few minutes. Once swarming was underway, 10 females from the low crowding condition were added to the cage (making a 3:1 male to female ratio). These females were simultaneously released into the cage using an aspirator. Mating activity was observed with a red light bulb. Pairs observed to form copula were immediately aspirated out of the cage and put together into a holding cup. On each evening of experiments, observation of mating was confined to an interval of 40-45 minutes. Observation ceased when all males had stopped swarming. At the close of the swarming session, unmated females were removed from the cage. The following morning, a fluorescent lamp was used to identify the larval rearing environment of each mated male. Females observed to have copulated with males were blood-fed on the morning following mating and moved into individual vials. Wet filter paper was placed on the bottom of these vials to act as an oviposition site. After five days in individual holding tubes, all eggs laid by mated females were collected and counted. Wing lengths of both males and females that mated, as well as a sub-sample of those from males that did not, were measured under a dissecting microscope.

Quantification of energy reserves

Batches of newly emerged males from each larval crowding regime were killed by shaking and transferred individually into glass test tubes for the quantification of lipids, sugars and glycogen. Once in tubes, mosquitoes were crushed using a glass rod. One hundred micro-litres (µl) of 2% sodium sulphate (which adsorbs glycogen) and 600 µl of a 1:2 chloroform-methanol mixture (which dissolves lipids and sugars respectively)

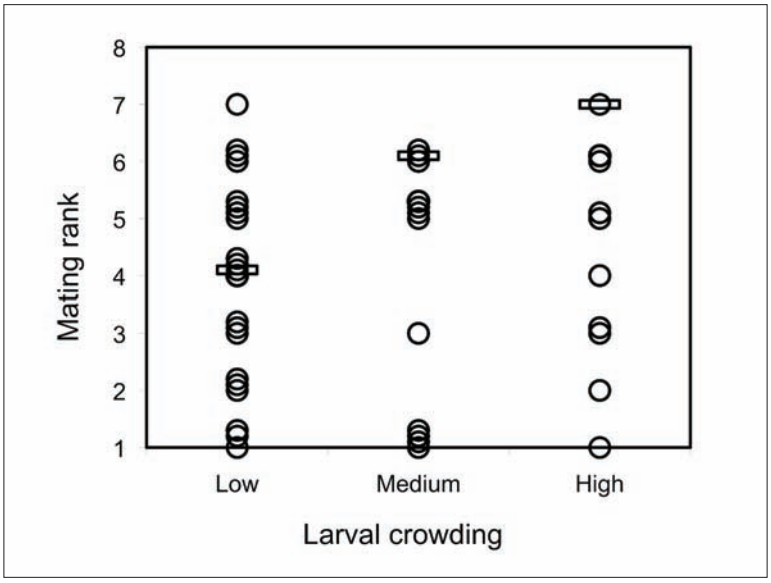

Figure 3. Mating rank of males from low, medium and high crowding treatments as observed in 14 mating trials. The dark line in each treatment gives the median mating rank

were added to each tube. Tubes were then covered and incubated for 24 hrs at room temperature. For each batch of males that was analysed, one blank was prepared by adding the same chemicals to a tube that had no mosquito. Lipids, glycogen and sugars of each male, were then quantified using a colorimetric technique adapted for mosquito analysis (Van Handel, 1988).

Longevity of unmated males

In a separate series of experiments, males emerging from each larval rearing regime were denied access to females but held in cages to monitor their longevity. These males were provided with a 10% glucose solution for sustenance until death. All dead males were removed and counted daily.

Statistical analyses

The main aim of our statistical analyses was to test for differences in the mating competitiveness, energy reserves, and longevity of *An. gambiae* males reared under different crowding conditions. Three analyses were conducted to assess mating competitiveness. First, we restricted our analysis only to the first male to mate in each of 28 trials. We considered the first male to mate to be the fittest in the group (the first place 'winner'), and we used a chi-square test to examine how larval crowding treatment influenced a male's probability of being a winner. Secondly, we tested whether the total number of copulations in all nights was influenced by larval crowding treatment, again using a chi-square test. Finally, we examined whether the order in which males mated during a night (1st, 2nd, 3rd etc.) was influenced by larval crowding treatment. For this, we restricted analysis to data from the 14 trials (out of 28) where at least five matings occurred in a night. Males that mated were given a rank that corresponded to the order in which they mated during the trial (e.g. 1st to mate got '1', etc). A Kruskal-

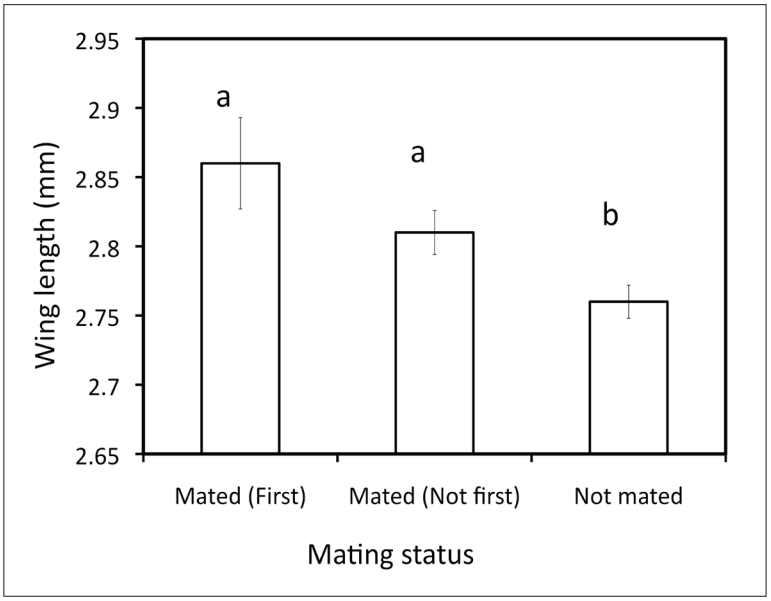

Figure 4. Body sizes (as indexed by wing length) of males who were the first to mate, who mated but were not the first, and that did not mate at all. Bars with the same number of asterisks (*) are not statistically different, but bars with differing numbers are.

Wallis test was then used to test the relationship between larval crowding treatment and mating rank (dependent variable). General Linear Models (GLM) were used to test whether larval crowding treatment influenced male wing length, or the abundance of lipids, glycogen and sugars they had on emergence. GLM were also used to test whether the number of eggs laid by a female was influenced by the larval crowding condition of the male that inseminated her. Finally, Kaplan-Maier survival analysis was used to test whether the survival of males depended on the crowding condition under which they were reared. All statistical analyses were done using the SPSS for windows and SAS system for Windows (version 8).

Results

Mating competitiveness

A total of 1120 *An. gambiae* mosquitoes were used in 28 nights of mating experiments (280 females and 840 males). Restricting consideration to the first male to mate, we observed that males from low crowding environments were much more likely to succeed ($\chi_2^2 = 13.61$, $p = 0.01$).

Males from the low crowding treatment won approximately 11 times (Odds ratio [95% CI] = 11.17, [2.7-50]) more often than those from the high crowding treatment, while those from the medium crowding condition won approximately 4 times more often (O.R [95% CI] = 3.51, [0.9-16.7], Figure 1). Analysis of all copulations (not just the first) in all 28 nights trials showed no statistically significant difference in mating frequency between males from different crowding treatments ($\chi_2^2 = 4.99$, $p = 0.08$), however there was a trend towards a higher mating frequency at low crowding condition, similar to that demonstrated in the 'first-to-mate' analysis (Figure 2). In the subset of 14 trials where at least five males mated, there was a weak tendency for males from the least

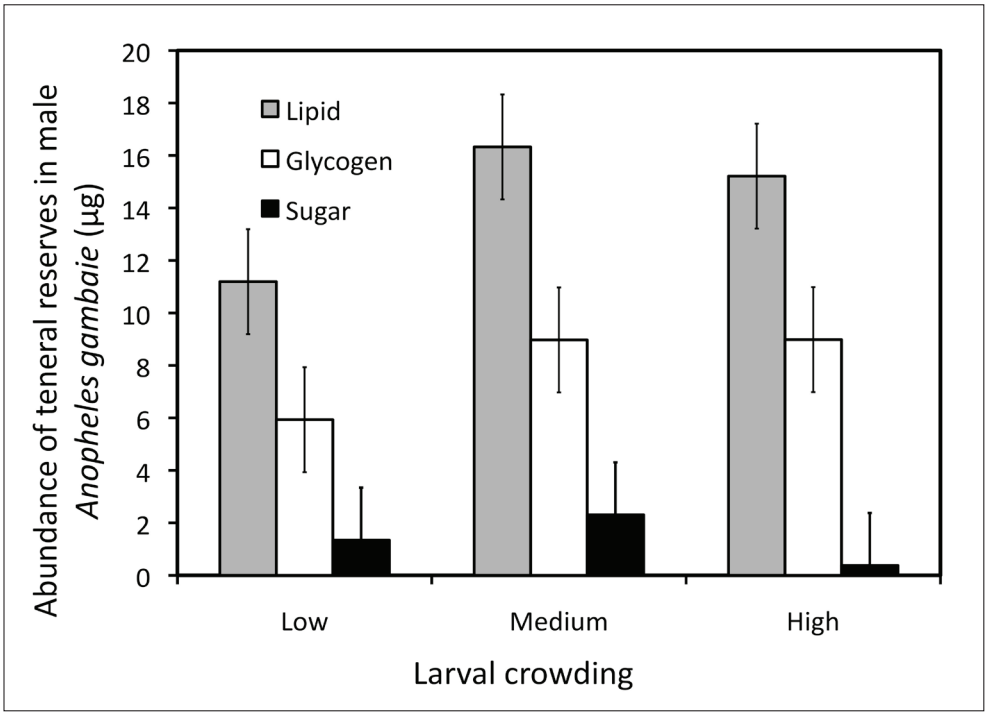

Figure 5. The mean mass of lipids, glycogen and sugar in newly emerged *An. gambiae* males reared in low, medium and high larval crowding conditions.

crowded larval condition to mate before those from more crowded conditions, but it was not statistically significant (χ^2_2= 5.09, p= 0.08, Figure 3).

The average size of male mosquitoes did not vary significantly between larval crowding treatments ($F_{2,397}$=2.43, p=0.09, mean body size: 2.75 ± 0.23 mm, 2.79 ± 0.12 mm and 2.79 ± 0.12 mm for low, medium and higher crowding conditions respectively). However, of those that were measured (n=398), males who successfully obtained a female were larger than those that did not ($F_{1, 397}$ = 6.97, p = 0.01, mean body size: 2.82 ± 0.02 mm and 2.76 ± 0.01 mm respectively, Figure 4). There was no difference between the body size of males who mated first, and those who mated later in the evening ($F_{1, 115}$ =1.79, p = 0.18, but both groups were larger than males who did not mate, Figure 4).

Only 15 out of 52 mated and subsequently blood-fed females oviposited their eggs. Amongst this subset, we found no association between egg batch size and paternal crowding condition ($F_{2,12}$ =0.67, p= 0.53) or maternal wing length ($F_{1, 11}$=1.98 p =0.19). Additionally, we found no association between the probability that females would oviposit and the larval crowding condition of her mate (χ_2^2= 0.91, p= 0.63).

Male teneral reserves and longevity

Pooling all treatments, the mean amounts of teneral reserves in newly emerged males were 14.3 (±1.3, SE) µg of lipids, 1.3 (±0.7 SE) µg of sugars and 8.0 (±0.4 SE) µg of glycogen. There was no evidence that larval crowding conditions influenced the abundance of these reserves in newly emerged adult males (lipids: $F_{2, 66}$ = 1.36, p = 0.26,

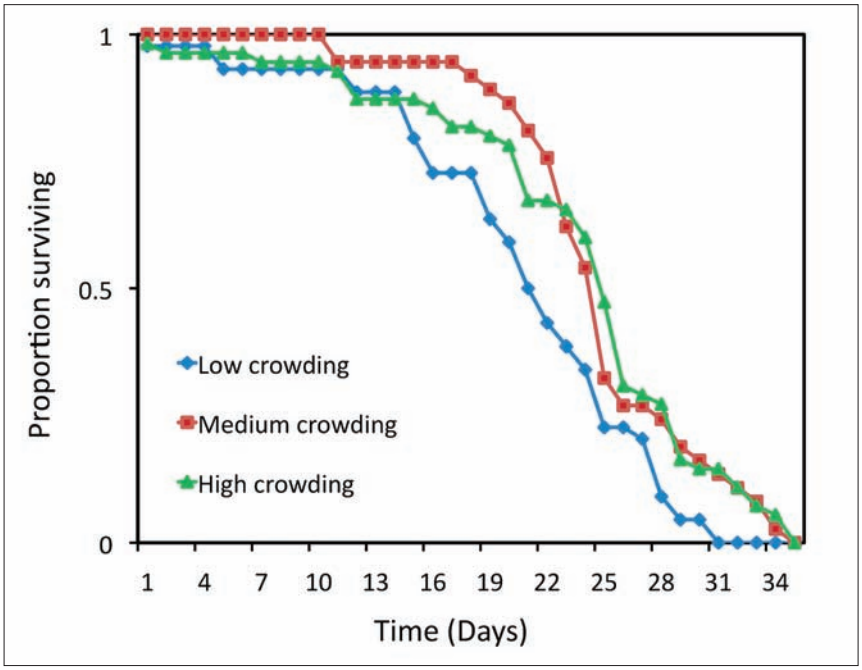

Figure 6. Survival of adult male *An. gambiae* s.s., from low, medium and high larval crowding treatments.

sugars: $F_{2, 66} = 2.16$, $p = 0.12$ and glycogen: $F_{2, 66} = 2.12$, $p = 0.13$, Figure 5).

The survival of 132 male *An. gambiae* was observed ($N_{low}= 44$, $N_{medium}= 37$ and $N_{high}= 51$). The survival of adult males varied in response to the crowding conditions under which they were reared (Log-rank= 10.79, df =2, $p < 0.01$, Figure 6), with the median survival of males equaling 21, 25 and 26 days for low, medium and high crowding conditions respectively. Males from low larval crowding conditions had poorer survival than those from medium (Log-rank= 7.14, df=1, $p < 0.01$) and higher crowding treatment (Log-rank 8.14, df=1, $p < 0.01$). The survival of males from medium and higher crowding conditions did not differ (Log-rank=0.12, df=1, p=0.73).

Discussion

We have shown that larval crowding influences the mating competitiveness of male *An. gambiae* mosquitoes. Results from 28 replicated experiments indicates that males reared under low crowding conditions are eleven times more likely to be the first in a swarming group to obtain a female than those reared at high crowding conditions. However, when all matings were considered (not just the first in each night), there was no evidence that the frequency of copulations obtained by males varied in response to larval crowding conditions.

Thus we have shown that larval crowding conditions influences a male's chance of beating his competitors in order to obtain the first female, but not his chance of getting a female in general. What does this say about the role of larval crowding as

a determinant of male fitness? We propose that a male mosquito's ability to obtain the first available female is more likely to reflect their lifetime reproductive potential than their success in eventually getting a mate; especially under controlled laboratory conditions. We have several reasons for this hypothesis. The first is that during mating, male *An. gambiae* implant a mating plug in females which presents a temporary physical barrier to further insemination(Bryan, 1968; Charlwood and Jones, 1979; Craig, 1967; Giglioli and Mason, 1966). Presuming females do not leave the swarm as soon as they are mated, males who hesitate may find themselves at a greater risk of encountering unreceptive females than those who mated first. Secondly, mating in *An. gambiae* is thought to be confined to a 15-20 min period (Charlwood *et al.*, 2002; Charlwood *et al.*, 2003; Yuval *et al.*, 1993). Within this period, some males have been observed to return to the swarm after they have mated and continue seeking females (Yuval *et al.*, 1993). Those who obtain the first females that enter the swarm are more likely to have sufficient time to return to the swarm after mating to look for additional females than those who mate later in the night. In our study, males were removed from the mating arena as soon as they obtained mate, so we could not test whether earlier maters were also more likely to mate repeatedly during the evening or not. However, this possibility is worth further study. A third reason for believing that those males who mated first have the highest mating competitiveness is that in nature, males are exposed to predation risks from insect predators such as dragonflies while swarming (Downes, 1969; Subramanian, 2002). Those who mate first can leave the swarm and escape this risk, or even if they remain in the swarm, will have had the advantage of passing on their genes before being preyed upon. If we could have introduced predators into our laboratory experimental cages, the ones that mated first might have exhibited an additional survival advantage. Our final reason for hypothesizing that males who were the first to mate in our experiments would be the most competitive in nature is that the conditions under which male *Anopheles* compete for females in the field are much more intense than those created here. For example, while here we experimentally created a ratio of 3 males to each female, in the field, males outnumber females at the mating site, in the range of 10:1 up to 600:1 (Charlwood and Jones, 1980; Reisen *et al.*, 1977). Under such skewed conditions where males dramatically outnumber females, it is extremely important for a male to seize a female at the earliest opportunity, as there is no guarantee another female will turn up before the end of the evening. Thus we believe any factor that increases a male's chance of being the 'first-to-mate', as we demonstrate with larval crowding here, will be strongly correlated with their lifetime reproductive successes under natural conditions.

We evaluated several possible mechanisms that could explain the differences in mating patterns between crowding treatments. The first was body size, which influenced the total number of males that mated, with larger males being more likely to obtain a mate than smaller ones. A similar finding was reported in *An. freeborni* (Subramanian, 2002) whereas no size-dependency for mating was observed in *An. gambiae* by Charlwood (Charlwood *et al.*, 2002). Although body size influenced mating, in general it did not explain treatment-associated differences in males who were the first-to-mate. This is because there was no difference in body size between males who mated first or later and no systematic difference in body size between crowding treatments. Thus we conclude that both body size and larval crowding can independently influence male mating success, and that the effect of the latter is not exclusively driven by variation in the former trait.

The amount of teneral reserves in males did not differ between crowding treatments, and thus could not explain this difference in mating success. Eliminating these possibilities, we hypothesize that the observed differences in mating success between crowding conditions could be due to the detrimental effects of chemicals (Moore and Whitacre, 1972; Roberts, 1998) and/or waste products that are released in crowded conditions, with larvae grown in dense conditions suffering more from exposure than those at low crowding.

When held at high density, some mosquito larvae release 'crowding chemicals' that retard the growth of their conspecifics (Roberts, 1998). This phenomenon has been recorded for *Aedes aegypti,* but not to our knowledge for *Anopheles*. In *Aedes,* chemical growth retardants are released by larvae when their density increases, even if each larva receives a constant ration of food (Moore and Whitacre, 1972). There is however, a certain food ration threshold above which no chemicals are produced regardless of the number of individuals (Moore and Whitacre, 1972; Reisen, 1975). When food rations are below this threshold, however, the release of these chemicals may regulate the number of adults that emerge (Roberts, 1998). Although we did not assay for the presence of such chemicals here, its existence would explain why in the absence of food limitation, mosquitoes grown in highly crowded conditions performed poorer than those from low crowding. The mechanism through which such chemical factors could have influenced mating success is not clear, as it was not associated with between-treatment variation in body size or teneral reserves. We thus assume exposure to these chemical factors may have led to subtle differences in size, behaviour or physiology not detected here (i.e. changes in male flight ability or reaction time) that ultimately influenced mating competitiveness. Further experiments are required to confirm whether such chemical factors exist in *Anopheles*, and how they operate.

Larval crowding also influenced the survival of *An. gambiae* adult males (Figure 4). Whereas males from low crowding conditions were generally the first to mate, and thus probably the most competitive for mates, they also had the poorest survival. This observation suggests the existence of an energetic trade-off between reproduction and survival in male *Anopheles,* such as has been observed in other insects (Hunt *et al.*, 2004). In male *An. gambiae*, such a trade-off could arise because males that are the first to mate are those that are the most active, and spend more time flying and swarming than those with lower mating success. As flying is energetically costly (Briegel, 2003; Charlwood and Jones, 1979), an increased tendency to do so may lead to both an enhanced mating competitiveness and reduced long-term survival, as we observed in males from the low crowding condition here. As we did not observe the proportion of time that males from different crowding conditions were flying in this experiment, we do not know if differential activity could explain the between-group variation in mating success and survival. Further study is required to measure whether flight activity is linked to mating success, and it whether influences the rate at which a male's energy reserves and longevity decrease.

The reduced survival of males from low crowding conditions may not necessarily compromise their long-term reproductive fitness. The benefits of being the first to mate during the early part of their adult life, as discussed above, may compensate for having a reduced number of mating opportunities in the longer term due to poorer survival. If so, our findings are consistent with the theoretical claim that longevity may

not be a reliable measure of male reproductive fitness (Hansen and Price, 1995; Hunt *et al.*, 2004). Further experiments in which males are given multiple opportunities to mate during their natural life are required to confirm whether being the first to mate on any given evening is indeed the best predictor of male mosquito lifetime reproductive success. Ideally these experiments would be carried out in larger semi-field systems (Ferguson *et al.*, 2008; Knols *et al.*, 2002; Knols *et al.*, 2003) as well as in natural populations, so realistic costs of activity (i.e. exposure to predation, energetic drain) can be incorporated.

Conclusions

These novel findings have direct application to genetic control strategies for malaria that seek to reduce transmission by releasing sterile or malaria-refractory *Anopheles* males. The reported poor competitive success of transgenic male mosquitoes (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004) could be enhanced by rearing males in conditions of low crowding and high food abundance. This could create a cohort of highly competitive yet relatively short-lived males for release. Ideally, transgenic males should be both highly competitive and long-lived. However, should an energetic trade-off exist between their competitiveness and longevity as suggested here, we argue it would be more useful to focus on increasing their short-term mating competitiveness by methods such as those discussed here.

To increase the competitiveness of mass-reared males, we advocate: 1) maintaining males at low densities and/or regular changing of rearing water to avoid the build up of crowding of chemicals that might result in disadvantaged males, and 2) supplying larvae with sufficient amounts of food. This finding therefore, may help overcome some of the mating-related hurdles that impeded early genetic control trials (Ferguson *et al.*, 2005). We propose that the fitness of all current genetically modified *Anopheles* constructs (Ito *et al.*, 2002; James, 2003; Moreira *et al.*, 2004) be re-assayed after under ideal larval conditions in order to show how substantially ecological manipulation could increase their mating success relative to the wild type.

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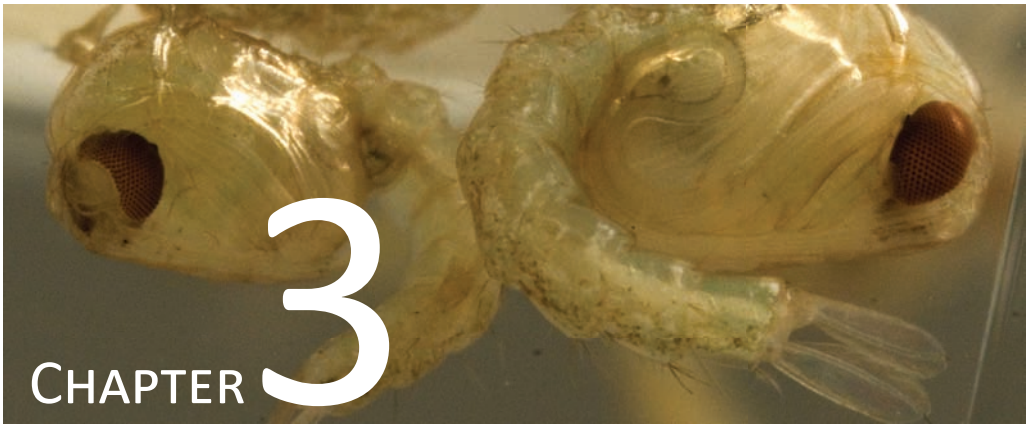

TIBOR BUKOVINSZKY

Effect of larval diet on the mating competitiveness of male *Anopheles gambiae* s.s

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> **Abstract**

Here we tested the hypothesis that the competitive ability of male mosquitoes within aerial mating swarms is not random, but a function of environmental conditions experienced during early life history that impact their adult phenotype. By manipulation of larval nutrition conditions (low, intermediate and high food allocation), we generated cohorts of male *An. gambiae* mosquitoes that differed in three key fitness traits, energetic reserves, body size and survival, and tested whether they influenced mating success under competitive swarming conditions. Males from the intermediate food treatment were 6 times more successful and 2 times at acquiring mates than those from the high and low food treatment respectively. Males from the intermediate group also had intermediate values of energetic reserves, body size and survival compared to males in the high and low group. Thus we conclude that, mating success in *Anopheles gambiae s. s.* males is influenced by larval food allocation, but not in a positive direction, with males grown in intermediate food conditions out competing those from less and more well provisioned environments.

Introduction

Sexual selection theory predicts that organisms most likely to spread their genes to subsequent generations are those possessing traits that give them a mating advantage over others of the same sex (Andersson and Simmons, 2006; Emlen and Oring, 1977; Hauber and Sherman, 2001; Jennions *et al.*, 2001). Selection for male traits that increase sexual competitiveness is expected to be strongest within mating systems where large groups of males congregate to mate with a limited number of females (Andersson, 1982; Clutton-Brock and Parker, 1992; Clutton-Brock and Parker, 1992; Darwin, 1871; Emlen and Oring, 1977). Evidence of sexual selection on male traits that increase attractiveness to females has subsequently been documented in a wide number of vertebrate and invertebrate species (Andersson, 1994; Andersson and Simmons, 2006; Hall *et al.*, 2004; Maan *et al.*, 2004; Skroblin and Blows, 2006). Characters under sexual selection range from traits that enhance a male's ability to fight (Clutton-Brock *et al.*, 1993; Hastings, 1994; Leboeuf, 1974; Malo *et al.*, 2005; Savage *et al.*, 2005; Shackleton *et al.*, 2005; Wolff, 1998) to those that signal the quality of the bearer to the opposite sex (Doucet *et al.*, 2005; Limbourg *et al.*, 2004; Siefferman and Hill, 2005; Zuk *et al.*, 1990). In species where male mating success depends primarily on non-aggressive competition; sexual selection has been demonstrated for traits such as energetic reserves (Hunt *et al.*, 2004), ornamentation (Hausmann *et al.*, 2003), visual (Costanzo and Monteiro, 2007; Elias *et al.*, 2005), auditory (Gerhardt, 2005; Gridi-Papp *et al.*, 2006; Kelley, 2004) and chemical signaling (Gleeson *et al.*, 2005; Uetz and Roberts, 2002).

Aerial insect swarms present a possible exception to the general rule that strong sexual selection should occur when large groups of males congregate to find female mates. Unlike communal courtship grounds (leks), in which groups of males congregate on a fixed habitat feature or area and compete for territory within it (Kruijt and Hogan, 1967), an aerial swarm is a three-dimensional arena whose physical structure is determined by the flight activity and abundance of males themselves (Downes, 1969; Yuval, 2006; Yuval *et al.*, 1993). These swarms are frequently maintained in a relatively stationary position above a visual marker (Charlwood *et al.*, 2002; Charlwood *et al.*, 2003; Reisen *et al.*, 1977), by the constant 'figure-eight' flight motion of males within them (Diabate *et al.*, 2003; Downes, 1969), with the size of swarms fluctuating as males fly in and out. It is speculated that a female is attracted to this swarm from a relatively long distance (Clements, 1992), and on approach, a few males dart out towards the female before she reaches the 'body' of the swarm, during which she is grabbed by a male and quickly mated (Charlwood *et al.*, 2002; Charlwood *et al.*, 2003; Lehmann *et al.*, 2006; Reisen *et al.*, 1977). The long-range attraction cue that draws females to swarms is not yet known, but it is plausible that visual detection of the swarm is involved. Thus males must cooperate to form the mating arena, and it has been suggested that they must also behave the same within a swarm for it to maintain its structure and position over a fixed marker (Lehmann *et al.*, 2006). If males must adopt similar behaviour in a swarm, and females fly randomly through them, it may be difficult for differences in male quality that fuel sexual selection to be manifested.

Most investigations of sexual selection in insects have focused on systems where mating does not occur in aerial swarms. In these systems, there is strong evidence that male mating success increases with phenotypic traits such as body size

(Alcock, 1996; Blanckenhorn *et al.*, 2004; Crean *et al.*, 2000; Partridge and Faquhar, 1983) and energetic reserves (Yuval *et al.*, 1994), which are often direct correlates of other measures of fitness measures such as survival and resistance to starvation and desiccation (Jennions *et al.*, 2001). The very limited investigation of species which mate in flight has yielded conflicting results, with some studies showing large size also yields a mating advantage (Yuval *et al.*, 1993), others that mating is random with respect to size (Charlwood *et al.*, 2002), and others that being small is advantageous as it may increase movement speed and maneuverability (Fyodorova and Azovsky, 2003; Lebas and Ritchie, 2004; Marshall, 1988; Neems *et al.*, 1998; Vencl and Carlson, 1998). What is lacking is systematic experimental investigation and identification of what, if any, traits are correlated with male mating success in a mating swarm, and whether these traits are positively or negatively correlated to other measures of male 'quality' such as survival and resource accumulation. Such information is vital to understand the determinants of mating success within swarms, and to confirm whether these systems actually do represent an exception to the generality that sexual selection on male traits should occur when the operational sex ratio is heavily skewed towards males.

Here we tested the hypothesis that the competitive ability of male *Anopheles gambiae* mosquitoes within mating swarms is not random, but a function of environmental conditions experienced during early life history that impact their adult phenotype. Larval nutrition was experimentally manipulated by altering food availability in order to generate adult males that varied in respect to three key indices of their fitness: body size, energetic reserves, and survival. These males were then competed against one another in a mixed swarm for access to a limited number of females, as would occur in nature. As *An. gambiae* mosquitoes are the principal vectors of malaria in sub-Saharan Africa (WHO, 1993), and main target of control programmes based on the mass release of genetically modified and/or sterile males (Andreasen and Curtis, 2005; Ito *et al.*, 2002; Moreira *et al.*, 2004; Tabachnick, 2003), information obtained here has direct relevance to malaria control. The prospects of using transgenic / sterile males for malaria control, mating has to occur at first stage in order that refractory genes can be transferred from males to wild females. The understanding the nature of mating behaviour, and elucidating the social and physical conditions which may limit sexual selection from acting on large groups of competing males, is thus important.

Materials and methods

Rearing

Anopheles gambiae sensu stricto mosquitoes from a population at the Ifakara Health Institute (IHI), Tanzania, were used in this study. This population was established from a wild population near Njage village, Tanzania, in 1996. First instar larvae were randomly assigned to one of three food quantity treatments: 0.1 mg, 0.2 mg, or 0.4 mg of dried fish food (Tetramin®, Melle, Germany) per larvae per day (Lyimo *et al.*, 1992). Larvae of the same food treatment were housed together in groups of one hundred in standard rearing trays (37× 14 ×13 cm). Each tray was filled with 1 litre of river water and supplied with a total daily mass of fish food sufficient to feed all 100 larvae (a total of 10 mg, 20 mg, and 40 mg of Tetramin® added to low, medium and high food treatment trays respectively). River water was used to mimic larval developmental

condition with that of wild mosquitoes (in each rearing try water was changed after every two days). To coincide their pupation time, cohorts of larvae in the lowest food treatment were established four days before the other two feeding treatments (time gap chosen on the basis of pilot studies of pupation rates). Once established, larval trays were inspected twice a day for the presence of pupae. When detected, pupae were collected and held individually in vials to allow for emergence. Batches of males from all three food treatments that emerged on the same day were competed against one another in mating trials using females from the medium food treatment. Only females from medium treatment were used to control female size and allow males of different sizes to compete for females of similar size..

Marking

From emergence, males were pooled according to larval food treatment and held in separate cages. On the second day after emergence, cohorts of adult males from two of the three food treatments were marked with green or pink fluorescent powder respectively. The third group was left unmarked. Marking treatments were alternated between food treatments across trials to ensure no systematic bias in performance due to dusting. Males were dusted one day before experiment and the fact that their main contacts are females, thus no possibility of color contamination between males was observed. Also to further narrow this possibility, all females who mated were siphoned out of the swarm during copulation, thus was no possibility for marking dust to be transferred between males via females.

Mating experiments

On the third day after emergence, 30 males (10 males from each treatment) were put together in one cage (15 × 15 × 10 cm). The cage was exposed to natural light a few hours before dusk. A black plastic disk was put in the bottom of each cage to act as a swarm marker (Ng'habi *et al.*, 2005). In nature, *An. gambiae* males form mating swarms approximately 15-20 min before sunset (Charlwood and Jones, 1980; Charlwood *et al.*, 2002; Charlwood *et al.*, 2003; Diabate *et al.*, 2003), a behaviour that is also observed in our laboratory population (Ng'habi *et al.*, 2005). We began monitoring activity inside each experimental cage approximately 10 min before sunset.

Once swarming was underway, 10 females from the medium food treatment were simultaneously released into to the cage (making a 3:1 male to female ratio). Mating activity within cages was observed under a low-intensity red light bulb. Pairs observed to form copula were immediately aspirated out of the cage and put together into a holding cup. Polyandry was not expected to bias our study, due to the fact that female anopheles mosquitoes have low rate polyandry in nature (Tripet *et al.*, 2003) as they mate once and use their stored sperm in the spermatheca to fertilize eggs during subsequent gonotrophic cycles (Craig, 1967). (On each evening of experiments, observation of mating ended after all males had stopped swarming (usually 40-45 min after first initiation). The following morning, a cotton wad soaked with chloroform was put on top of each holding cup, and all mosquitoes captured in copula were anesthetized and killed. Males were then observed under a fluorescence lamp to identify which larval food treatment they originated from. The wing lengths of both male and female partners were then measured under a dissecting microscope. Females that did not mate were also sacrificed and their wing lengths measured for comparison with those

caught in copula. Eight replicates of this competition experiment were conducted.

Quantification of energy reserves

A sub-sample of newly emerged males from each larval feeding regime were killed and transferred individually into glass test tubes for the quantification of lipids, sugars and glycogen. Once in tubes, mosquitoes were crushed using a glass rod. One hundred micro-litres (µl) of 2% sodium sulphate (which adsorbs glycogen) and 600 µl of a 1:2 chloroform-methanol mixture (which dissolves lipids and sugars respectively) were added to each tube, then incubated for 24 hrs at room temperature. We analyzed 93 males; 28 from low, 26 from medium, and 39 from high feeding regimes. For each batch of males analysed, one blank was prepared by adding the same chemicals to a tube that had no mosquito. Lipids, glycogen and sugars of each male were then quantified using a colorimetric technique adapted for mosquito analysis (Van Handel, 1988).

Longevity experiments

In a separate series of experiments, males emerging from each larval feeding regime were denied access to females but held in groups of 32-47 in standard holding cages (15 × 15 ×10 cm) to monitor their longevity. At least 2 cage replicates of each larval food treatment were monitored. While in cages, adult males were provided with a 10% glucose solution ad libitum. Daily observation was made of each cage, and all dead males found within it were removed and counted.

Statistical analysis

The main aim of our statistical analysis was to test for differences in male mating competitiveness within a swarm as a function of the larval nutrition-set adult phenotype. We considered whether male larval nutrition treatment was a predictor of: (1) the first (most competitive) male to mate, and (2) the total number of males (competitive) that obtained copulations across all trials. In both cases, chi-square tests were used to evaluate the relationship between the dependent variable (proportion of matings) and larval nutrition treatment. General linear models (GLM) were used to test whether larval nutrition treatment influenced male mosquito body size (as indexed by wing size), and/or the abundance of energy reserves (sugars, glycogen and lipids) on emergence. Finally, Kaplan-Meier survival analysis was used to test whether the survival of adult males varied in response to larval nutrition treatment. The SPSS (Chicago, USA) statistical package (11.5 for Windows) was used for all analyses.

Results

By varying larval nutrition, we succeeded in generating three distinct adult male phenotypes. These phenotypes differed with respect to each of the male traits we measured. First, body size varied substantially between nutrition groups ($F_{2, 234}$= 94. 128, P< 0.01, Fig 1), with males from high food treatment being 18% and 9% bigger than those in the low and medium food treatment respectively. Field studies have reported varying sizes of free foraging males similar to that created in the laboratory. For example Huho et al, (2007), reported that free foraging males body sizes ranges from (1.9-3.1 mm). The observed size ranges in this study (2.2-3.1 mm) is within the reported field size range. The average male body sized generated by these treatments

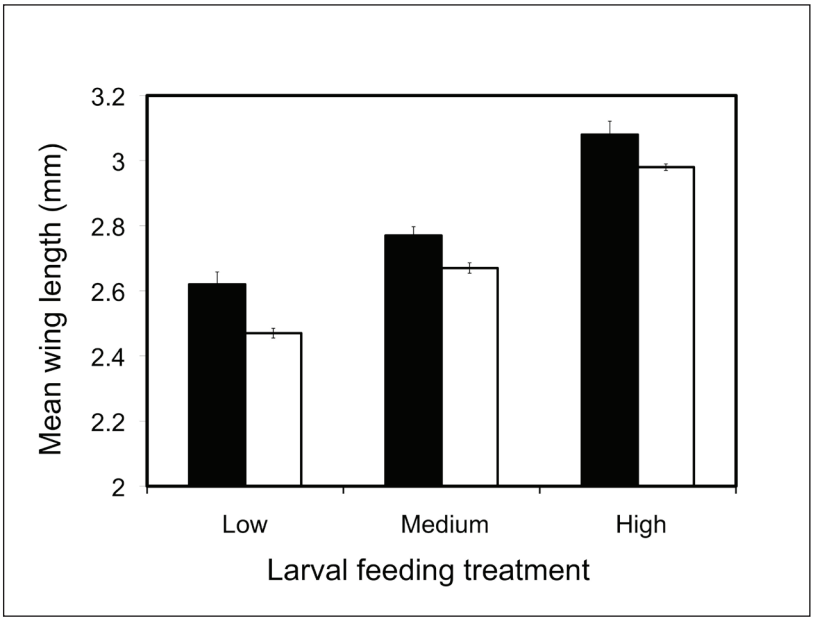

Figure 1. The mean wing length (an indicator of body size) of *An. gambiae s. s*. males that mated (dark bars) and those that did not (white bars) from each larval nutrition treatment. Bars represent one standard error (se).

(X_{ow} = 2.49 (0.15), X_{med}=2.69 (0.15), X_{high}=2.94 (0.01)) fell within the range observed in natural populations from the surrounding Kilombero valley (Huho et al 2007; Lyimo et al, 1993). The abundance of teneral energy reserves in adult males also varied in response to larval treatment. As with body size, males emerging from the highest food treatment had the highest abundance of lipids, glycogen and sugars (Lipids: $F_{2, 26}$ = 15.76, P<0.001; Sugars: $F_{2, 24}$ = 22.05, P<0.001; Glycogen: $F_{2, 37}$ =27.05, P<0.001, Fig. 2). Furthermore, male survival was similarly influenced by the abundance of food larvae received during their development (Log rank =136.69, df =2, P<001, N=294, Fig. 3). The median survival of males from the lowest food regime was 8 and 11 days lower than males from the medium and high larval food treatment respectively (median survival =12 (± 1), 20 (± 1), and 23 (± 1) days for the low, medium and high groups respectively). Thus the larval nutrition treatment we imposed yielded three distinct phenotypic groups of males that varied substantially in quality as indexed by body size, teneral reserve, and survival.

Female mosquitoes presented to males in these experiments were all taken from the medium larval food treatment, in order to control size variations in females and maximize in males These females differed in body size from all three male groups ($F_{3, 316}$ = 166.98, P< 0.01) (X_{female} = 2.83 mm $\pm$ 0.19). Mated females also differed in body size from two mated male group (High; P = 0.02; Lower; P < 0.01), except for males from the medium group (Medium; P = 0.77). Studies have reported a size range of 2.34 - 3.62 mm (Mean: 2.92 mm) of female *An. gambiae* in Ifakara region (Lyimo and Takken, 1993). Our observed female size ranged from 2.33 mm to 2.93 mm (Mean: 2.83 ± 0.19 mm) Thus, the generated female body size is usual and range within the reported field observations and unlikely to be overestimated. With regard to sexual dimorphism in *Anopheles* mosquitoes, studies have shown that females are generally bigger than males (Grech *et al.*, 2007; Lehmann *et al.*, 2006; Lounibos *et al.*, 1996), although some reports that there is no difference (Lounibos *et al.*, 1995).

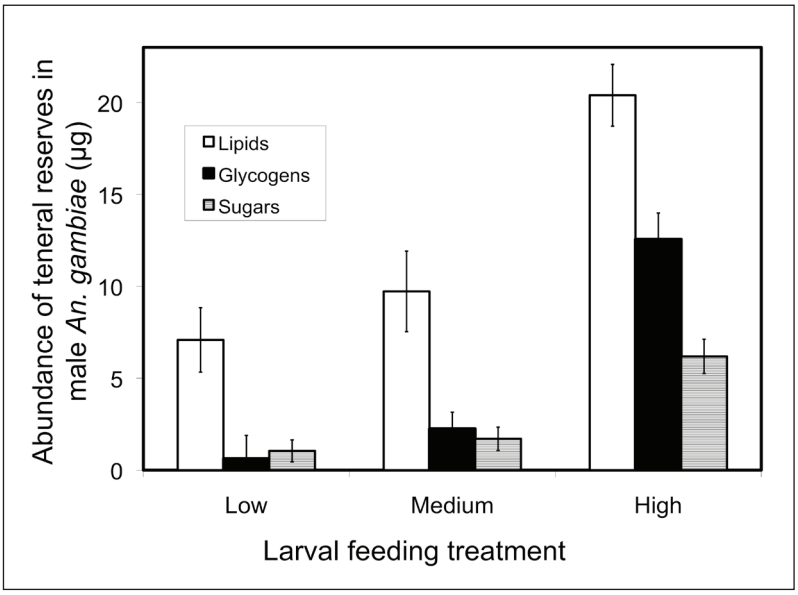

Figure 2. The mean amounts of lipid, glycogen and sugars in newly emerged *An. gambiae s. s.* males reared in low, medium and high larval feeding conditions. Bars indicate one standard error (se).

Mating experiments in which a total 240 males competed for 80 females over 8 replicates were conducted to assess whether these proximate measures of male quality also predicted their mating competitiveness. We identified males on the basis of the fluorescent dust applied to them prior to mating experiment, with no any ambiguity or mixed coloration was observed. Males originating from the medium feeding treatment with intermediate body size, energy reserves and survival, were the first to obtain a mate in 7 out of 8 experimental replicates (χ^2_2= 18.50, P< 0.01). Considering all mating events and not just the first one to occur in an evening, we found that copulations were not distributed randomly amongst males from different larval treatment groups (χ^2_2=13.59, P<0.01, Fig. 4). Males from the intermediate larval food treatment monopolized mating activity; being 6 times more likely to acquire a female mate than males from the higher food regime (Odds ratio [95% CI]= 6.33 [2.05-19.52], P < 0.01). Males from the medium group also accrued more mating than those in the low larval nutrition treatment, although this difference was not statistically significant at the 0.05 level (Odds ratio [95% CI] = 2.09 [0.93 - 4.71], P = 0.08).

Within each of the three larval nutrition treatments, we found that males who obtained female mates were larger than those that did not ($F_{1, 234}$ = 29.49, P<0.01, Fig 1). This observation contrasts with between-treatment observation that males from the group having intermediate body size were more successful than males from the higher and lower larval nutrition groups. In our statistical analysis of the total number of mating, inclusion of male body size as an additional explanatory factor did not diminish the significance of larval treatment (χ^2_1= 40.64, P<0.01); indicating that adult body size and larval nutrition are important independent predictors of mating competitiveness.

Discussion

Here we demonstrate that male mating success within swarms (at least under laboratory settings) is not random, but linked to nutrient uptake during larval development. Contrary to expectation, we observed that the 'best male' with respect to frequently

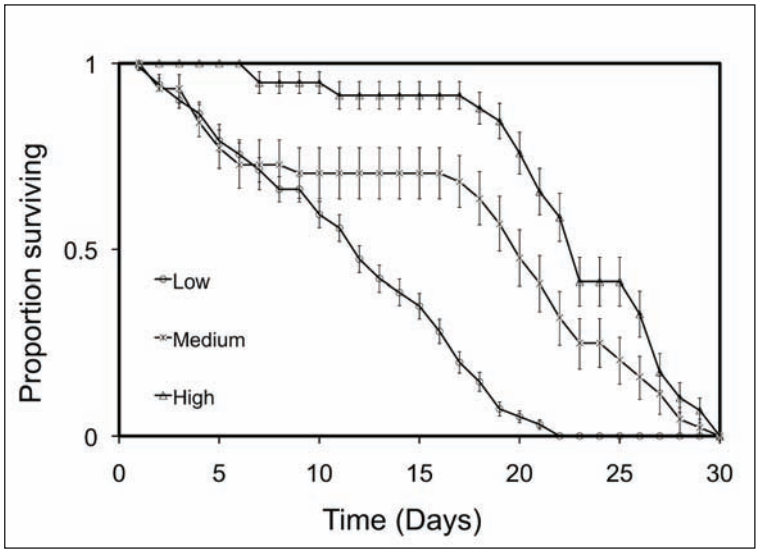

Figure 3. The survival of adult male *An. gambiae s. s.* maintained on high (n=58), medium (n= 44) and low (n=192) food provision during larval development. Circles are for males from the low larval food treatment, crosses for the medium group, and triangles for males from the highest larval food treatment.

observed predictors of male insect fitness (longevity, body size and energetic reserves) is not the one who wins females under the competitive conditions of within a mating swarm. Instead, we observed that males most likely to win females were those with intermediate values of these fitness traits in comparison to their competitors. The non-random nature of mating competitiveness observed here suggests that sexual selection on male phenotypic traits can occur in swarms. This is unexpected observation as males in swarms tend to behave exactly in the same way to maintain the position of the swarm and the visual cue. Here we demonstrate the existence of both intense competition for female mates and cooperation within mating insect swarms, as they are able to exhibit differences in mating success.

Two of the phenotypic traits studied here, male body size and adult survivorship, are known to be influenced by genetic as well as environmental factors in nature (Lehmann *et al.*, 2006). As these traits show moderate levels of heritability in the field (Lehmann *et al.*, 2006), it is possible that male-male competition occurring in mating swarms could influence the direction of body size and longevity evolution in male *Anopheles* populations. Further evaluation of the relative contribution of genetic and environmental factors to variation in these traits is required to further test this hypothesis.

The nutritional conditions and resource allocation experienced during early periods of development has been reported to affect individuals fitness during adults stage. This effect, referred to as silver-spoon effect' has widely been reported in vertebrate models (Madsen and Shine, 2000; Metcalfe and Monaghan, 2001; Van de Pol *et al.*, 2006). The expression of males traits that give them a mating advantage, is therefore controlled partly by genes and partly by the environmental factors (Jennions and Blackwell, 1998; Zuk *et al.*, 1990). Also resource availability has a greater effect on the priority of resource investment. Males have been found to invest on sexually traits that are less costing but important to females when resources are limiting (Basolo, 1998). Males in the well provisioned group lost out despite having larger body sizes. Since resources were not limiting, it is plausible that males prioritised to invest

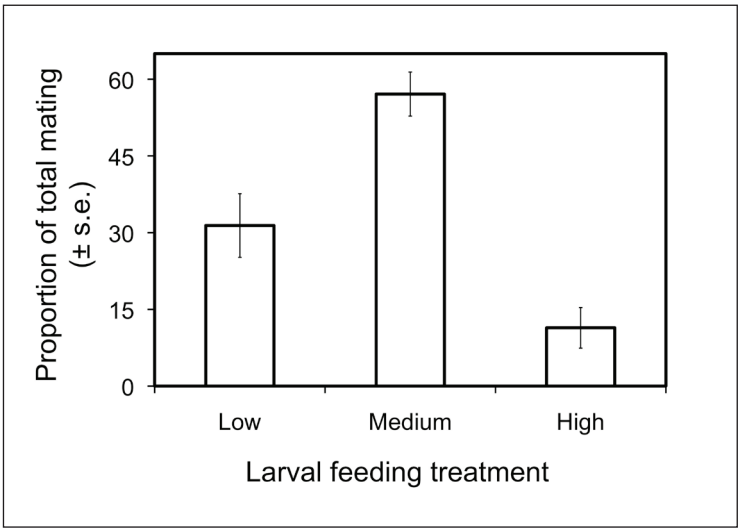

Figure 4. Proportion of total matings (n = 35) going to adult male *An. gambiae s. s.* from low, medium and high larval feeding treatments, pooled over 8 experimental replicates.

resources on body size, a traits that is costing and less was invested on other sexual traits that give them mating advantage. This phenomenon has been reported in a range of vertebrates, where males tend to invest much resources on traits that maximize their reproductive fitness when resources are limiting (Basolo, 1998; Wiens, 2001). The low nutrition group were not the most competitive group either despite limiting resources. The medium group where resources were limiting, but not as limiting as in the low nutrition group, were the most successful group of all. Why should this happen? If closely look at the results, it clearly show that the intermediate group were 6 time more competitive than males from high nutrition group, (Odds ratio [95% CI]= 6.33 [2.05-19.52], $P < 0.01$) and 2 times competitive than low nutrition males but this difference was not statistically significant at the 0.05 level (Odds ratio [95% CI] = 2.09 [0.93 - 4.71], $P = 0.08$). With respect to the observed differences in mating success in this study, we hypothesize that, resource availability during early developmental stages has strong effect on males mating success but not in a positive manner. For this matter the intermediate group in this study provided an optimal condition under which traits determining mating success were fully expressed. This phenomenon could be widely spread and strongly operating in nature where resources are unpredictable and in most cases limiting (Ye-Ebiyo et al, 1993; Minakawa et al, 2005). Thus, females chose to mate with intermediate sized males not because they are abundant in nature but because traits that attract females are more expressed and most probably they became more manoeuvrable than larger males during swarming with much more reserve than smaller males to sustain their flight during swarming. We cannot yet conclusively identify these phenotypic trait(s) that drive this segregation, but hypothesize that they are directly liked to nutrient uptake by individuals at early developmental stages. Another possible explanation is that, as males from the intermediate nutrition condition were closer in body size to the available females (Post hock observation), males genitalia corresponded most closely to that of the females giving them the highest mating success (Arnqvist, 1997). Although our experimental setup wasn't designed so and the current logistic didn't allow us to test this hypothesis, it is a valuable inquiry for the future direction.

Being larger has been reported to have mating disadvantage in other insects due to reduced manoeuvrability and unable to deliver gifts to their patners (Lebas and Ritchie, 2004). Field studies have reported the average body size of male *An. gambiae* in naturally occurring swarm (Mean: 2.63 ± 0.16), (Charlwood *et al.*, 2002) in which the mean body size of males in our low, medium and high group corresponds to, by being less than 2 standard deviations of this naturally occurring range, thus within the unusual range. Further more, the mean body size of swarming males reported from field studies (Mean: 2.67 ± 0.16), (Charlwood *et al.*, 2002), is close to the average body size of males in our most successful intermediate group (2.69 ± 0.13). This observation indicates that individuals of this size originated from environment with optimal nutrition, not enough to invest in more costing traits (Such as body size) which maximizes their survival but enough to invest on much traits that will enable them to successfully mate and perpetuate their species. Thus, studies that report male mating success is random, may be due to the abundance of intermediate males in the swarm originating from one breeding habitat with intermediate food availability (Charlwood *et al.*, 2002). Further studies are needed to substantiate this fact by observing natural swarms at different localities and different times.

Here we found that being large yielded a mating advantage when comparing males from a similar larval background, but not when comparing across different larval backgrounds that generated much more substantial variation in body size. This provides evidence this trait and traits related to body size are important in determining male mating success in An. gambiae mosquitoes, but the extent at which one more expressed depends no the availability resources at early developmental stages. The generated body variation were similar with the natural body size ranges observed in field studies (Charlwood *et al.*, 2002; Huho *et al.*, 2007). This evidence may help explain discrepancies in previous studies of mosquitoes and other flying insects that have conflictingly reported that male body size is positively, or negatively correlated with their reproductive success (Blanckenhorn *et al.*, 2004; Gomulski, 1985; Partridge and Faquhar, 1983). Experimental studies are needed to explore male mating success across the full range of phenotypic traits observed in nature.

We conclude by hypothesizing that male mating success as observed in our experimental swarms is not random but governed by environmental conditions experienced during early larval life history, although in a manner not similar to silver-spoon effect (Grech *et al.*, 2007; Madsen and Shine, 2000; Metcalfe and Monaghan, 2001). Thus in male *An. gambiae* mosquitoes larval nutritional level influences their reproductive fitness, not in a positive manner but a shift in resource allocation on sexually selected traits (Basolo, 1998). Since early nutritional conditions do not affect all fitness components positively therefore, most of the traits that are positively correlated are the ones that gives individuals from similar environment a mating advantage, and subjected to sexual selection (King, 2002; Olsson and Shine, 2002). This suggests therefore that even in extreme systems where individuals cooperate to form a 'supra-organism' (the swarm) to attract females, a male- male competition for female mates is manifested and selection for traits that maximize mating success is more likely to occur.

As mosquito larval habitats and resulting adult males phenotypes vary greatly in the wild (Koenraadt *et al.*, 2003; Minakawa *et al.*, 2004; Mutuku *et al.*, 2006; Ye-

Ebiyo *et al.*, 2003), such a phenomenon could maintain divergent male sexual traits (Hoffman and Parsons, 1991; Nur and Hasson, 1984). Additionally, these findings have several practical implications for the application of insect control programs based on the mass release of laboratory-reared sterilized males (Benedict and Robinson, 2003; Ferguson *et al.*, 2005; Knols *et al.*, 2003; Lounibos, 2003; Scott *et al.*, 2002). The application of this new approach ultimately relies on the natural behavior and ecology of the mosquitoes, specifically mating Behaviour. The possible and one way this gene will be transferred from males carrying the desired gene to females is through mating between released males and wild females to introduce the desired gene. Therefore, all factors that may reduce or impact male mating success, such as sexual selection needs to be well addressed to pave way for their implementation. To enhance the success of such control programs, these results suggest that 1) thorough evaluation of traits that give males a mating advantage is needed. 2) The condition under which these traits are fully expressed need to be optimized and incorporated in mass rearing schemes of males destined for release.

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CHAPTER 4

HANS SMID

Physiological fitness of free-living and laboratory-reared male *Anopheles gambiae* s.l.

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> **Abstract**

Laboratory experimentation forms the basis for most of our knowledge of the biology of many organisms, in particular insects. However, the accuracy with which laboratory-derived estimates of insect life history and behaviour can predict their fitness and population dynamics in the wild is rarely validated. Such comparison is especially important in cases where laboratory-derived information is used to formulate and implement strategies for the genetic control of insects in nature. Here we conducted a comparative study of the reproductive potential and life-history of male Anopheles gambiae Gilies sensu lato mosquitoes from both standardized laboratory conditions and from natural field settings. We measured three indirect indicators of male mosquito fitness: energetic reserves, body size, and survival, in a bid to determine whether the demographics and energetic limitations of wild males can be correctly predicted from their laboratory counterparts. Crucially, the body size and lipid reserves of wild males were substantially greater than those reared under standard laboratory conditions. We caution that the energetic limitations of insects as identified in the laboratory may underestimate their resilience in the wild, and discuss the implications of this phenomenon with respect to vector-borne disease control programmes based on genetic control of mosquitoes.

Introduction

An inherent feature of laboratory-based experimentation is the quest to limit extraneous sources of variation that may obscure detection of relationships between an outcome variable and hypothetical causative or correlated factors of interest. This usually requires studying organisms in simplified environments, potentially creating bias generally deemed acceptable when weighed against the powerful hypothesis testing that these simplifications permit. However, when the aim of laboratory experiments is to estimate parameters to guide the implementation of interventions aimed at natural populations, whether to protect or suppress them, it is essential to know how closely the physiology and behaviour of laboratory-maintained individuals represent those from the wild. The task of contrasting the responses of laboratory-reared and free-living organisms has held a low profile, but must now be reprioritized to assist numerous animal and plant population control programmes which rely on releases of captive-reared individuals.

Much of our knowledge of insect ecology and evolution comes from laboratory experimentation. However, the accuracy with which these laboratory-derived estimates of insect life history and behaviour can predict the fitness and population dynamics of insects in the wild is uncertain. Unlike homeotherms, the development and demography of insects is heavily dependent on climate and other environmental variables (Carey, 2001), and can also vary substantially in response to subtle differences in diet (Chang, 2004; Gary, Foster, 2001; Held, Potter, 2004; Jorgensen, Toft, 1997; Straif, Beier, 1996). Given their dependence on environmental variation, behavioural and life-history traits documented under standardized laboratory conditions could grossly misrepresent the complexity and norms of insect behaviours. Critically, laboratory studies using insects reared in captivity may not represent the resilience of their populations to natural disturbances and/or human interventions.

In the case of insect vectors of disease, inappropriate extrapolation of laboratory results could have substantial economic and public health implications. The recent development of genetically-modified (GM) *Anopheles* mosquitoes that block the development of malaria parasites (Capurro *et al.*, 2000; Christophides, 2005; Ito *et al.*, 2002; Tabachnick, 2003), and the use of sterile insects to suppress pest population growth (Benedict, Robinson, 2003; Dyck *et al.*, 2005), serve as excellent examples of this issue. Both these approaches require the release of laboratory-reared individuals in the wild; with the GM approach seeking to reduce malaria by introducing a parasite refractory gene into natural populations, and the Sterile Insect approach to suppress population growth by inducing wild females to mate with infertile laboratory-reared males. Ethically, only male mosquitoes could be released in such programmes as the release of more blood-feeding females would at best increase the biting nuisance, or at worst, the transmission of other vector-borne pathogens and possibly even malaria itself if transgenic females are not 100% refractory.

The mating ability and survival of laboratory-reared, GM or sterile males when released into the wild is thus critical to the success of these enterprises. However, comparisons of the fitness of genetically-modified and wild type mosquitoes have thus far been made only under laboratory conditions (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Moreira, 2000; Moreira *et al.*, 2004). Colonization can alter the mating behaviour of laboratory-reared mosquitoes and generate selection for assortative

mating traits. The evolution of assortative mating preferences reduces the ability to mate with wild type female conspecifics, and can occur in as few as three generations of laboratory maintenance (Reisen, 2003). Direct field tests of the competitiveness of laboratory-reared genetically modified mosquitoes when pitted against wild males must necessarily wait until concerns regarding the ethics, biosecurity and efficacy of this approach are resolved (Knols *et al.*, 2007; Mshinda *et al.*, 2004). In the meantime, substantial progress towards assessing the effectiveness of the GM and Sterile Insect approach could be made by contrasting the fitness of male mosquitoes when mass-reared in the laboratory, and allowed to forage freely in nature; to our knowledge has never been conducted on male African *Anopheles*.

The reproductive potential and fitness of male mosquitoes can be indirectly measured by their energetic reserves as adults (Briegel, 1990; Van Handel, 1984). These reserves, accumulated during larval development and/or from blood or sugar-feeding as adults (Briegel, 2003; Foster, 1995), are critical determinants of adult survival and mating ability (Briegel, 1990; Timmermann, Briegel, 1999; Van Handel, 1988). Three key energetic reserves of adult mosquitoes are lipids, glycogen and sugar. Lipids are required for long-term maintenance (e.g. survival), and are primarily acquired through feeding during larval development, and sugar feeding as adults (Briegel *et al.*, 2001; Van Handel, 1984). Flight is a requirement for mosquito mating, an activity fuelled by sugars or glycogens derived from sucrose or its components fructose and glucose in nectar, honey-dew and fruit juices (Briegel, 2003; Foster, 1995; Nayar, Sauerman, 1987; Rowley, Graham, 1968; Van Handel, 1984). Body size is another indirect measure of mosquito reproductive success, with several studies showing that larger individuals have greater reproductive success (Ng'habi *et al.*, 2005; Takken *et al.*, 1998; Yuval *et al.*, 1993).

It is generally assumed that free-living insects are subject to much harsher environmental conditions than those being reared in a laboratory, and thus will generally be smaller in size and have lower levels of energetic reserves than those reared in standardized, controlled environments. This suggests that mosquitoes reared in standardized laboratory conditions should be better provisioned to out-compete wild individuals upon release. If this is not true, any fitness cost conferred by a refractory gene (Catteruccia *et al.*, 2003; Irvin *et al.*, 2004; Moreira *et al.*, 2004), or irradiation (Helinski *et al.*, 2006) in the case of the sterile insect technique, will be further inflated by the poorer physiological condition of laboratory-reared mosquitoes. Here we investigate how key nutritional resources and body size vary between laboratory-reared and free-living male mosquitoes from southern Tanzania. We focused on male *An. gambiae s.s.* Giles and its sibling species *An. arabiensis* Patton because little is known about the biology of this sex (Ferguson *et al.*, 2005), and because these species are the most important vectors of malaria in Africa (Gillies, DeMeillon, 1968; White, 1974) and thus a leading target for control measures based on the release of genetically-modified (Ito *et al.*, 2002; Moreira, Jacobs-Lorena, 2003) and/or sterile males (Helinski *et al.*, 2006). Consequently there is an urgent need to understand the life-history and performance of free-living male *An. gambiae s.l.,* and evaluate the extent to which their behaviour, physiology and reproductive potential can be inferred from laboratory observation.

Materials and methods

Field collection and dissections

Anopheles gambiae Giles *sensu lato* were collected in Lupiro village in the Kilombero valley of Tanzania (Charlwood *et al.*, 1995). Over a 4-week period in 2005 (mid May- mid June), we conducted daily resting catches in the morning (6-8 am.) in approximately 10 houses and outdoor toilets to collect *Anopheles* mosquitoes. Males visually identified as belonging to the *An. gambiae s.l.* species complex (Gillies, DeMeillon, 1968) were kept for dissection which was done within one hour after collection. Mosquitoes were killed by shaking them in a holding cup ; thereafter one leg was removed from each male *An. gambiae s.l.* and stored in an eppendorf tube containing silica gel for genotypic identification to sibling species level using PCR (Scott *et al.*, 1993).

Additionally, one wing was removed and measured under a dissecting microscope fitted with an ocular micrometer (1 unit = 0.35 mm). Mosquito wing length is often used as a proxy for body size as it is a fixed, relatively easy to measure trait, and is positively correlated with body mass in most species (Koella, Lyimo, 1996; Nasci, 1990; Siegel *et al.*, 1992). The relationship between wing length and body weight is variable, and its exact nature can differ between mosquitoes of different species, strain, and rearing background (Nasci, 1990; Siegel *et al.*, 1992; Siegel *et al.*, 1994). Despite this limitation, Anopheline mosquito wing length has consistently been shown to be a significant predictor of traits such as fecundity and survival (Ameneshewa, Service, 1996; Hogg *et al.*, 1996; Kittayapong *et al.*, 1992; Lehmann *et al.*, 2006; Lyimo, Takken, 1993), and thus was selected as a useful approximator of mosquito fitness here.

After wing removal, the remainder of the mosquito body was placed in a drop of PBS on a cavity microscope slide. Under a dissecting microscope (10×), the reproductive system of males was removed using dissecting pins and examined under a compound microscope (50×). Three key features of the male reproductive system that have been associated with male *An. gambiae* age (Huho *et al.*, 2006) were observed and scored: the number of spermatocysts in the testes, proportion of the testes occupied by the sperm reservoir, and presence or absence of a clear border surrounding the edge of the accessory gland. Remaining male body parts and fluids were washed into a test tube using 100µl of 100% ethanol. In the field, these tubes were heated at approximately 90^0C for 10 minutes over a heating block in order to temporarily fix and preserve energetic reserves for subsequent biochemical analysis in the laboratory. Following this protocol, samples can be stored for up to two weeks at room temperature before being processed (H. Briegel, personal communication).

Mosquito species identification

DNA was extracted from legs of individual wild-caught male *An. gambiae* by placing them individually in an eppendorf tube containing 15µl of Tris-EDTA (TE) buffer, and then crushing them using a micropestle. Three microlitres of this solution were used for DNA extraction. A master mix containing DNA templates for the *An. gambiae* species complex was prepared, and added to each DNA sample to initiate the PCR (Scott *et al.*, 1993). Only two *An. gambiae s.l.* species were represented within our field sample namely *An. arabiensis* and *An. gambiae s. s.* Giles.

Laboratory reared mosquitoes

Male *An. gambiae s. s.* from the insectary at Ifakara Health Research Development Centre, were used for comparison with wild mosquitoes. These mosquitoes originated from a rearing colonized from wild individuals collected at Njage village, Kilombero in 1996. Since then, these mosquitoes have been reared in laboratory conditions perceived as ideal for survival and reproduction. As larvae, they are maintained on a standard diet of TetraMin® fish food at densities of 150-200 larvae per 100 ml of water in a larval tray (32 cm × 12 cm × 15 cm). Upon emergence, adult males were pooled in a separate cage and maintained on 10% glucose solution, at ambient conditions (approximately 28-30°C, 70-80% relative humidity and a photoperiod of 14:10 h (L:D). From these cohorts of males, groups of different age (1-20 days) were randomly sampled and subjected to biochemical analysis to assess their energetic reserves. Their body size was also estimated from their wing length as described above. As with the wild-collected mosquitoes, laboratory-reared males had one leg, one wing and their reproductive system removed before their remaining parts were fixed in ethanol and stored for further biochemical analysis. *Anopheles gambiae s. s*. was the only captive reference strain available in the laboratory group.

Laboratory quantification of sugars, glycogen and lipids

We determined the contents of three key energetic reserves in field and laboratory collected mosquitoes using a spectrophotometric method originally devised by Van Handel (Van Handel, 1985; Van Handel, 1985). Standard curves were made by measuring the absorbency of known concentration of cooking oil (lipids) and glucose (sugars and glycogen). Then the standard curve was used to obtain the strait line equation from which the amount of lipids and sugars present in mosquito bodies were estimated.

Age grading of wild male mosquitoes

Previously we have shown that an age-grading method based on male reproductive morphology originally devised for *Anopheles stefensi and An. culicifacies* (Mahmood, Reisen, 1982; Mahmood, Reisen, 1994) can be successfully adapted for male *An. gambiae* s.s (Huho *et al.*, 2006). Information on the number of spermatocysts, relative size of the sperm reservoir, and presence of a clear area surrounding male accessory glands was used to classify male *An. gambiae s.s.* into age categories of 'young' (≤ 4 days post emergence) and old (> 4 days) with 89% accuracy (Huho *et al.*, 2006). We applied this model here to age-grade wild-collected males, and test for any association between age and reserve abundance.

Data analysis

Preliminary analysis of the total glycogen, sugar, and lipid content of male mosquitoes indicated that these reserves did not follow a normal distribution (Kolmogorov-Smirnov normality test, $p < 0.001$). Consequently we used the non-parametric Mann-Whitney U test for two independent samples to test for differences in reserve levels between the following treatment groups: 1) laboratory-reared and field-collected males, 2) field-collected *An. gambiae s.s.* and *An. arabiensis* males, and 3) males of different age categories (two age groups: ≤ 4 days post emergence, or older). Laboratory-reared males were excluded from the analysis of between-species variation in reserve levels

to avoid confounding species differences with those generated by rearing condition (as only one species, *An. gambiae s. s.* was represented in the laboratory group). Relationships between male body size and reserve levels were investigated using Spearman's correlation coefficient (non-parametric), and analysis of variance was used to test if there were differences in body size between treatment groups that could account for observed differences in reserve levels. All data were statistically analyzed using SPSS (version 11.5). Unless otherwise stated, numbers in parentheses following means represent one standard error.

Results

A total of 482 male *Anopheles gambiae s.l.* were captured over 28 days of resting catches. Of these, 459 were successfully identified to species level by PCR. *Anopheles gambiae s. s.* was the dominant species in this sample (86.7% versus 13.3% *An. arabiensis).* A sample of 190 *An. gambiae s. s.* males was obtained from our laboratory colony and analyzed for comparison with this field sample.

Energetic reserves in laboratory reared and wild male An. gambiae s. s.

The majority of males collected in the field had no detectable sugar levels (70%) and many also tested negative for glycogen (30%). In contrast, 83% and 90% of laboratory-reared males tested positive for sugars and glycogen respectively. Males from the laboratory were 12 times more likely to test positive for sugar than the field group (χ^2 = 158.15, p<0.01, Odds ratio = 12.93, 95% CI: [8.31-20.13]**),** and 4 times more likely to test positive for glycogen (χ^2 = 34.29, p<0.01, Odds ratio = 4.32, 95% CI: [2.57-7.24]**)**. In contrast, lipids were detected at a much higher frequency in wild than in laboratory-reared males (97.3% Vs 78% prevalent in wild and lab males respectively, χ^2 =54.78, p<0.01, Odds ratio = 9.98, 95% CI: [5.0-19.91]).

Not only the prevalence, but also the abundance of sugars was higher in laboratory-reared male *An. gambiae s. s.,* than in their wild conspecifics (Mann Whitney U= 13397.0, P < 0.01, $Median_{LAB}$=8.01 μg, $Median_{FIELD}$=0.00 μg, Figure 1A, B). Similarly, glycogen content was higher in laboratory-reared males; being on average three times greater than the amount found in wild males (Mann Whitney U= 19783.5, P < 0.01, $Median_{LAB}$ =15.26μg, $Median_{FIELD}$= 4.21μg, Figure 1C, D). In contrast, lipid content in wild *An. gambiae s. s.* males was more than twice that of laboratory-reared individuals (Mann Whitney U =23035, P < 0.01, $Median_{LAB}$ =4. 6 μg, $Median_{FIELD}$= 9.6 μg, Figure 1E, F).

Adult body size also varied significantly between laboratory-reared and field-collected *An. gambiae s. s.* ($F_{1, 554}$ = 436.77, P > 0.001). Wild males were approximately 17% larger than laboratory-reared individuals ($Mean_{LAB}$=2.17 mm [0.011], $Mean_{FIELD}$=2.54 mm [.010]). Body size was substantially more variable in field-collected males (range 1.86-3.14 mm) than in the laboratory-reared males (range 1.89 - 2.57 mm). Male body size was positively correlated with lipid stores in both laboratory-reared and wild male *An. gambiae s. s.* (Table 1; Figure 2C). In contrast, the amount of glycogen and sugars in males was not associated with the body size of either laboratory or field-collected males (Table 1; Figure 2A, B).

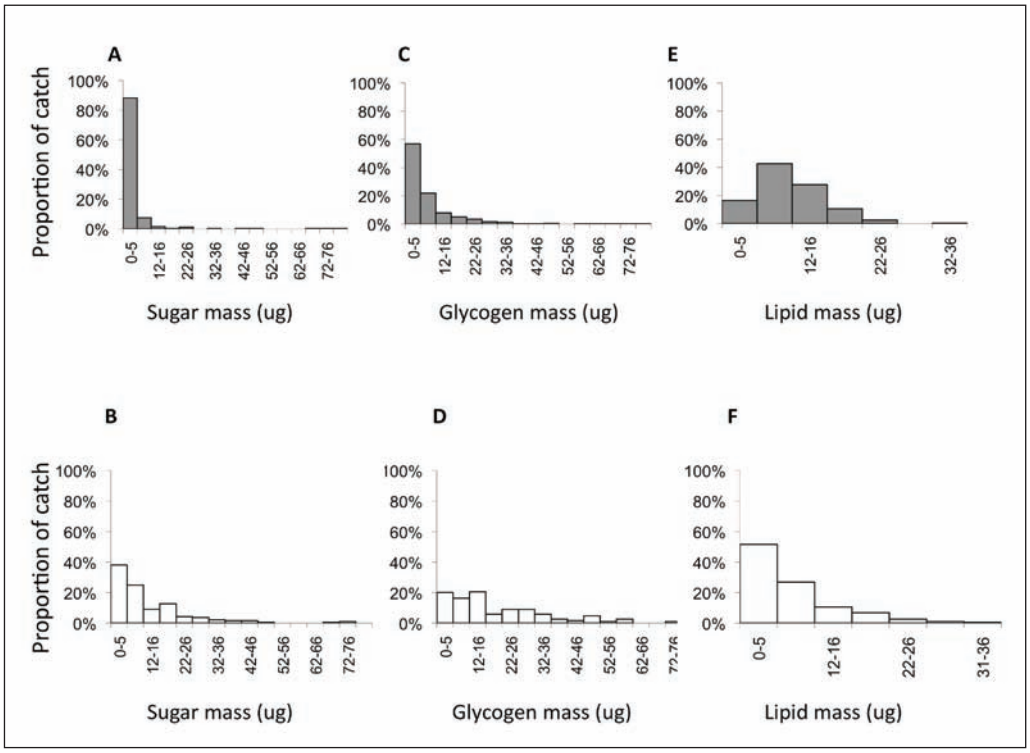


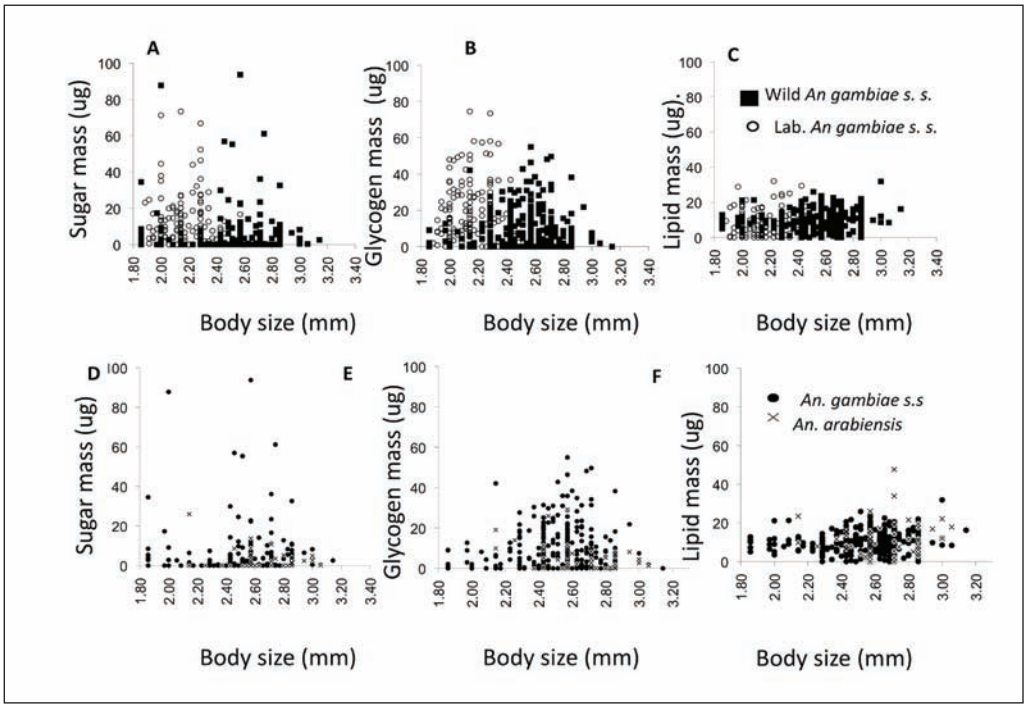
Figure 1: Frequency distribution of masses of three key energy reserves detected in laboratory-reared (white bars) and wild (grey bars) male *An. gambiae* s. s.

Between-species differences in energetic reserves of wild collected mosquitoes

Restricting the analysis to field-collected mosquitoes, the quantity of stored reserves did not vary between *An. arabiensis* and *An. gambiae s. s.* (Table 2). Despite the lack of a statistical difference in reserve abundance between mosquito species, *An. arabiensis* males were significantly larger than *An. gambiae s.s.* ($F_{1,457} = 11.38$, $P < 0.01$, $Mean_{ARABIENSIS}$=2.63 mm [0.024], $Mean_{GAMBIAE}$=2.54 mm [0.010]). Thus for a given unit of body length, *An. gambiae s. s.* contained a higher abundance of energetic reserves than *An. arabiensis*. Body size was positively correlated with sugar abundance in *An. arabiensis*, but not in *An. gambiae s. s.* as detailed above (Table 1, Figure 2D). Lipids were positively correlated with body size in field-collected *An. gambiae s. s.,* but not *An. arabiensis* (Figure 2F). Neither species showed any association between body size and glycogen (Table 1; Figure 2E).

Variation in energetic reserves with age

The age of laboratory-reared males was known with certainty because this was tracked from emergence. To facilitate comparison with age-grades available for the field sample, we pooled our laboratory-sample into two age groups of 'young' (≤ 4, days post emergence) and 'old' (> 4 post emergence. The morphologically-based method we applied to age-grade our sample of wild males into similar categories has an accuracy of approximately 89% (Huho *et al.*, 2006). Our aim was to test whether energetic provisions change with age in both wild and laboratory-reared males. One



Figure·2: Relationship between body size and three key energetic reserves in (1) laboratory-reared (A, C, E; open circles) and free-living male *An. gambiae* s. s. (A, C, E; Black squires) and (2) *An. gambiae* s. s. (B, D, F; black circles) and *An. arabiensis* males (B, D, F; croses)

potentially confounding factor when testing for age-related changes, or lack thereof, is size-selective mortality. If small males die earlier than large males, the older age group both in the laboratory and the field may be disproportionately represented by large males who inherently have greater reserve levels; a phenomenon that could obscure any decline in reserve abundance with age. To rule this out, we first tested whether the body size of young and old males varied. We found that indeed the average body size of 'old' males was greater than that of 'young' males in both field ($F_{1, 389}$=12.11, p<0.01) and laboratory samples ($F_{1, 157}$=4.78, p=0.03), indicating that size selective mortality is operating in both populations. We then sub-selected from within our field-collected *An. gambiae s. s., An. arabiensis,* and lab-collected *An. gambiae s. s.,* to obtain samples of 'young' and 'old' males of approximately equal body size. This was done by calculating the mean body size for males in each of the three groups, and eliminating individuals whose body size fell outside one standard deviation of this mean. Subsequent statistical analysis revealed no statistical difference in body size between 'young' and 'old' males within these sub-samples (p = 0.43 for field *An. arabiensis*, p = 0.79 field *An. gambiae s. s.* and p = 0.77 laboratory *An. gambiae s. s.).* Within these size-restricted groups, there was no difference in sugar or lipid mass between 'young' and 'old' males (Table 3). However, there was a substantial increase in glycogen content in older males within field-collected the *An. gambiae s. s.* sample (Table 3); an observation that was not evident within the laboratory group or wild *An. arabiensis* males.

Table 1: Correlations between male mosquito body size and energetic reserves. Asterisks (*) indicate correlations that were statistically significant (P < 0.05).

Background	Species	Spearman's correlation with body size		
		Sugars	Lipids	Glycogen
Laboratory	*An. gambiae s.s*	-0.03	0.18*	-0.13
Field	*An. gambiae s.s*	0.04	0.12*	-0.02
	An. arabiensis	0.29*	0.14	-0.24

Discussion

Here we show that despite the apparent harshness and stochastic nature of environmental conditions in the wild, male *Anopheles* mosquitoes allowed to forage freely in nature outperform individuals reared in apparently 'ideal' laboratory conditions with respect to at least two key determinants of adult survival: body size and lipid reserves. In contrast and perhaps unsurprisingly given their *ad libitum* glucose diet, laboratory-reared males had substantially greater reserves of sugar and glycogen than wild males. These findings challenge the notion that measures of insect fitness and reproductive potential will be upwardly biased in laboratory studies, and stimulate re-evaluation of the optimal rearing conditions for mosquito development and maintenance. Interestingly, these conclusions differ from those reported for female mosquitoes, in which levels of sugar, glycogen and lipid were always higher in laboratory-reared than field collected individuals (Day, Van Handel, 1986). This discrepancy between studies of males and females suggests the existence of sex-specific variation in mosquito energetic budget, and that males may require a broader range of nutritional resources to maximize their energetic reserves than females.

While the higher sugar and glycogen content of laboratory-reared mosquitoes was expected, the greater lipid reserves of wild male *Anopheles* was not. Unlike wild male mosquitoes, *An. gambiae s. s.* maintained in the laboratory had a guaranteed supply of sugar at all times of day, and was rendered largely inactive due the limited confines of their cages. The higher accumulation of sugars and glycogen under these conditions in contrast to free-living mosquitoes is thus not surprising, and suggests that wild males do not sugar-feed to repletion, probably due to limitations in the availability of sugar sources. Alternatively, free living mosquitoes may sugar-feed less during the night than laboratory males, resulting in a lower detectable of carbohydrate reserves when they were sampled in the early morning hours.

We hypothesize that the larger lipid stores of field-collected males is a by-product of their larger body size. Female anophelines are known to accumulate lipids in a size dependent manner (Briegel, 1990), an observation supported here for males. As mosquito body size is determined almost entirely by larval nutrition and microclimate (Briegel, 1990; Timmermann, Briegel, 1999), the artificial larval habitats we created in the laboratory must have been of lower quality to males than those in the wild. Identifying the key features that account for the higher quality of natural larval habitats would not only assist in improving laboratory rearing methods, but also widen our understanding of larval ecology.

Table 2: Median value of energetic reserves in two Anopheline species with different rearing backgrounds. In all cases, reserve levels in field-collected *An. gambiae* s. s. were statistically different from reared in the laboratory (Mann-Whitney U test, p<0.05). Reserve levels were not statistically different between field-collected *An. gambiae* s. s. and

	Median value of reserve (µg)		
	An. gambiae s.s		*An. arabiensis*
	Laboratory	Field	Field
Sugars	8.01	0	0
Glycogen	15.26	4.21	2.62
Lipids	4.54	9.67	10.36

In light of these findings, what can we conclude about the likely success of laboratory-reared versus wild male mosquitoes when competing against each other in nature? Ultimately the relative success of male mosquitoes is determined by their lifetime mating success; a composite measure depending on both their ability to obtain mates on a particular swarming event, and the number of swarming events in which they can participate (correlated with survival). With respect to the first component of male reproductive success, sugars and glycogen are known to determine male mating success in a swarm; with the ability to initiate and sustain swarming being positively associated with carbohydrates reserves (Briegel *et al.*, 2001; Nayar, Sauerman, 1987; Rowley, Graham, 1968; Yuval *et al.*, 1994). Thus the higher abundance of sugars and glycogen in laboratory-reared males may predispose them towards greater competitive success in a swarm. However, males from the field have substantially greater body size than those from the laboratory; a trait that has also been associated with greater competitive success in a swarm in some (Ng'habi *et al.*, 2005; Yuval *et al.*, 1994) but not all studies (Charlwood *et al.*, 2002).

In terms of the second component of male mosquito lifetime reproductive fitness, adult survival, free-living males should have an advantage because they have greater lipid stores than laboratory-derived males. Several studies have shown long-term survival is positively associated with lipid abundance in mosquitoes (Briegel, 1990; Service, 1987; Van Handel, 1984) and in other insects such as *Drosophila melanogaster* Meigen (Service, 1987). Adult body size is also positively associated with survival, with this study and others showing that larger mosquitoes live longer (Ameneshewa, Service, 1996; Hawley, 1985; Reisen *et al.*, 1984). Thus both the body size and lipid provisioning of wild males incline them towards substantially greater survival than laboratory-reared individuals. If this advantage outweighs the possibly shorter term benefit of relatively higher sugar content, it is likely that free-living males will have higher physiologically-determined reproductive potential than their laboratory-reared counterparts.

It is generally assumed that in nature, male mosquitoes depend upon sugars from plant juices for longevity and other reproductive functions (Foster, 1995; Van Handel, Day, 1990; Yuval *et al.*, 1994). However in this study, we found only small amounts of free sugars in field-collected males; with the vast majority having no detectable levels of sugar. This contrasts with studies of *Anopheles freeborni* Aitken, that found substantial levels of sugars in males sampled in resting catches (Yuval *et al.*, 1994).

An. arabiensis (described in text).

Table 3: Median values of reserves in three sub-samples of male mosquitoes, selected to generate groups in which the body size of 'young' and 'old' individuals were similar. N^R gives the number of males included in these size-restricted samples. 'F' indicates males that were collected in the field, and 'L' males obtained from a laboratory colony. An asterisk

Group	Age	Median reserves (μg)			
		N^R	Sugars	Glycogen	Lipids
An. gambiae s. s. (L)	Young	51	8.37	15.72	4.95
	Old	49	9.70	15.32	4.36
An. gambiae s. s. (F)	Young	110	0	3.77*	9.34
	Old	196	0	5.59*	9.92
An. arabiensis (F)	Young	10	0	3.74	7.66

Anopheles gambiae males may have a lower dependence on sugar feeding or may replenish their reserves at different times of day than *An. freeborni*. Consequently, the importance of sugar-feeding for male *Anopheles* remains an open question and likely varies substantially between species, populations, and habitats (Foster, 1995). Further comparative analyses of the physiology of *Anopheles* species in different environments with different floral sugar resources will help resolve this issue.

There were no measurable between-species differences in the abundance of energetic reserves in wild *An. gambiae s. s.* and *An. arabiensis*. Interestingly, reserve levels were constant across these two species despite the fact that *An. arabiensis* was significantly larger than *An. gambiae s.s.* As lipid levels, both in this study and others, are known to increase with body size (Yuval *et al.*, 1994), it is unclear why *An. arabiensis* did not gain an energetic advantage from its increased body size. One possibility stems from the observation that *An. arabiensis* generally store more water than *An. gambiae s.s.* (M. Kirby, personal communication), a feature which may explain why they are capable of tolerating drier conditions than *An. gambiae s.s.* (Coluzzi *et al.*, 1979). Thus *An. arabiensis* may devote a smaller proportion of its total body volume to the storage of energetic reserves than does *An. gambiae s. s.* in order to increase its capacity for water storage.

Energetic reserves changed little with male mosquito age; with the only observed difference being an age-related increase in glycogen in field-collected *An. gambiae s. s.* It is unclear why this species' laboratory-reared counterparts did not exhibit a similar increase in this resource with age. One possibility is that sugar resources were so readily abundant to laboratory males that this resource became saturated in their tissues early in life, and simply could not increase further as they aged. Further analyses of male mosquito resource use and energy budget in nature will help identify additional proximate physiological markers of their survival and reproductive success. We note that glycogen is used primarily to fuel mosquito flight (Briegel *et al.*, 2001).

The fact that this resource increased with age in the *An. gambiae s. s.* field groups suggests that older males should be equally or even more capable of swarming and dispersal than young males; and thus male reproductive fitness may not decrease with age.

Our findings highlight the importance of validating laboratory-derived estimates of insect physiology and fitness within a field-realistic context. We have shown that indirect estimates of male mosquito fitness as obtained from measurement of body size and energetic reserves differ between field and laboratory populations and not in a consistent direction (e.g. laboratory mosquitoes do not always have higher or lower reserve levels than field mosquitoes). Specifically, our findings suggest that if one is to release laboratory-reared male mosquitoes of this stature (small and with lower lipid reserves) their likeliness to survive as long as its wild counterparts may be reduced unless they can build up lipid reserves rapidly. However, it remains unknown whether the physiological fitness differences also translate in the Darwinian fitness (i.e. the number of granddaughters produced), mating competition experiments need to be done with field and laboratory collected males to unravel this phenomenon. We caution that these conclusions have been reached by considering only physiological determinants of survival, and acknowledge there may be behavioral factors that alter the relative performance of these phenotypes in nature. Previous control efforts based on releasing laboratory-reared males suggest these behavioral factors would give an advantage of field males (Benedict, Robinson, 2003; Ferguson *et al.*, 2005), a conclusion that does not differ from what we predict from physiology. Further studies to explore the intrinsic determinants of the mating success and survival determinants of wild versus laboratory reared *insect* vectors, especially those that are the target of genetic control for disease control, are strongly encouraged.

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HANS SMID

Establishment of a self-propagating population of the African malaria vector *An. arabiensis* under semi-field conditions

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To be submitted as: Ng'habi, K.R., Mwasheshi, D., Knols, B.G.J. & Ferguson, H.M. Establishment of a self-propagating population of the African malaria vector *An. arabiensis* under semi-field conditions.

> **Abstract**

The successful control of insect disease vectors relies on a thorough understanding of their ecology and behaviour. However, knowledge of the ecology of many human disease vectors lags behind that of plant pests. This is partially due to the paucity of experimental tools for investigating their ecology under natural conditions without the risk of exposure to disease. Assessment of vector life-history and demographic traits under natural conditions has also been hindered by the inherent difficulty of sampling these seasonally and temporally varying populations with a limited range of sampling tools. Consequently much of our knowledge of vector biology comes from the studies of laboratory colonies, which may not accurately represent the genetic and behavioural diversity of natural populations. Contained semi-field systems (SFS) have been proposed as more appropriate tools for the study of vector ecology. SFS are relatively large, netting-enclosed, mesocosms in which vectors can fly freely, feed on natural plant and vertebrate host sources, and access realistic resting and oviposition sites. Vertebrate hosts used in these systems can be maintained parasite-free, ensuring that vector populations exposed to them will remain uninfected. Despite the advantages of such systems, few have been successfully established and shown capable of maintaining vector populations over many generations. This study reports on the first successful establishment and maintenance of a population of the African malaria vector, *An. arabiensis* under SFS conditions for multiple generations (>24). This population was established within a large field cage (21 X 9.1 X 7.1 m) at the Ifakara Health Institute, Tanzania, and set up to mimic the typical village environment where these vectors naturally occur. The observed zoophagy, time from blood feeding to oviposition, larval development time, adult outdoor resting, plant-sugar feeding and swarming within the SFS match closely with what has been reported under natural conditions, suggesting that the ecology of *An. arabiensis* was accurately represented. This development presents proof-of-principle that populations of important African malaria vectors can be established within environmentally realistic, contained semi-field settings. Such SFS will be valuable tools for experimental study of vector ecology and for the assessment of their short and longer-term evolutionary responses to existing (e.g. insecticide treated nets) and new (e.g. genetically modified and sterile insects) interventions.

Background

In Africa, current frontline strategies for reducing malaria transmission and their impact on morbidity and mortality rely on the use of residual insecticides through application on Insecticide Treated Nets (ITNs) and indoor residual spraying (IRS) inside houses. The contribution of these strategies to reduce child mortality and morbidity has been considerable (Abdulla *et al.*, 2005; Fegan *et al.*, 2007; Hawley *et al.*, 2003; Lindblade *et al.*, 2004; Müller *et al.*, 2006). However, these approaches are facing challenges and limitations as the mosquito vectors they target are increasingly becoming resistant to insecticides (Müller *et al.*, 2008; Ranson *et al.*, 2009) and many exhibit behavioural plasticity (e.g. biting and resting outside of houses, or early in the evening before people are under nets) that limits their contact with insecticides (Geissbühler *et al.*, 2007), indicating that these strategies alone may not be sufficient and that new control strategies are needed to supplement them (Ferguson *et al.*, 2010).

One of the challenges undermining contemporary vector control strategies is our limited understanding of the ecological complexities that allow vector populations to persist and evade control approaches. For example with African malaria vectors, there is insufficient understanding of mosquito life-history processes that occur outside of the domestic environments (e.g. houses) where they usually bite, including their oviposition, larval development, sugar feeding, and dispersal; all of which have crucial implications for their survival, population dynamics and vulnerability to control interventions (Ferguson *et al.*, 2010; Lyimo and Ferguson, 2009; Müller and Schlein, 2006). Most vector control studies are understandably focused at developing and evaluating specific control interventions within a given ecological context. While such studies provide the ultimate evidence necessary for evaluating whether to adopt a particular strategy, failure to concurrently measure the ecological parameters of the target vector population during the trial means that little evidence is available to interpret why an intervention failed, and what aspects of its implementation could be modified to achieve greater success in the longer term. Paying explicit attention to mosquito ecology is vital not only for interpretation of why some otherwise well proven interventions are less effective than expected (e.g. ITNs, if vectors do not bite indoors), but also for identifying other vulnerabilities in the mosquito life cycle that could be targeted by novel methods. Examples of the latter include the demonstration that insecticides can be applied to plant sugar sources to reduce anopheline populations (Müller and Schlein, 2006; Müller and Schlein, 2008), or mosquitoes themselves can be used to spread insecticides as they disperse between oviposition and resting sites (Devine *et al.*, 2009).

Gaining insight into ecological processes of malaria vectors such as these is both logistically difficult and expensive in natural field settings because of the lack of sampling tools for reliably measuring the abundance and behavioural diversity of the different species, sexes and life-history stages of mosquito vectors inside and outside of domestic environments, and the substantial heterogeneity in their density over time and space (Lindsay *et al.*, 1998; Magbity and Lines, 2002). These inherent challenges resulted in many researchers adapting a laboratory experimental approach to quantify key aspects of mosquito life-history and demography (Armbruster and Hutchinson, 2002; Lyimo *et al.*, 1992). However it is widely recognised by these researchers

and the wider vector biology community that laboratory conditions are unlikely to adequately represent vector fitness and behaviour in nature, nor the range of selective pressures acting on mosquito life history and demographic parameters they encounter. Furthermore, the artificial feeding and rearing regimes used in laboratory colonies have been associated with the appearance of behaviours (Lefèvre *et al.*, 2009; Pates *et al.*, 2001) and phenotypic traits (e.g. body size and energetic reserves) that are atypical of corresponding field populations (Huho *et al.*, 2007). Consequently in order to progress understanding of vector ecology beyond the limitations of current field and laboratory approaches, there is an urgent need for more environmentally realistic and larger-scale experimental systems where mosquito vector behaviour, ecology and population dynamics can be studied in a natural context, over multiple generations.

Contained semi-field systems (SFS) have been proposed as more realistic and reliable experimental tools for the characterization and manipulation of mosquito vector ecology and population dynamics (Ferguson *et al.*, 2008; Knols *et al.*, 2007; Knols and Louis, 2006; Knols *et al.*, 2002; Knols *et al.*, 2003). An SFS is defined as an experimental mesocosm, situated within the natural environment of the target vector population exposed to similar climatic conditions, within which all (natural) dietary and habitat resources for vector life-cycle completion are present (Ferguson *et al.*, 2008; Knols *et al.*, 2002). The movement of insect vectors either in or outside of the SFS is typically prevented by netting which blocks their dispersal but not natural airflow or other climatic influences. A key benefit of SFS is that they can permit the maintenance of large vector populations in a situation where mating and other behavioural activities can occur more naturally than in traditional laboratory cages, and inbreeding may be less likely to occur (Chapter 7). SFS can be set up to mimic the natural climatic and habitat features of natural mosquito vector environments, including ambiently varying climate/light conditions, and realistic resting microhabitats, dietary resources and predators (Ferguson *et al.*, 2008; Knols *et al.*, 2003). It is thus expected that the demographics, genetic composition, behaviour and life-history of vectors maintained under such semi-field conditions will be much more representative of wild populations than is the case in typical laboratory colonies. Another advantage of such systems is that in contrast to open field studies, the exposure of workers to pathogens such as the malaria parasites can be eliminated. In the absence of malaria risk, researchers can provide a wider range of experimental manipulations within the SFS, including deliberate exposure to mosquito biting, than would be ethically permissible in the field. Finally, by facilitating detailed study of a defined vector population over time, SFS provides a unique opportunity to investigate their evolutionary as well as ecological dynamics in response to experimental manipulations that can be designed to mimic the effect of current or predicted environmental change (e.g. introduction of control intervention); a feat very difficult to achieve under natural conditions.

Although the value of SFS as a broad-ranging experimental tool for research on vectors of malaria and other diseases is being increasingly recognized (Ferguson *et al.*, 2008; Knols *et al.*, 2007; Knols and Louis, 2006; Knols *et al.*, 2003; Stone *et al.*, 2009), very few such systems have been successfully established (Knols *et al.*, 2002), and none so far have reported the successful maintenance of a vector population under SFS for multiple generations, as would be required to monitor phenotypic and evolutionary changes in their behaviour, genetic structure and population dynamics through time. Here we report the first successful long-term establishment (>24

generations) of an African malaria vector population under SFS conditions within an area of endemic transmission in Southern Tanzania (Ferguson *et al.*, 2008). The study focused on the establishment of *Anopheles arabiensis,* a widespread and increasingly important vector of malaria in Africa (Bayoh *et al.*, 2010; Coetzee, 2004). As the abundance of *An. gambiae s.s*, historically attributed as the most prolific African vector, is shrinking in many parts of the continent following the widespread use of ITNs, *An. arabiensis* now plays the primary role in malaria transmission in many areas (Bayoh *et al.*, 2010; Lindblade *et al.*, 2006). Given the growing importance of *An. arabiensis*, there is increased interest in obtaining more knowledge of its ecology and behaviour to stimulate new approaches for its control. The successful establishment of long-term *An. arabiensis* populations under SFS conditions where their behaviour and life-history are relatively natural, would provide an invaluable tool for gaining such knowledge.

Methods

Experimental set up

A large netting-enclosed semi field system (SFS) was constructed for the study of malaria vector ecology and behaviour at the Ifakara Health Institute (IHI) in southern Tanzania (Ferguson *et al.*, 2008). This 1800 m^3 facility is situated on the main campus of the IHI, which is within the Kilombero valley (7'44"- 9'29"S/ 35'33" -36'56" E), an area of high malaria endemicity where intense levels of transmission are maintained year round (Drakeley *et al.*, 2003; Smith *et al.*, 1993; Smith *et al.*, 1995). Although all of the three main major African vector species are found in this area (*An. arabiensis, An. funestus* and *An. gambiae s.s.*) transmission is largely dominated by *An. arabiensis* which composes >85% of the vector population in most areas (Charlwood *et al.*, 1995;

Figure 1: The inside of the SFS experimental chamber where the *An. arabiensis* population was established, showing; (A) natural vegetation and planted food crops. (B) clay pots used as resting sites, and (C) artificial larval habitats with a moat to exclude ants.

Figure 2: (A) Large, medium and small larval habitats of different sizes placed inside the SFS, (B) a calf host used to provide blood meals to adult females, and (C) a trap for collecting adults emerging from a larval habitat.

Killeen *et al.*, 2006; Takken *et al.*, 1998).

One large experimental chamber (21 x 9.1 x 7.1 m) of the SFS was set aside for the establishment of a long-term *An. arabiensis* population. This chamber is enclosed from the surrounding environment by PVC coated polyester netting (346 holes, per inch^2 Polytex UK) with the interior set up to mimic the natural habitat features of the rural village environment where *An. arabiensis* are typically found in Kilombero and other regions of East Africa. The floor of the chamber was covered with 30 cm of soil obtained from the nearby area, and the vegetation emerging from seeds therein was allowed to grow naturally (e.g. grasses, Figure 1a). In addition, a variety of other food crops which are normally cultivated around rural homesteads were also planted in the system (e.g. banana plants (*Musa paradisiacal*), papaya (*Carica papaya*), and sweet potatoes (*Ipomoea batatas*). Furthermore, Castorbean (*Ricinus communis* L.) plants, on which anopheline mosquitoes have been observed to frequently rest/feed in parts of East Africa (Impoinvil *et al.*, 2004; Manda *et al.*, 2007) were also planted. Although the four walls of the experimental chamber were separated from the surrounding environment only by netting, the decision was taken to cover the roof with polyethylene plastic (plastic film, Filclair Serren Industry N.V.) to provide flexibility to experimentally manipulate rainfall in future research, and also protect the area from the rare but extremely heavy rains and flooding that occasionally occur in the Kilombero area. Consequently, vegetation within the system was watered by sprinklers three times each week. A traditional mud walled house, cattle shed and a chicken coop were constructed following local design to provide adult resting sites (Figure 1) (Ferguson *et al.*, 2008). Clay pots (23 total) partially filled with water (to provide humidity) were also distributed throughout the compartment and positioned at an oblique angle (Figure 1b) to provide additional refuge sites to adult mosquitoes (Odiere *et al.*, 2007). Clay pots were locally made and are commonly used for water storage and cooking.

In the wild, *An. arabiensis* typically lay their eggs in small, shallow sunlit pools of water ranging in size from puddles and hoof prints to large swamps and can include man-made as well as naturally-occurring water storage bodies, free from canopy cover with less turbid water (Minakawa *et al.*, 2005; Munga *et al.*, 2005). To mimic natural sites, artificial larval habitats (Figure 1c) of variable sizes (large, medium and small) were made by half filling plastic basins with soil (as a base) and then covering them with water (Figure 2a). This design was used to give females the opportunity to land and lay their eggs on the wet soil or shallow water as they are observed to do in nature (Huang *et al.*, 2007). Twenty large artificial larval habitats (diameter 43.5, depth 0-5 cm) were made by burying plastic containers to ground level, and partially filling them with local soil before adding water. The soil layer acted as a source for microbial and/or algal growth which provides food for larval growth and development. In addition, five medium-sized (diameter 19.5, water depth 0-4 cm) and five small (diameter 13.5, water depth 0-3 cm) artificial larval habitats (Figure 2a) were also distributed in the compartment. Water depth was maintained through daily replenishment with tap water.

Establishment of An. arabiensis

The population of *An. arabiensis* established in the SFS was founded from the eggs of a wild population in the nearby village of Sagamaganga (~20km from the IHI, -8.0667 S; 36.8000 E). This village is situated along the flood plains of the Kilombero River where anopheline larval habitats are abundant. Most residents of this village are pastoralists who keep livestock year round. As *An. arabiensis* has a preference for feeding on cows over humans when both are available (Highton *et al.*, 1979), and is usually found in close association with livestock (Hadis *et al.*, 1997), we anticipated that this vector would be the dominant member of the *An. gambiae* species complex in this area. Sagamaganga was selected as the source population because pilot work showed that *An. arabiensis* constituted approximately 90% of the *An. gambiae s.l.* complex in this area (Mayagaya & Ferguson, pers. comm)

Live blood-fed females that were morphologically identified as *An. gambiae s.l.* were collected from houses and animal sheds using mouth aspirators in Sagamaganga, May 2008. Collections were made continuously until enough females were obtained to produce the target number of larvae to be released into the SFS was achieved (approximately 3000 released into artificial large larval habitats over one week in May 2008). On the day of their capture, blood-fed females were transported to the IHI semi-field insectary where they were transferred into individual cups containing wet filter paper to allow them to oviposit. After oviposition, wild-collected females were killed and subjected to PCR analysis to confirm their species identity (Scott *et al.*, 1993). Larval offspring of all females identified as *An. arabiensis* were pooled and added to the artificial larval habitats in the SFS (Figure 1c).

Prior to egg and larval release in the SFS, a variety of other invertebrates that are naturally found in and around houses in rural Tanzania (e.g. praying mantids (Mantidae), grasshoppers (Acridoidea), ants (Formicidae) and important *Anopheles* predators such as jumping spiders (Salticidae) (Jackson *et al.*, 2005) were observed in the SFS. These predators probably entered the system in soil and building material during the chamber set-up (prior to mosquito introduction), and were allowed to establish within it. Once *An. arabiensis* was introduced, any further movement of

insects and larger organisms into or out of the system was prevented by the netting enclosing all walls and doors, and the closed roof. After introduction, larvae were not provided with any source of food other than the micro-organisms growing naturally within larval habitats.

Adult maintenance and blood feeding

Adults emerging from these larval habitats were allowed to fly freely in the SFS and feed on sugars from a variety of available plants. In nature, both male and female adult *Anopheles* are known to feed on plant sugars, which has been shown to enhance their survival in the wild (Clements, 1999; Yuval, 1992) as well as in semi-field conditions (Okech *et al.*, 2003). A major issue when establishing long-term, free-flying mosquito populations in SFS is to ensure containment and prevent the accidental introduction of malaria parasites that could infect mosquitoes and pose an infection risk to researchers. To achieve this, four steps were routinely undertaken. First, mosquito containment was ensured by the installment of a triple door entry-system into the chamber which prevented direct entrance or accidental mosquito escape (each door is opened and closed independently). Second, the integrity of the outer netted walls and roof was checked thrice weekly during inspections. Third, blood meals were provided only from calves (which *An. arabiensis* feed on commonly under natural conditions; Figure 2b). As cattle save as dead-end host for human malaria parasites, mosquitoes that have fed only on them are incapable of becoming infected or transmitting parasites to humans. Host blood was provided to mosquitoes by introducing a calf into the SFS every evening from 7.00 PM - 7.00 AM for five consecutive nights each week. Two different calves were used on alternate nights. Finally, all research staff working in the area were screened for malaria parasites on a weekly basis using a Rapid Diagnostic Kit (Moody, 2002) before being allowed to enter the system. Any staff that tested positive for malaria was immediately given a full course of antimalarial treatment (artemisinin combination therapy, ACT), and restricted from entering the SFS for at least 2 weeks after their infection was cleared.

Regular entomological monitoring

Daily temperatures inside microhabitats within the SFS was monitored by placing data loggers (Tiny tag™) in aquatic larval habitats and potential adult resting sites (e.g. inside the mud walled house and clay pots). Water temperature was monitored only in large and small larval habitats by submerging data loggers (Tiny tag™). After the introduction of *An. arabiensis* eggs, larval habitats were inspected daily for the presence of larvae and pupae. Larval development was monitored for eighteen days from the day they were released as first instar into ten larval habitats. Emerging adults were monitored by setting up emergence traps (Figure 2c) over all large larval habitats in the SFS. Emerging adults captured in these traps were counted and then released into the SFS. *Anopheles arabiensis* population growth was assessed for the first five consecutive generations. The number of adults emerging (as assessed by capture from emergence traps) was used to assess the growth of *An. arabiensis* population over time in the SFS.

The duration between consecutive generations was also estimated in the SFS. This was done by first estimating the number of days it took for first instars of a new generation (e.g. F2) to appear after females of previous generation (e.g. F1), had taken

their first blood meal. This was followed by estimating the number of days it took for first instars to emerge as pupae. The median larval development time was also assessed. This was achieved by daily inspection of larval habitat and recording number of pupae emerging in each habitat. Therefore, the estimated generation length was estimated by taking the median larval development time plus the number of days it took from blood feeding to the appearance of first instars.

An experiment was set up to assess the effect of larval habitat size on larval survival. Here a subset of large, medium and small larval habitats were covered with netting to prevent oviposition from freely flying females, and 100 first instar larvae (from gravid females collected in the SFS) were experimentally released in small, medium and large larval habitats (Figure 2a). This experiment was run from the 5th to the 14th generation to achieve a total of twenty replicates from each larval habitat size class. All emerging pupae were collected and counted using pipettes and transferred to another larval habitat in the SFS for emergence.

The resting behaviour of both males and females was assessed by counting the number of adults inside mud-walled houses (indoors) and clay pots (outdoors) with the aid of a flash light and counter. The assessment was conducted for three consecutive days at each generation from generation 2 to 7 and was repeated again at generation 24. The number of males and females observed were recorded. The age structure of males and females surviving in the SFS was assessed by randomly collecting 35 males and 176 females from generation 20. Ovaries were dissected to determine parity (Charlwood *et al.*, 1985) and male gonads were dissected to determine age class (Huho *et al.*, 2006).

Data analysis

Analysis of variance (ANOVA) was used to compare the temperatures differences between larval habitats and resting sites inside the SFS using SPSS statistical package (13.0 for windows). Generalised Linear Models were used to test whether larval survival (as assessed by the proportion of pupae emerging from 100 instars) varied between larval habitats of different size classes (R statistical software). Here 'habitat size' was treated as a main effect, and the generation on which observations were made as a random effect. The daily larval survival rate in large larval habitats was also estimated using the formula $S = P^{1/t}$, where p is the proportion of larvae that survived to pupation and t is the mean time to pupation in days (Paajmans *et al.,* 2009). Similarly, generalised linear models were used to test whether the proportion of *An. arabiensis* adults resting inside (combining those found in the houses, cow shed and chicken coop) versus outside varied between sexes.

Results

Microclimatic conditions

Temperature is an important factor in determining larval development time and adult mosquito survival. Water temperatures in large and small larval habitats were statistically different ($F_{1, 10612}$= 31.92, P<0.001, Table 1). The air temperature inside the SFS were

Table 1: Recorded mean temperatures (oC) from mosquito resting sites and larval habitats inside an SFS (Based on recordings made every 1 min over the period from March to October 2008).

Description	Mean temperature (^{o}C)
Larval habitat	
Large size	24.9 ± 0.05 (S.E.M)
Small size	25.3 ± 0.03 (S.E.M)
Resting site	
Inside mud house	28.48 ± 0.06 (S.E.M)
Outdoor clay pot	25.48 ± 0.06 (S.E.M)
Air temperature in SFS	34.23 ± 0.43 (S.E.M)

significantly higher than inside the mud-walled house ($F_{1, 2739}$ = 509.56, P<0.001) and clay pots ($F_{1, 2763}$ = 1189.8, P < 0.001, Table 1). The mean air temperature inside the mud- walled house was significantly higher than air temperature inside clay pots ($F_{1, 4261}$ = 1301.44, P< 0.001, Table 1).

Larval development and population growth

Five days after the calf had been introduced into the SFS, new first instars were observed in mosquito larval habitats confirming that a new generation had been produced inside the SFS. The larval pupation time from the ten large habitats ranged from 6 to 17 days (Figure 3). The median larval development time was estimated to be 11.5 days. Therefore, the time taken for new adults of the F2 generation to emerge was approximated from day 11 to day 22 since their F1 mothers had their first blood meal. Although the exact number of generations that passed since the founder generation could not be precisely established for each member of the population at a given point due to overlapping generations, we conservatively estimated the average time between successive generations to be 22 days. The number of individuals was observed to increase during the first five generations indicating that the SFS population was growing (Figure 4). Larva pupa survival varied significantly between different larval habitat size classes (χ_2^2=62.44, p<0.001 Figure 5). Specifically, the pupation rate in the large-sized habitats (69%) was almost 4 times greater than in the small and medium habitats (Figure 5). The daily larval survival rate in the large larval habitat was estimated to be 0.962 per day.

Larval predation by ants was also observed in the SFS. An attempt was made to reduce ant-related predation by placing larval habitat bowls within a second water-filled outer bowl to provide a protective moat (in order to give mosquitoes the best chance of becoming established, Figure 1c). Initially, larvae were only observed within the designed artificial larval habitats. However, at 3rd generation, female mosquitoes began ovipositing in moat barriers and inside the shallow water pools within clay pots (Figure 1b), thus, ant-predation prevention was not fully successful.

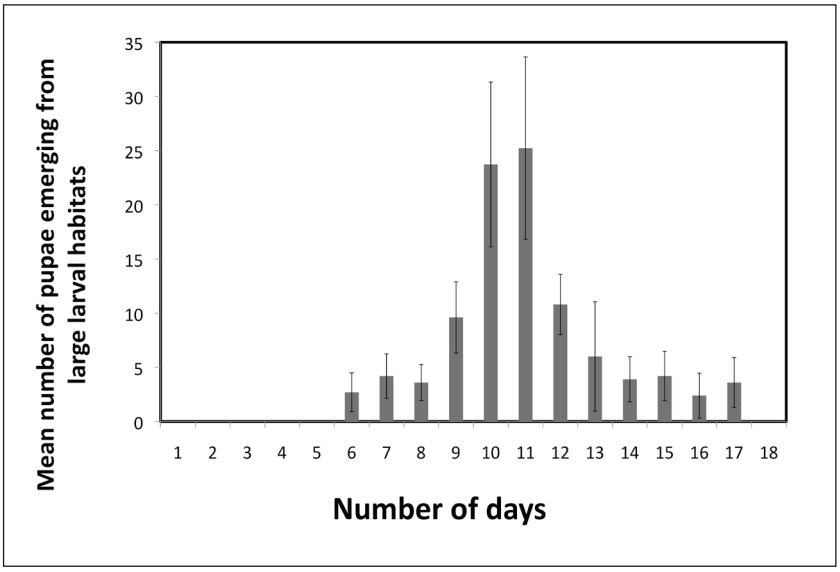

Figure 3: The mean number of pupae observed in large habitats in SFS since the first day larvae were released, for a period of 18-days.

Adult feeding and survival

Of the 35 dissected males, which were collected on the same day in the SFS (generation 20), only four males (11.4%) were classified as being in the ≤ 4 days old age category (Huho *et al.*, 2006). The remaining 31 males (88.8%) had a number of spermatocysts ranging from 0-2 which are estimated to correspond to an age of more than 4 days post-emergence. Of the 176 females collected, 42 and 26 were observed to be either blood fed or gravid respectively. The ovaries of the remaining 108 females were dissected to determine their reproductive history. Within this sample, 38 (35.2%) were virgins, 40 (37.04%) nulliparous and 30 (27.8%) previously laid eggs. However, we could not precisely age-grade the parous class (such as 1st, 2nd and 3rd gonotrophic cycles).

Resting behaviour and reproduction

Despite variation in the mean temperature between resting sites (Table 1), both sexes of the *An. arabiensis* mosquitoes were observed to rest in all resting sites (Figure 6). While both sexes were more frequently found resting in outdoor resting pots than in indoor environments, males were substantially more exophilic than females (χ_3^2=223.05, P<0.001, Figure 6). There was significant difference between male and female resting preference in the four different resting sites (χ_3^2 = 222.67, P<0.001, Figure 6). Swarming activity was also occasionally observed. Observed swarms consisted of 50-100 males that formed at dusk, (approximately around 7.30 PM in one side of the SFS where the horizon was visible, and lasted for 15-20 minutes.

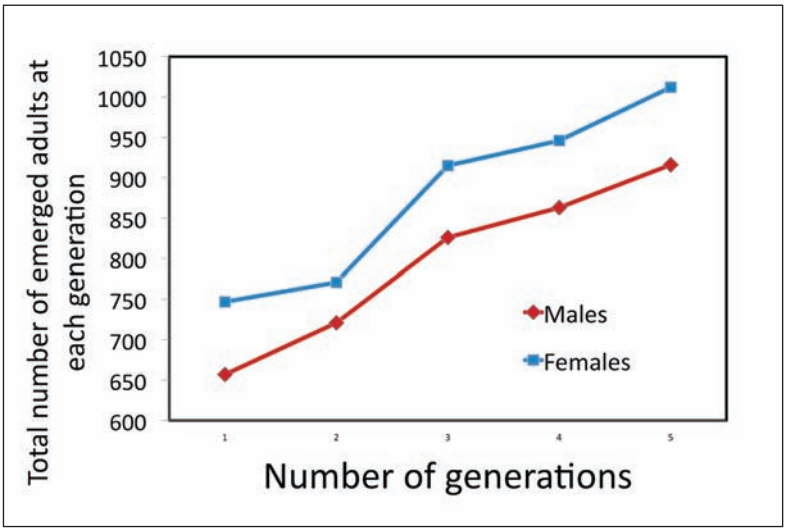

Figure 4: The total number of adult mosquitoes collected in emergence traps from large larval habitats for five consecutive generations.

Discussion

The mean daily temperatures recorded in all larval habitats, was within the range of recorded natural *An. gambiae s.l.* larval habitats (20°C -35°C) and did not exceed the upper tolerance limit of 40°C for *An. gambiae s.l.* (Haddow, 1943; Huang *et al.*, 2006; Huang *et al.*, 2007). In nature larvae are known to feed on microbes that promote growth and development. Thus, it is most likely that algae and microbes were the main source of larval food in SFS habitats (Gimnig *et al.*, 2002; Kaufman *et al.*, 2006; Walker and Merritt, 1993). The larval development time observed in the SFS (6 to 17 days), falls within the range of larval development time reported for *An. gambiae s.l.* mosquitoes (Pfaehler *et al.*, 2006). Also the larval survival estimated in large habitats was in line with what has been reported in other studies (Paaijmans *et al.*, 2009). Larval pupation rate in large larval habitats was 4 times higher than medium and small habitats which is in agreement with field reports that, in the absence of predators and pathogens the amount of food available to larvae at a particular habitat, determines the number of emerging adults (Gimnig *et al.*, 2001; Ye-Ebiyo *et al.*, 2003). Therefore, the observed poor larval survival in both medium and small larval habitats in the SFS could potentially be a result of lower amount of algae/microbes available for larval growth and development (Minakawa *et al.*, 2005; Rejmankova *et al.*, 1993). Since larvae ingest particles indiscriminately (Ye-Ebiyo *et al.*, 2003), and because no additional food was added, lower microbial growth in medium and small habitat may have caused larvae to ingest inedible particles such as clay and silt that may have killed most of the larvae by damaging their intestines (Paaijmans *et al.*, 2009). As water temperatures in large and small larval habitats was inline with the range of *An. gambiae s.l.* reported larval-habitat temperatures in nature (Huang *et al.*, 2006), the variation in larval survival between habitats could be explained by resource limitation rather than temperature variation.

Mosquitoes were found to rest in all designed resting sites despite differences in mean temperatures, which confirms the ability of this species to tolerate varying temperature ranges (Gray *et al.*, 2005; Kirby and Lindsay, 2004). The daily mean

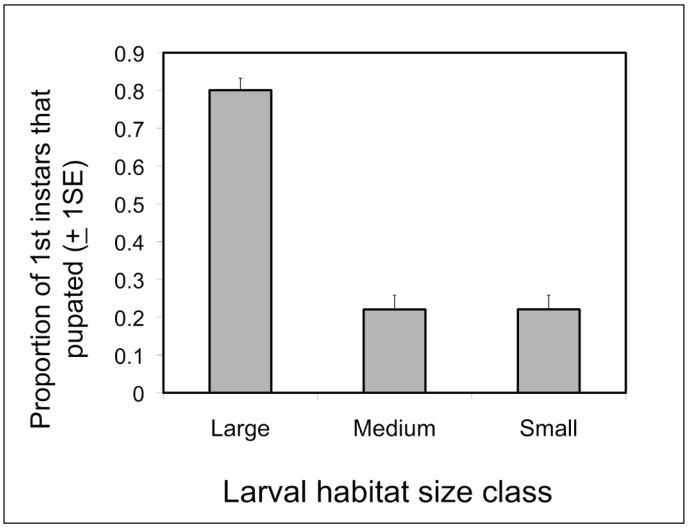

Figure 5: The proportion of first instar larvae (n=1465) that pupated in large, medium and small larval habitats in the semi-field system (error bars indicate S.E.).

temperatures recorded in all resting sites were within the reported temperature range and did not exceed the *An. arabiensis* adult temperature tolerance limit (Kirby and Lindsay, 2004). This may have allowed adult mosquitoes to survive and behave naturally. For example, the observation that female *An. arabiensis* in the SFS readily blood-fed on the cow host and oviposited thereafter is inline with the widespread observation that this species, especially from East Africa is facultative zoophilic (White *et al.*, 1972) and can maintain populations in the absence of human hosts. However, exceptions have been reported from West Africa and Madagascar where this species has been reported to be anthropophilic (Duchemin *et al.*, 2001; Tirados *et al.*, 2006). This study also observed that the period between females taking their first blood meal to the time when first instars appeared was 4-5 days (including approximately 1-2 days when first instars become visible after hatching), which has been reported in natural field populations (Bruce-Chwatt, 1985).

In nature plants are the major source of sugar for both male and female mosquitoes (Clements, 1999; Gary and Foster, 2006; Impoinvil *et al.*, 2004; Manda *et al.*, 2007) and these can readily imbibe and digest plant juices and nectar to enhance their survival (Foster, 1995; Gary and Foster, 2004). Because adult mosquitoes can rarely survive for more than 48 hours without the availability of an energy source (Foster, 1995), plants inside the SFS must have served as the major energy source for male mosquitoes, and may have served as a nutritional supplement to females. Studies to analyse plant sugars in mosquito gut content are needed to verify this (Junnila *et al.*, 2010). As males rarely survive for more than 48h without a sugar source, the presence of large proportion of males 80% (n=37) survived for more than four days, provides further evidence for successful survival on plants and that plants in the SFS system provided suitable sugar sources for their survival (Gary and Foster, 2004). However, further studies such as gut content analysis should confirm whether they have been feeding on plants nectar and the type of plant they have been feeding on. It can also be deduced that females readily fed from calf-hosts and oviposited, with at least 28% surviving their first gonotrophic cycle. A high proportion of nulliparous females (37%) could be the result of freshly emerged females on that day. The observed mosquito

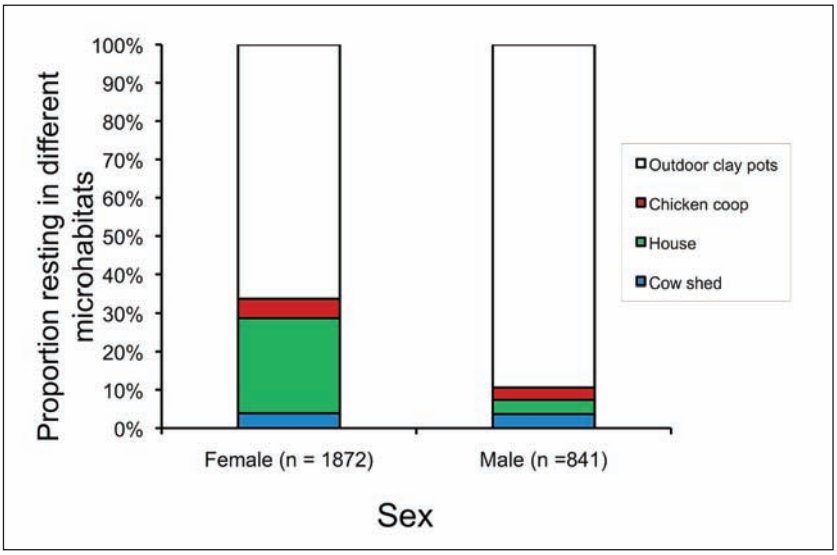

Figure 6: The proportion male (n = 841) and female (n = 1872) mosquitoes resting in different resting sites inside the SFS.

survival in the SFS is in line with the reported mean survival of field mosquitoes in nature which is about 6-9 days and that 65% of them die within the first week after emergence (Gillies, 1988). Although the age-grading methods used in this study provide a general indication of adult mosquito age, we could not precisely estimate how long individuals survived beyond 4 days and recommend further age-grading studies. More precise chronological age estimation methods will be required to estimate the life span (Mayagaya *et al.*, 2009). Swarming behaviour has been suggested to be typical in mosquitoes with the main purpose of reproduction (Charlwood and Jones, 1980; Clements, 1992; Diabate *et al.*, 2003). However, in East Africa, male anopheline swarms have been difficult to observe in nature, possibly due to the fact that they occur at dusk when visibility is poor (Charlwood *et al.*, 2003; Diabate *et al.*, 2003; Marchand, 1984) or deploy other mating strategies like indoor mating (Dao *et al.*, 2008). Due to its sporadic occurrence, we could not clearly establish whether swarming was the primary mating strategy employed by mosquitoes in the SFS. Further investigation is needed to quantitatively describe swarming behaviour to unravel the mating strategy that *An. arabiensis* uses in the SFS.

There are several advantages of this SFS approach: characterization of mosquito demography, life-history and behaviour under field conditions can be difficult and time consuming given the relatively low efficiency of some sampling tools for some mosquito phenotypes such as resting and mating (Lindsay *et al.*, 1995; Magbity and Lines, 2002; Ribeiro *et al.*, 1996; Smith *et al.*, 1995). Therefore, SFS populations provide a readier and easier-to-observe source of vectors whose behaviour and other ecological parameters can be monitored with greater precision than in the open field. Another major advantage of this development is that a range of valuable experimental manipulations that present some ethical concerns in the field can be performed with relatively low risk in SFS settings. For example, whereas human landing catches, the gold standard for assessing human biting rates (Geissbühler *et al.*, 2007; Killeen *et al.*, 2006), are becoming increasingly difficult to justify in the field because of the exposure risk they present to participants, they can be conducted safely within a SFS.

Furthermore, techniques such as mark-release-recapture which can provide valuable insights into mosquito demography and habitat use can be applied with low risk in the SFS because there is no chance of releasing potentially infectious mosquitoes into the community.

Although we argue that biological inferences generated in semi-field systems may provide a much more accurate representation of field populations than laboratory colonies, we caution that they also have limitations. For example in the SFS, there were no interaction between *An. arabiensis* and other mosquito species in the larval habitat (Koenraadt *et al.*, 2004) and ample breeding sites probably minimized cannibalism which is known to occur (Koenraadt and Takken, 2003). Also, unlike in field settings, pressure from insecticides or other vector control interventions was absent and the diversity of natural predators was limited. Humans (research staff) were generally not present within the regular host-seeking activity period of this vector, usually from 10 pm to 5 am (Gillies, 1957), thus human-mosquito interaction was limited. Although the absence of malaria parasites within the *An. arabiensis* population in the system may have eliminated another source of selection pressure acting on natural mosquito populations, this has a very minimal effect on mosquito reproduction and survival as only 1-2% of individuals get infected in natural field conditions (Chege and Beier, 1990; Ferguson *et al.*, 2003; Ferguson and Read, 2002). Thus semi-field studies should be seen as bridging ground and not replacement of field studies and wherever possible observations from these studies should be further verified under fully natural conditions.

In the context of time, cost effectiveness of research and outputs, enclosed semi-natural ecosystems offer a safer and more logistically feasible alternative than field studies. These enclosed mosquito ecosystems provide a suitable intermediate between laboratory-based research and those of field conditions. Studies of vector ecology and fitness conducted on laboratory maintained colonies are likely to oversimplify genetic, life-history and behavioural heterogeneity of mosquitoes in their natural environment, and may be poorly representative of mosquito population and evolutionary dynamics in the field (Knols *et al.*, 2002). For example, laboratory conditions alter energetic reserves (Huho *et al.*, 2007), genetic variation (Mukhopadhyay *et al.*, 1997; Norris *et al.*, 2001) as well as host preference (Lefèvre *et al.*, 2009) of maintained species. However, not all vector species have been able to adapt to laboratory conditions. It has proven difficult to colonize some important mosquito species such as *An. funestus* and *Mansonia annulata* under laboratory conditions (Casimiro *et al.*, 2006; Samung *et al.*, 2006), presenting an obstacle to investigation of their biology and their disease transmission cycles. The relatively more natural ecological conditions within the SFS may prove more amenable for the establishment of such vector populations.

In addition to the above benefits, this tool has a much wider range of potential uses including the evaluation of a whole range of different vector control interventions. SFS research has the potential to develop and evaluate new field sampling tools and trapping methods within a short period of time, such as repellants/attractants (Okumu *et al.*, 2010; Okumu *et al.*, 2009) as well as insecticide auto-dissemination (Devine *et al.*, 2009). The long-term establishment of vector population allows the testing of some very critical ecological questions that are almost impossible or even too risky to address under open field conditions. The prediction of behavioural and

physiological resistances of vectors against specific interventions such as ITNs and the quantification of the strength of selection pressures imposed by specific intervention, may appropriately be measured under controlled semi-field environment (Ferguson *et al.*, 2010). The use of sterile and genetically transformed mosquitoes for control of malaria and other vector borne diseases has largely been confined under laboratory findings. The concept behind these approaches is that the mass-release of sterile and/or transgenic males would lower pathogen transmission either by reducing vector population density and/or making a wild vector population refractory to parasite infection, respectively (Catteruccia, 2007; Helinski *et al.*, 2006; Ito *et al.*, 2002; Kim *et al.*, 2004; Lobo *et al.*, 2006). Because field release is irreversible, a semi-field mosquito population can be an intermediate testing ground to evaluate the feasibility of these approaches prior to their field application.

Conclusions

We report the successfull establishment of a self-propagating vector population in an enclosed semi natural environment, thereby providing a valuable new research tool for conducting experimental investigations of the vector species' behaviour and ecological characteristics under environmental conditions that are largely representative of natural conditions. The finding of this study shows that, climatic conditions within the SFS are broadly similar to those within natural *Anopheles* transmission settings, and that mosquito in the SFS exhibited similar life-history and behavioural traits as those in the field. Additional analyses not presented here indicate that SFS populations maintain a higher degree of genetic diversity than those established in the laboratory, and maintain a closer genetic relatedness to field populations through time than laboratory colonies (Chapter 7). This development will therefore help to move the long-awaited transfer of new genetic control technologies from bench confinement to field application. We expect that, with this development, more studies will be planned to assess the behaviour and other ecological parameters of both natural and genetically-engineered mosquitoes to assess their demography, competitiveness as well as vulnerability to interventions under semi-natural conditions. We hope that the demonstrated feasibility of this approach will stimulate the development of new interventions and optmise available control approaches to cover wider range of vectors species in various settings.

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HANS SMID

A new robust diagnostic polymerase chain reaction (PCR)-based method for determining the mating status of female *An. gambiae* mosquitoes

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> **Abstract**

The principal mosquito vector of human malaria in Africa, *Anopheles gambiae* contains two pairs of autosomes and one pair of sex chromosomes. The Y-chromosome contains a male determining factor and other Y-chromosome specific DNA sequences, which are transferred to females during mating. A reliable tool to determine the mating status of dried wild *An. gambiae* females is currently lacking. DNA was extracted from dried virgin and mated females and used to test whether Y-chromosome specific PCR markers can be successfully amplified and used as a predictor of mating. Three male-specific primers were used in this study. Here we report a new PCR-based method to determine the mating status among successfully inseminated and virgin wild *An. gambiae* females. This dissection-free method has the potential to facilitate studies of both population demographics and gene flow studies from dried mosquito samples routinely collected in epidemiological monitoring, and aid existing and new malaria-vector control approaches.

Introduction

Current knowledge suggests that sex chromosomes of many groups of animals and plants evolved from a pair of ordinary autosomes that acquired a major sex-determining locus (Charlesworth and Charlesworth 2000; Muller 1932). The Y-chromosome, frequently associated with sex-determination, and its associated genes, have been well explored in a range of vertebrate organisms (White 1973). Natural selection is believed to have suppressed the ability of the Y-chromosome to recombine with its counterpart (Skaletsky et al. 2003). Recombination suppression is considered to be responsible for a gradual Y-chromosome genetic degradation (Bachtrog 2003a). The silencing of most of the genes present on the Y-chromosome (Bachtrog 2003b; Steinemann and Steinemann 2000) as a result of degradation explains why only a few male fertility-determining genes remain functional on this chromosome (Carvalho et al. 2001; Carvalho et al. 2000). Among invertebrates, Y-linked gene studies have been limited to *Drosophila* and flea beetles (Steinemann and Steinemann 1998; Nielsen, 1999) and were recently extended to *An. gambiae* (Krzywinski 2004; Krzywinski et al. 2005).

The *An. gambiae* mosquito has a karyotype consisting of two pairs of autosomes (Chromosome 2 and 3) and one pair of sex chromosomes (Chromosome X and Y). The Y chromosome constitutes ~10% of the whole genome and contains a male determining factor which, when present in an XX/XY system, induces male development (Clements 1992). Y-chromosome linked DNA fragments have been characterized and Y-chromosome specific PCR markers have been developed (Krzywinski 2004; Krzywinski et al. 2005). This finding has been viewed as a milestone towards the application of novel vector control strategies for which a thorough understanding of population structure and gene flow among *An. gambiae* populations is necessary.

Traditionally, detection of mating success among females relied on microscopic dissection of female ovaries or examination of sperm in the female spermatheca. This method is reliable and robust but with the limitations that it is time consuming, labour intensive, and requires fresh specimens. A simple and rapid method to determine the mating status of dried female *An. gambiae* is therefore required in order to analyse large sample sizes within a short period of time. In this study we tested the hypothesis that Y-chromosome specific PCR markers can be successfully amplified from male sperm DNA present in recently inseminated females of *An. gambiae sensu stricto* and *An. arabiensis*. We present this technique as a new dissection-free (PCR based) method to determine the mating status of samples collected during routine entomological surveys.

Materials and Methods

Experimental design

Mosquitoes used in this study were obtained from laboratory colonies obtained from the Malaria Research and Reference Reagent Resource Center (MR4) and maintained at the University of California, Davis. This included a colony of *Anopheles gambiae s. s.* originally collected from Kisumu (East Africa) and a colony of *An. arabiensis* (Dongola

strain) originating from northern Sudan. Pupae of both *An. gambiae s. s.* and *An. arabiensis* were isolated in cages allowing males and females to emerge separately. In this experiment 100 female pupae and 50 male pupae were isolated to provide an appreciable number of virgin females and males respectively. Following adult emergence, 25 virgin females were mixed with 25 males for three days and exposed to natural sunlight (dusk), which stimulated mating. Another 25 virgin females were kept in a separate cage for three days as a control. On day three, 15 virgin females, 15 mated females, besides 10 males were killed and kept in the fridge. After five days, when the samples had dried, DNA was extracted from the whole body of males, virgin and mated females, following the DNAzol extraction protocol (Chomczynski et al. 1997). A sub-sample of 10 mated females was dissected under the microscope to confirm insemination by examination of the spermatheca. The experiment was done in triplicate.

PCR reactions

The PCR reactions consisted of the following concentrations. Five µl of 10x reaction buffer containing 1.5 mM of $MgCl_2$, 0.2 µl of each DNTPs containing 2.5 mM, 0.25 µl of each primer and 0.5 µl of *Taq* polymerase in a total of 50 µl. Primer sequences to amplify rDNA have been published for *An. gambiae* (Krzywinski 2004). Primers were S23 (F: 5'-CAAAACGACAGCAGTTCC-3'; R: 5'-TAAACCAAGTCCGTCGCT-3'), 128125B (F: 5'-AGCGTGGAGGACATACAA-3'; R: 5'-ATGGCAATTTCGTTTTCA-3') and 128125I (F: 5'-GGCCTTAACTAGTCGGGTAT-3'; R: 5'-TGCTTTCCATGGTAGTTTTT-3') as described by Krzywinski (2004). Thermal cycling for all reactions was performed using an MJ Research PTC-225 tetrad thermocycler. Conditions were the same for all primers (S23, 128125B and 128125I): 94° for 3min, 35 cycles of 94° for 20 sec, 55° – 64° for 30 sec, and 72° for 1 min, followed by 72° for 10 min. Four (4) µl of 6x loading buffer was mixed with 8 µl of PCR product and loaded onto 3% agarose gel. The agarose gel was then submerged in the electrophoresis chambers, 2/3 filled with 10x TBE running buffer. The gel was run for 1:30 hrs at 85 mA.

Results

We performed experiments to determine whether a multicopy locus (rDNA) can be amplified in inseminated females. The experiment determined whether the DNA from the male partner of mated *An. gambiae s.s.* and *An. arabiensis* females could be amplified by PCR. A total of 50 virgin females, 57 mated females and 28 males of both *An. gambiae* and *An. arabiensis* were used in the study. Primers S23 and 128125B gave strong amplification in *An. gambiae s.s.* females, while primer 128125I gave the strongest amplification in *An. arabiensis* females. DNA from dried specimens amplified in a similar way as those from fresh specimens. All dissected females were found to have sperm in their spermathecae. Figures 1A&B illustrate that male DNA from inseminated females of both species can be amplified by Y-chromosome specific PCR markers with similar efficiency as in the males themselves. As expected, no amplification was obtained from virgin females.

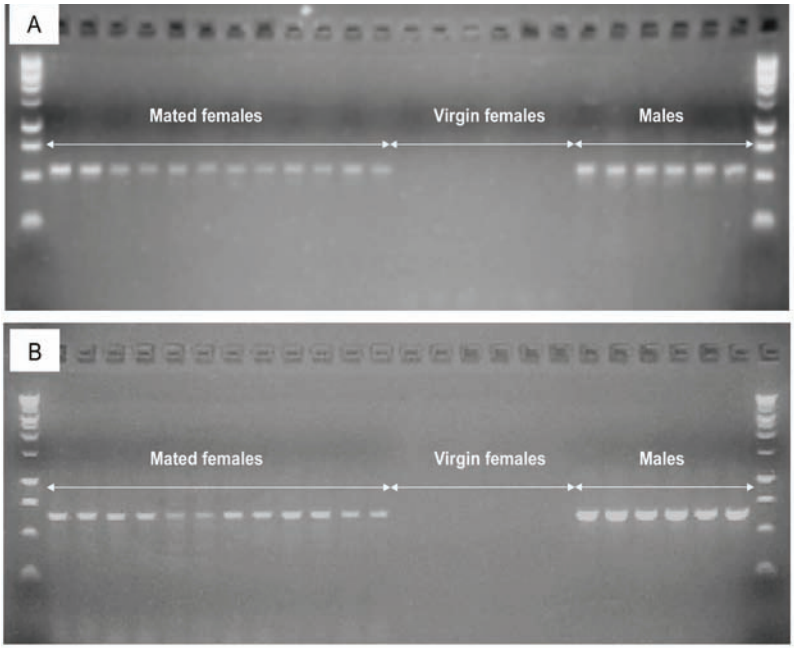

Figure 1: Agarose gel electrophoresis showing amplification of Y chromosome sequences in (A) *An. arabiensis* (primer 128125I) and (B) *An. gambiae* s. s. (Primer S23) males and mated females. There was no amplification in virgin females.

Discussion

We have shown that this method is suitable for distinguishing mated females from virgin females for both *An. gambiae s.s.* and *An. arabiensis* specimens and that this approach lends itself for screening large numbers of recently mated and dried specimens. This finding has useful implications for novel vector control strategies based on the release of large numbers of factory-reared male mosquitoes to mate with virgin wild females to confer sterility or drive parasite-refractory genes into wild populations. In these cases, the transfer of sterile or transgenic sperm from the released population to wild con-specifics will depend on the frequency of mating between males carrying the trait (to be introduced) and the females that do not - even if target genes are linked to transposable elements (Tabachnick, 2003). As mating is ultimately the most appropriate mechanism to deliver the desired traits or genes into a target population (Catteruccia, 2007), an effective method for evaluating mating success is of critical importance. Thus this new, dissection-free technique has potential useful application to novel vector control approaches.

Additionally, this technique will help improve our understanding of gene flow within *An. gambiae* populations by assessing mating frequency. As opposed to methods based on microscopic dissection and direct observation which are conventionally used to assess male *An. gambiae* mating success, this method will make it possible to screen a substantially greater number of wild-collected females either in fresh or dried state. Contained semi-field systems are now becoming a central focus for bridging laboratory confined findings to full field application, in which the feasibility of transgenic and/or sterile male techniques can be assessed (Ferguson *et al.*, 2005; Ferguson *et al.*, 2008; Helinski *et al.*, 2006; Knols *et al.*, 2007; Knols, Louis, 2006). The method developed here can thus make large-scale evaluation of mating success of released males by

repetitive female sampling and screening possible. Although the time since mating can be longer in field samples prior to analysis, it is important to further evaluate the sensitivity of the developed PCR-method against time since mating. This may be due to the depletion of stored sperms in the female spermatheca through fertilization during different gonotrophic (oviposition) cycles. However such shortfall can be minimized by the fact that, few females in the field survive up to three gonotrophic (oviposition) cycle after mating where stored sperms in the spermatheca can be depleted (Gordon and Gordon, 1971).

In conclusion we have demonstrated that mating can be determined by PCR amplification of specific parts of rRNA from inseminated female. The speed and ease of this technique, and the fact that dried specimens can easily be evaluated, indicates this process should allow robust and extensive analysis of large field-collected samples.

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CHAPTER 7

The impact of laboratory versus semi-natural colonization on genetic heterogeneity in the malaria mosquito *Anopheles arabiensis* Patton

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> **Abstract**

The genetic diversity of a population depends on the degree of heterogeneity of the environment it inhabits. The understanding of promoters of genetic variation within populations has critical importance both to test fundamental predictions of evolutionary biology and for applied disciplines such as conservation biology and disease vector control. In this study we aimed to demonstrate that populations maintained under more heterogeneous environmental conditions exhibit and maintain higher genetic diversity than those from more homogeneous laboratory (cage) environments. We simulated a natural ecosystem in a semi-field system in which a self-replicating malaria vector population was established besides two parallel lines of laboratory cage colonies that originated from the same founding population collected in the field. The genetic changes in these populations were observed over time and compared to those of the field population from which they originated. The simulated environmental conditions within the semi-field system presented a more realistic ecosystem which appeared to maintain a higher level of genetic variation (50%) than that observed in cage colonies (45%). Cage colonies experienced higher inbreeding than semi-field individuals. The number of alleles for cage colonies declined more than that of the semi field population. We recommend that colonization of insect vector species designated for research purposes should be undertaken in semi-field systems. Also feasibility studies to evaluate new control approaches can be carried out in semi-field systems such that the results obtained are more representative of a field population than is the case for studies based on cage populations.

Background

The ability of a population to evolve in a changing environment depends on the presence of genetic variation for ecologically relevant traits (Charlesworth and Hughes, 2000; Hoffman and Parsons, 1997). The maintenance of genetic variation in a population depends on factors, such as the long-term effective population size (Kimura, 1983) and the degree of environmental heterogeneity (Gooch and Schopf, 1972; Kassen, 2002). The motivation to understand and prove the relationship between environmental heterogeneity and genetic variation (Schmidt and Conde, 2006) has been fuelled by the discovery of molecular markers such as allozymes (Harris, 1966) and microsatellites (Zheng *et al.*, 1996) which can be used to estimate the level of standing genetic variation maintained in a population. This has critical importance both to test fundamental predictions of evolutionary biology, and for applied disciplines such as conservation biology (Levene, 1953; Popadin *et al.*, 2007) or disease control (Catteruccia, 2007; Lanzaro *et al.*, 1998). The aim here, being to alternatively enhance or decrease the potential of target populations to respond to natural or man-made environmental change. An excellent example of the latter is the study of genetic diversity within insect vectors that transmit human and animal diseases such as malaria (Donnelly *et al.*, 1999; Lanzaro *et al.*, 1998).

The maintenance of insect vectors under laboratory conditions for study purposes is intended to provide a good representation of the field population from which they originated, such that the study of laboratory-colonized vectors can provide valid insights into the biology of their corresponding field population. An unavoidable side-effect of the colonization process is that a substantial proportion of extant natural variation is lost (Matthews and Craig, 1987). Consequently the genetic composition of laboratory cage populations often exhibits significant departures from the original founder population as evidenced by the representation of fewer alleles and lower mean heterozygosity (Mukhopadhyay *et al.*, 1997; Munstermann, 1994; Norris *et al.*, 2001) even when such laboratory colonies are maintained as large populations (≥ 5000 individuals)(Briscoe *et al.*, 1992). Such changes in genetic structure are often accompanied by the appearance of undesired phenotypic traits, such as poor mating ability (Benedict and Robinson, 2003), a phenomenon that could be either a cause or symptom of reductions in diversity (Reed and Frankham, 2003). Given the vital importance of using insect vectors that are behaviourally and genetically representative of natural populations for studies of their biology and susceptibility to control, there is a clear need to develop an improved colonization process that would avoid such effects by maintaining a realistic level of genetic variation.

A contained semi-field system (SFS) (Ferguson *et al.*, 2008; Knols *et al.*, 2002), is proposed as an intermediate to move laboratory-based research into full field application (Knols and Louis, 2006; Knols *et al.*, 2002). Individuals in semi-field systems can mate and behave more naturally than individuals from a cage-based population and are exposed to a more realistic range of environmental heterogeneity including ambiently varying climate/light conditions (Ferguson *et al.*, 2008; Knols *et al.*, 2002; Knols *et al.*, 2003), and a wide range of microhabitats (e.g. resting sites) and dietary resources (e.g. plant sugar sources). It is thus predicted that vectors maintained in a SFS should be much more representative of the genetic structure and behaviour of their field counterparts than those typically reared in confined laboratory cage conditions. This prediction however, has not yet been confirmed, probably due to the scarcity of

semi-field populations available for comparative analysis. Although the importance of semi-field populations has been stated repeatedly (Knols *et al.*, 2007; Knols and Louis, 2006; Knols *et al.*, 2003), very few vector populations, have actually been established, specifically malaria vectors (Knols *et al.*, 2002) and none of them have been established for several generations, as would be required to observe genetic changes over time.

In this study, we present the first analysis of the long-term population genetic dynamics of *Anopheles arabiensis* Patton, one of the most important vectors of human malaria on the continent (Dyck *et al.*, 2005; Knols *et al.*, 2007; Moreira *et al.*, 2004; Tabachnick, 2003). Despite intensified control efforts, malaria is still accounting for an estimated 243 million cases and 863, 000 deaths world wide each year, 85% of which occur in Africa (WHO, 2009). The devastating public health and economic toll imposed by this disease has stimulated efforts to develop alternative means of control to complement traditional front line methods (insecticide-treated nets, indoor residual spraying, and drugs), such as the mass release of genetically-modified GM or sterile males into natural populations where they would mate successfully with wild females. The need to develop a rearing system that would maintain the natural phenotypic and genetic characteristics of males destined for release under such programmes motivated this study.

Working in a highly endemic area in southern Tanzania where *An. arabiensis* is the most important vector of malaria, we simultaneously established self-replicating populations of this species both under standard laboratory cage conditions (2 lines) and in a large SFS (Ferguson *et al.*, 2008) and compared their genetic variability over time with each other and to the field population from where they originated. Our aim was to demonstrate that viable, self-replicating populations of important disease vectors can be maintained under realistic semi-field conditions to provide an alternative to artificial laboratory cage conditions, and test the fundamental prediction from evolutionary theory that populations maintained under more environmentally heterogeneous conditions will exhibit and maintain higher genetic diversity than those from more homogeneous laboratory (cage) environments.

Material and Methods

Live blood-fed females, morphologically identified as *Anopheles gambiae sensu lato*, were collected from 16 houses and 5 animal sheds in Sagamaganga village (Southern Tanzania, 8.0667 S; 36.8000 E) using mouth aspirators, for two weeks in May 2008. Human habitation was in clusters of 2-5 houses, and the distance between clusters was 0.5 -1 km. The distance between a house and animal shed within a cluster was 5-10 m. Collected females were maintained in a cage at the Ifakara Health Institute (IHI) for 1 day before being transferred into individual cups (4.5 x 7 x 7.5 cm) for oviposition. After egg laying, all females were subjected to PCR analysis to confirm their species identity (Ferguson *et al.*, 2008). Eggs of all of those females identified as *An. arabiensis* were pooled and added to larval habitats to initiate populations within laboratory cages and the SFS as described below.

Small cage colony establishment and sample collection

Anopheles arabiensis cage colonies were maintained in an established insectary within a large enclosed SFS (Lanzaro *et al.*, 1998; Zheng *et al.*, 1996), in which temperature and humidity were not controlled and varied depending on the ambient conditions. Two lines of cage colonies were established by gradual release of F1 larvae in rearing trays to approximately 1800 (F1) per line for one week. Larvae were reared inside plastic rearing trays (diameter 43 cm; water depth 5-5.5 cm) and maintained on fish food (Tetramin™). Pupae developing in these rearing trays were randomly moved into small cages (35 x 35 x 35 cm) and maintained on an *ad libitum* diet of 10% glucose.

Blood meals were provided to females in the cage populations by a human volunteer inserting a forearm in the cage for 10 min 3 times per week. Generations were separated by moving all "new eggs" into new rearing trays and emerged adults into new cages. All new rearing trays and cages were labeled according to the number of the generation. For each generation, a total of 100 individuals (50 males + 50 females) were randomly collected from each adult cage population using a mouth aspirator and stored individually in tubes containing silica gel for microsatellite analysis.

Semi-field system colony establishment and sample collection

A contained semi-natural environment of 21 x 9.1 x 7.1 m was installed within a 700 m^2 *Anopheles* mosquito SFS built at the IHI in southern Tanzania (Zheng *et al.*, 1996). The inside of this chamber was designed to mimic the natural habitat of *Anopheles* mosquitoes. Habitat features included vegetation that emerged from seeds that were introduced in the compartment with the soil. Other plants that are common in natural habitats such as Plantain banana (*Musa paradisiacal*), Castor bean (*Ricinus communis*) and Sweet potato (*Ipomoea batata*) were planted (Figure 1). Artificial larval habitats

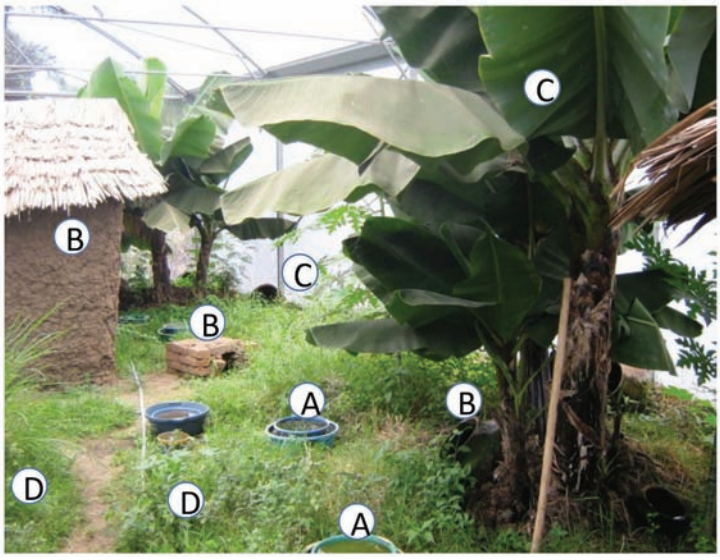

Figure 1: The simulated natural environment inside the semi field system in which a colony was established. (A) artificial breeding site, (B) resting sites, (C) planted crops, (D) naturally emerged plants.

were constructed using round plastic trays (43 cm diameter) similar to those used for rearing cage colony larvae (Figure 1). These were partially filled with soil to allow microbial and/or algal growth. Approximately 3000 *An. arabiensis* larvae, obtained from field-collected females were released into these larval habitats over a one-week period in May 2008. The conditions in the compartment of the SFS (temperature and humidity) varied depending on the ambient conditions. Larvae were not provided with any additional source of food other than the algae and micro-organisms naturally growing within artificial habitats. Adults emerging from these habitats were allowed to freely move throughout the SFS compartment. Females were given the opportunity to blood feed on a calf by placing it inside a cattle pen inside the compartment on most week nights.

In contrast to the cage populations, the more natural conditions within the SFS made it possible for generations to overlap. Consequently, the exact number of generations that had passed from the founder cohort could not be precisely assigned for each member of the population at any given point. In a pilot study conducted in the SFS we found that it takes approximately 12-23 days for adult offspring to emerge since the time their mothers had their first blood meal (Ng'habi *et al.*, unpublished data). Based on this data, we conservatively estimated the average period between generations length within the SFS to be at 20 days. Applying this guideline, a total of 100 emerging adults (50 males + 50 females) were sampled using emergence traps. Emerging adults were sampled at an average of 25 days sampling points for five (5) consecutive days (every 21st to 25th day) in the SFS every generation from the 1st to 10th generation and stored in tubes with silica gel for microsatellite analysis.

New field population samples were collected 10 months later after the SFS and cage colonies had completed 10 generations. Samples were collected from the same place and from same houses and animal sheds using mouth aspirators. Mosquitoes were stored in tubes with silica gel for microsatellite analysis.

Microsatellite DNA analysis

In this study only males from generations 1, 2, 5 and 10 from cage and SFS collected mosquito samples were screened for 11 microsatellite loci from chromosome 2 and 3 (Lanzaro *et al.*, 1995; Lehmann *et al.*, 1996; Walton *et al.*, 1998). The 11 microsatellite markers used were as follows: *AG2H175*, *AG2H85*, *AG2H164*, *AG2H197* and *AG2H675* on chromosome 2; and *AG3H127*, *AG3H249*, *AG3H812*, *AG3H311*, *AG3H811* and *AG3H93* on chromosome *3*. Microsatellite loci were PCR-amplified from individual mosquito DNA, using flanking primers identified by (Lanzaro *et al.*, 1998; Walton *et al.*, 1998). Each 11 µl PCR reaction consisted of 5 µl of Multiplex master mix, 1 µl primer mix and 4 µl of Rnase free water. The primer mix was made to a final volume of 250 µl, consisting of varying amounts (µl) of each primer and variable amount of TE buffer depending on the number and amount of primers mixed. The forward primer in each reaction was labeled with a fluorescent marker (FAM, NED or HEX) compatible with ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems). DNA amplifications were completed in MJ Research PTC-200 thermal cyclers (MJ Research, Watertown, MA). A 5 min denaturation step at 95°C, followed by 29 cycles of 20 s at 95°C, 30 s at 55°C and 30 s at 72°C. A final extension at 72°C was extended to 1 hr to alleviate problems associated with addition of non-template nucleotide (dA) to the PCR products. 0.5 µl

of PCR products was diluted in 20 µl of deionized H_2O before it was mixed with 0.5 µl of GeneScan (GeneScan™ 400HD ROX™, Applied Biosystems), size standard and 12 µl of Hi-Di formamide. Mixtures were denatured in MJ Research PTC-200 thermal cyclers (MJ Research, Watertown, MA), for 5 min at 95^0C, before being run on an ABI 3130 Genetic analyzer. Output was analyzed using ABI PRISM® 3130 Genemapper (Applied Biosystems).

Data analysis

This study aimed to compare the microsatellite polymorphism between laboratory, semi-field and field populations. We used *PopI* workbench (https://grassi2.ucdavis.edu/PopulationData) to store and manage our data. Data format conversion for *Arlequin* was done using the tool provided by *PopI* workbench. The polymorphism was evaluated by comparing estimates of mean heterozygosity and the number of alleles at each of the 11 loci from chromosome 2 and 3. We used the *Arlequin* (ver. 3.11) programme, to calculate the allele frequency and observed heterozygosity for each locus in each population. The Wilcoxon rank sum test was used to compare the differences between SFS and laboratory colonies in terms of mean observed heterozygosity. Linear regression analysis was used to examine the tendency for mean observed heterozygosity to decrease/increase with increasing number of generations. Also, analysis of molecular variance (AMOVA) was used to estimate inbreeding depression and 10100 permutations were performed to test for significance. We also calculated the genetic distance between populations (F_{ST}) and estimated the degree of inbreeding (F_{IS}).

Results

A total of 602 mosquitoes were subjected to microsatellite analysis. Fifty females were field collected, 384 (48 individuals from generations 1, 2, 5 and 10) from the two laboratory colonies and 188 (47 individuals from generations 1, 2, 5 and 10) from the SFS. The SFS population had a mean observed heterozygosity of 49.9% (range 48-50%) while laboratory cage colonies maintained a mean observed heterozygosity of 44.5% (range 38.7-47.5%). The field population had a mean observed heterozygosity of 52.3%. There was a significant difference between the mean observed heterozygosity of the SFS population and that of the two cage colonies (W =0, P = 0.028, Figure 2). The mean observed heterozygosity values of the two cage colonies were not significantly different (W =10, P = 0.69).

Analysis of molecular variance (AMOVA) (at 10100 permutations) indicated that individual variations in all populations was the major source of variation and accounted for 80% of the observed variation. Cage colonies exhibited a significantly higher degree of inbreeding than the semi-field populations and increased with time as opposed to the SFS population which decreased with increasing number of generations (W = 28, P = 0.0242, Figure 3). Analysis of the decline in the number of alleles indicated that the decline in the number of alleles was significant for cage colonies (P = 0.00007, Figure 4) but not for the SFS population compared to field population. Based on the F_{ST} estimates, the SFS population was genetically closer to the field population (F_{ST} = 0.078)

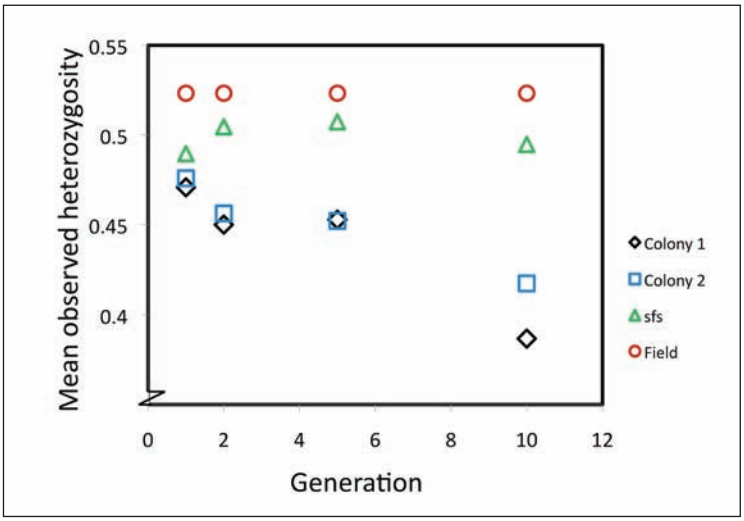

Figure 2: Mean observed heterozygosity of field, semi field and cage colonies for 11 microsatellite loci over ten generations.

while cage colonies were genetically more distant to the field population (F_{ST} = 0.167). The SFS population was intermediate between field and laboratory colonies.

Discussion

We observed that the contained SFS population maintained more genetic variation than colonies established in standard laboratory cages. There was a 50% chance for an individual in the SFS to be heterozygous while this was only 45% for cage individuals (*P* = 0.028). We conclude therefore that the SFS population is more representative of the field population than the cage colonies. There are four sources of evidence to support this statement: (1) The contained SFS population maintained significantly higher genetic variation (mean observed heterozygosity 49.9%) than laboratory cage colonies (mean observed heterozygosity 44.5%), (2) the two laboratory-established colonies were more genetically distant from the field population (F_{ST} = 0.167) than the SFS population (F_{ST} = 0.078), (3) laboratory colonies exhibited substantially more inbreeding in the first ten generations than the SFS population (W = 28, *P* = 0.0242). (4) The number of alleles significantly decreased in laboratory colonies over ten generations.

The mean observed heterozygosity of the field population (52.3%) was greater than that for the SFS (49.9%) and the laboratory colonies (44.5%). The mean observed heterozygosities of the field and SFS populations in this study, are within the range of heterozygosity estimated from microsatellite analysis of a variety of field populations of African *Anopheles* vectors (0.48 – 0.89) (Mukhopadhyay *et al.*, 1997; Munstermann, 1994). However, the mean observed heterozygosity for mosquitoes of the cage colonies in this study was higher than the reported mean heterozygosity in cage colonies of various insects including sandflies (Norris *et al.*, 2001), *Drosophila* (Bryant, 1976; Nevo, 1978) and *Anopheles gambiae* (Nevo, 1978; Schmidt and Conde, 2006). The cage colonies in this study were maintained within the SFS, such that natural light condition, temperature and humidity were not controlled but varied with ambient. This may explain why cage colonies in this study maintained a higher mean

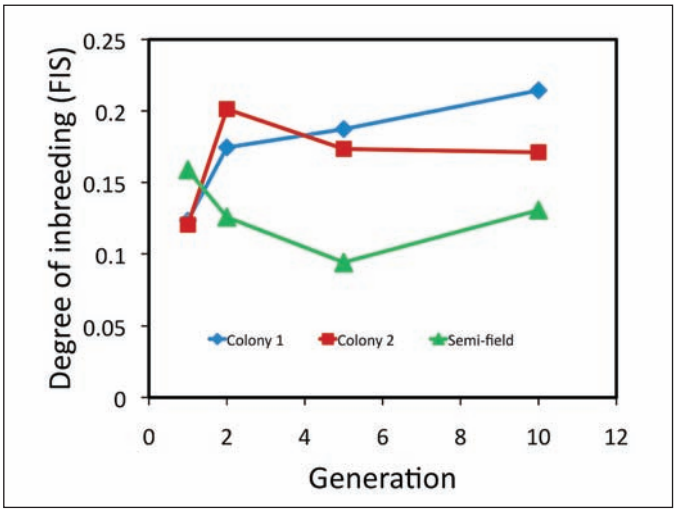

Figure 3: The observed degree of inbreeding (F_{IS}) in the cage and semi field populations over ten generations.

observed heterozygosity than reported from other laboratory cage colonies. It is likely that natural dusk and dawn light conditions exerted less selection pressures on the cage colony mosquitoes than regular laboratory conditions where transitions between light and dark are normally sharp.

It is generally accepted that at any ecological gradient, a population may continuously be distributed over several homogeneous micro-habitats, such that the overall local diversity is a result of incoming gene flow from neighboring micro-habitats which are differentially adapted (Bryant, 1976). Thus, micro-habitat heterogeneity in the SFS may be responsible for creating local diversity within each microhabitat and redistributed through mating over the entire SFS population, resulting in an increased overall genetic diversity of the SFS population (Dicke and Grostal, 2001; Griswold and Lounibos, 2005; Vitt *et al.*, 2007). A similar phenomenon has been reported in other animals and plants species whose level of gene flow is relatively low (Kimura, 1983). It is also plausible that the SFS may have supported a larger effective population size which might have increased the possibility for beneficial mutations to be maintained, contrary to cage colonies which experienced higher inbreeding as a result of reduced effective population size (Reed and Frankham, 2003). Also as mosquitoes were allowed to fly freely, locate and select mates of their choice using natural cues, there is higher possibility for random mating which may have increased the chances of gene reshuffling which lowered the degree of inbreeding in SFS population than in cage colonies (Lanzaro *et al.*, 1995; Lehmann *et al.*, 1996).

Our findings have relevance to a number of disciplines. These findings have potential relevance to the design and implementation of new vector control approaches of mosquito-borne diseases such as malaria (Allen *et al.*, 2001), dengue (Scott, 2002) and West Nile virus (Reisen, 2003) such as the mass release of laboratory-reared sterilized/genetically-modified male mosquitoes. One major drawback with this approach is that it does not account for the ecological and behavioural aspects of target vector species (Ferguson *et al.*, 2005; Huho *et al.*, 2007; Ng'habi *et al.*, 2008; Ng'habi *et al.*, 2005). Studies have indicated that poor understanding of male mating biology has been implicated in the failure of a number of release trials to control disease vectors in the early 1970s (Benedict and Robinson, 2003; Clayton, 2006; Knols and Louis, 2006),

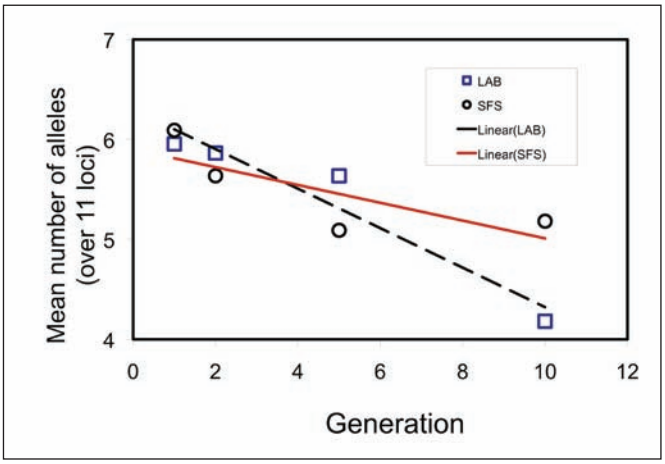

Figure 4: The mean number of alleles over 11 loci for laboratory and semi field populations over ten generations.

and little progress has been made to address this (Ferguson *et al.*, 2005). Furthermore, the development of multiple generations of a vector population in SFS, would allow the evaluation of gene drive systems. For example; the effectiveness of nucleic-acid vectors (Kidwell and Ribeiro, 1992) and bacterial symbionts (Curtis and Sinkins, 1998) to drive parasite-refractory genes (Catteruccia, 2000) into target mosquito population, can be evaluated in SFS.

Although it is clear from this study that biological inferences made from SFS populations may be more realistic than those from cage colonies, there may be other important ecological and biological processes that are not fully represented or could not be introduced in this ecosystem. For example the absence of the full range of mortality risks faced by mosquitoes in their natural environment, such as predation, flooding and attack by insecticides, which were not introduced in this ecosystem. We recommend further fitness studies to compare the three mosquito populations (field, cage and SFS) such as mating competitiveness, survivorship and physiological reserves to test how truly representative these systems are, both from a genetic and behavioural/ecological point of view.

Conclusions

The *An. arabiensis* population in the SFS has higher genetic variation than laboratory maintained colonies as indicated by a higher level of heterozygosity. This study presents the first report on the suitability of SFS to maintain extant natural variation which is frequently lost in laboratory cage colonies. The simulated environmental conditions within the SFS present a more realistic ecosystem, which supports higher levels of genetic variation than that observed in laboratory colonies. We recommend that the SFS approach should be prioritised for the colonization of insect vector species designated for research purposes, such that the insights obtained are more realistic than a cage colonized population, although not as similar as those of field populations. It is expected that SFS-maintained individuals will be more resilient than cage-maintained individuals. Therefore, male mosquitoes destined for release in sterile and/or transgenic disease vector control programmes can reliably be colonised in SFS

conditions to maintain the genetic variation which is frequently lost in cage colonies, and increase the chances for them to survive and compete with their counterparts for female mates in the wild. We suggest that the observed genetic diversity differences resulting from different colonization procedures may guide the design of appropriate rearing protocols, such that male mating success is enhanced, a key to the success of new approaches. In this study we did not directly link the observed loss in genetic variation to mating ability and other fitness measures, which is thus an open avenue for future work.

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Genetic population structure of sympatric malaria vectors *Anopheles arabiensis* and *Anopheles gambiae sensu stricto* in a malaria endemic region of Southern Tanzania

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> **Abstract**

Genetic diversity is a key factor enabling a natural population's adaptation and persistence at a particular environmental condition. It is influenced by the interaction of a natural population's dynamics and the environment it inhabits. *Anopheles gambiae s. s.* and *Anopheles arabiensis* are the two major and widespread vectors of malaria in most African countries. Several studies have examined the ecology and population dynamics of these vectors. The ecological condition along the Kilombero valley influences the distribution and population density of the two malaria vectors. It remains unclear whether the ecological diversity within the Kilombero valley has affected the population structure of *An. gambiae s. l.* populations. The goal of this study was to characterise the genetic structure of sympatric *An. gambiae s.s* and *An. arabiensis* populations along the Kilombero valley. Mosquitoes were collected from 7 locations in Tanzania: six from the Kilombero valley and one outside the valley (~700 km away) as an out-group. To archive a genome-wide coverage, 13 microsatellite markers from chromosomes X, 2 and 3 were used. Here, we report high levels of genetic differentiation within *An. arabiensis* populations as opposed to *An. gambiae s. s.* which were genetically undifferentiated across the 6650 km2 of the Kilombero valley landscape. It appears that the genetic differentiation is not attributed by physical barrier or distance but possibly by the ecological diversification within the Kilombero valley. The genetic divergence between *An. arabiensis* populations (F_{ST} = 0.066) was even higher than that of the well known M and S forms of the *An. gambiae s. s.* in West and Central Africa (F_{ST} = 0.035), suggesting that these populations are maintained by some level of reproductive isolation. We hypothesize that ecological diversification across the valley may be a driving force for the observed *An. arabiensis* genetic divergence. The impact of the observed *An. arabiensis* substructure to the prospects for new vector control approaches is discussed.

Introduction

The genetic structure of a population is shaped by the interaction between the behaviour of individuals and their prevailing environment (Waples and Gaggiotti, 2006). These factors in combination, influence the magnitude of gene flow within and between populations and the genetic structure of a species throughout its range (Clobert *et al.*, 2001). Ecological diversification has been suggested to be one of the factors that can interrupt the movement of genes by creating landscape or genetic barriers such as rapid chromosomal inversion that ultimately results in reproductive divergence and speciation (Schneider, 2000). Therefore the understanding of the relationship between individuals and their surrounding environment is the basis of population structure and can provide information about movement of genes from one individual / population to another. This information is important not only for species monitoring and conservation but also for their control.

The method commonly used to study the level of gene flow is to identify genetic units within an ecological landscape and the features that help to shape these units (Coulon *et al.*, 2006). This involves genetic structuring of pre-defined populations where the level of gene flow between them is measured using F_{st} values or other parameters. Then, deducing which features/factors are responsible for restricting or promoting movements of genes between or within these populations (Lanzaro *et al.*, 1998; Tourè *et al.*, 1998). Although this method has been useful, it suffers from drawbacks, as it is not informative for small areas and as it relies on prior reasoning and assumptions of population limits (Coulon *et al.*, 2006; Foster and Sharp, 2002; Kaeuffer *et al.*, 2007). The development of the Bayesian clustering method has been a great achievement because it uses individual genotypes as a sole source of information and individuals can be partitioned into genetic units at Hardy-Weinberg equilibrium (Pritchard *et al.*, 2000). The Bayesian clustering method has gained popularity and has been applied in population genetic studies of a range of organisms such as humans, animals (Coulon *et al.*, 2006; Perez-Espona *et al.*, 2008; Rosenberg *et al.*, 2002) and plants (Larson *et al.*, 2004).

Mosquitoes of the *Anopheles gambiae* complex, include the two primary vectors (*An. gambiae s.s.* Giles and *An. arabiensis* Patton) of human malaria in sub-Saharan Africa, that are responsible for an estimated 240 million cases and 280,000 deaths worldwide, with over 80% occurring in Africa (WHO, 2009). The two species are the most widespread members of the *Anopheles gambiae* complex and major vectors of malaria (Coluzzi *et al.*, 1979). Although the two species are mostly found occupying similar ecological niches, *An. gambiae s. s.* is associated with more humid climates than *An. arabiensis,* which has a greater tolerance for drier environments (Ayala and Coluzzi, 2005; Donnelly *et al.*, 1999). Additionally, *An. gambiae s.s.* are highly anthropophagic (Coluzzi *et al.*, 1979; White, 1974), endophagic and typically endophilic (White, 1974), whereas *An. arabiensis* are more zoophagic, exophagic (White *et al.*, 1972), and exophilic (Coluzzi *et al.*, 2002; Gillies and DeMeillon, 1968). There is a strong pre-mating isolation mechanism existing between the two species. Although post-mating isolating mechanism is incomplete, hybrids which are fertile (Besansky *et al.*, 2003) are competitively inferior as evidenced by rare hybrids in nature (0.02-0.76%) (Temu *et al.*, 1997; Touré *et al.*, 1998).

Population substructure is more pronounced in *An. gambiae s. s* and has mainly been influenced by environmental heterogeneity (Touré *et al.*, 1994; Touré *et al.*, 1998). For example based on chromosomal inversions, five distinct *An gambiae s.s.* subpopulations which exist in sympatry, have been revealed in West and Central Africa (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985; della Torre *et al.*, 2002; Toure *et al.*, 1998). Studies from north, south and western Africa have reported some degrees of genetic differentiation between *An. arabiensis* populations (Donnelly and Townson, 2000; Nyanjom *et al.*, 2003; Simard *et al.*, 1999). However, neither physical barriers nor geographic distance has been reported to be forces responsible for *An. arabiensis* population differentiation (Besansky *et al.*, 1997; Nyanjom *et al.*, 2003), except for island populations whose genetic differentiation has been associated with historical drifts (Simard *et al.*, 1999)

The *An. gambiae s. s.* subpopulations have intensively been studied and are of potential epidemiological importance as they have been suggested to undermine available malaria vector control efforts. For example the two molecular forms (Savannah and Mopti-forms) of *An. gambiae s. s*. have been reported to respond differently to the available control interventions, as the Savannah form is more resistant to pyrethroids (insecticides used for impregneting bednets) while the Mopti form remains largely susceptible (Reimer *et al.*, 2008; Tripet *et al.*, 2007). In addition to undermining current control interventions, such population subdivisions are expected to pose more challenges to the application of new genetic control approaches. Such population subdivision my require multiple genetic modification of males for successful introduction and spread of desired traits into wild populations (Coluzzi *et al.*, 2002; Lanzaro *et al.*, 1998; Powell *et al.*, 1999). Therefore, understanding the genetic structure and the relative amount of gene flow taking place within and among wild populations is an important component for effective planning and implementation of available insecticide-based vector control approaches. Additionally, poor understanding of the genetic structure and level of gene flow between target populations may possibly undermine proposed genetic control strategies, especially those that aim at reducing mating success of genetically-modified / sterile male mosquitoes from natural populations (Blandin *et al.*, 2004; Ito *et al.*, 2002; Osta *et al.*, 2004; Reisen, 2003; Shahabuddin *et al.*, 1998). The existence of genetic substructure within vector populations would create barriers that may restrict the spread of desired genes (Lanzaro *et al.*, 1998; Lanzaro and Tripet, 2003).

In this study we characterized the population structure of *An. gambiae s. s*. and *An. arabiensis* within the Kilombero valley (6650 km^2) located in southern Tanzania. This valley experiences some of the most intense malaria transmission in the world (Killeen *et al.*, 2006; Smith *et al.*, 1993). Epidemiological studies in this valley have revealed that malaria transmission intensities, as indexed by entomological inoculation rate (EIR, number of infective bites a person is exposed to each year) are very high and range between 100 to 1000s infective bites per annum (Killeen *et al.*, 2006; Killeen and Smith, 2007; Smith *et al.*, 1993; Smith *et al.*, 1995). It is still unclear whether such high levels of transmission are generally attributed to environmental factors that affect mosquito population density and distribution within the valley (Charlwood *et al.*, 1995; Charlwood *et al.*, 2000) or whether there are genetic factors that increase the vectorial capacity of some mosquito populations in this area. Several studies have examined the ecology and population dynamics of the malaria vectors within this valley (Charlwood *et al.*, 1995; Charlwood *et al.*, 2000; Killeen *et al.*, 2006), but there are no studies that

examined the population structure of *An. gambiae s. s.* and *An. arabiensis* along this valley, and how it maps with malaria epidemiology. Using Bayesian clustering analysis, we employed 13 polymorphic microsatellite loci originally designed for use in *An. gambiae s. s.* (Zheng *et al.*, 1996) to characterize the genetic structure of each of the two malaria vectors along the Kilombero valley. We tested the hypothesis that physical distance between collection sites may affect gene flow and create genetic units of the two species.

Material and Methods

Study site and mosquito collection

The Kilombero valley is located in southeastern Tanzania (Figure 1). The two major vectors of malaria, *An. gambiae s. s.* Giles, and *An. arabiensis* Patton, are widely distributed along the valley. The valley is oriented from the northeast to southwest (7°44'-9°26'S/35°33'-36°56'E), between the densely forested escarpment of Eastern Arc mountains covering an area of 6650 km^2 (Bonnington *et al.*, 2007). Estimated mean annual rainfall ranges from 1200 mm/yr to >2000 mm/yr (Charlwood *et al.*, 2000; IIED, 1992). Mosquitoes were collected from seven localities from January - May 2007. Two locations are situated in close proximity with the forested Eastern Arc mountains (Mkamba and Ilonga) and four locations were situated along the river (Malinyi, Ukindu, Lupiro, and Mikeregembe). The distance between these collection sites ranged between 20 to 50 km. Mosquitoes were also collected from Kaliua village located >700 km west of the Kilombero valley, as an out-group. In each village, five CDC light traps were placed in different randomly selected houses for three consecutive nights. Every morning, traps were retrieved and mosquitoes identified according to their morphological differences. Mosquitoes visually identified as belonging to the *An. gambiae* complex were individually stored on silica gel in Eppendorf tubes and taken for further molecular analysis to determine whether they belonged to either the *An. gambiae s.s.* or *An. arabiensis* species.

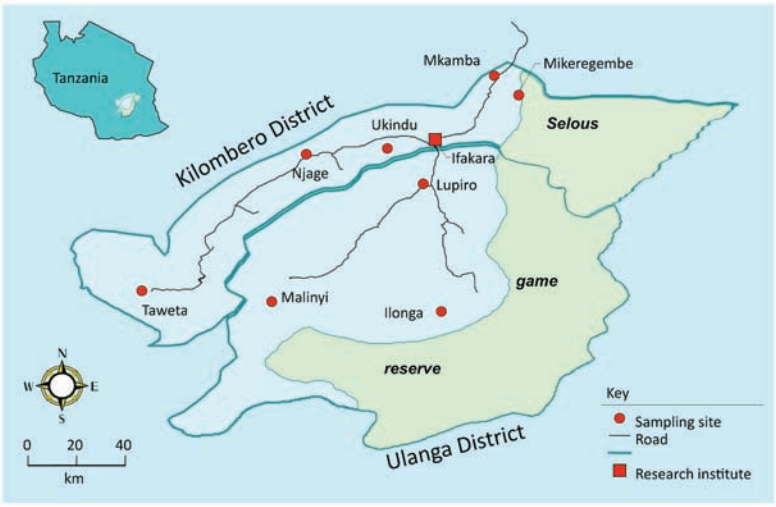

Figure 1: Map of the Kilombero valley, Tanzania showing mosquitoes collection sites

DNA extraction and species identification

Prior to DNA extraction, individual mosquitoes were rehydrated in 100 µl of ultra pure water overnight. The following day, 10 µl of proteinase K and 90 µl of ATL buffer were added to each individual tube, and a Tissue Lyser® then used to break mosquito tissues. DNA extraction was performed using the 96 Biosprint® work station in 96 well plates. Each individual of the *An. gambiae* complex was identified to species level using species-specific PCR primers (Scott *et al.*, 1993).

Microsatellite DNA amplification

We screened 20 microsatellite loci selected from the entire mosquito genome (Zheng *et al.*, 1996). These microsatellite markers were initially developed for *An. gambiae s. s.*, and only 13 gave good amplification in both *An. gambiae s. s.* and *An. arabiensis.* Consequently only these 13 markers were used in this study. These markers provide representative coverage of the entire genome as they are distributed relatively evenly throughout the genome as follows: X chromosome: *AGXH25*, *AGXH100* and *AGXH71*; Chromosome 2: *AG2H175*, *AG2H85*, *AG2H164*, *AG2H197* and *AG2H675*; and Chromosome 3: *AG3H127*, *AG3H249*, *AG3H311*, *AG3H811* and *AG3H9* (Zheng *et al.*, 1996).

Microsatellite loci were PCR-amplified for each mosquito sample. Each 24 µl PCR reaction consisted of 12.5 µl of Multiplex master mix, 2.5 µl primer mix and 9 µl of RNase free water. The primer mix was made to a final volume of 250 µl, consisting of 10 pmol of each primer. The forward primer in each reaction was labeled with a fluorescent marker (FAM, NED or HEX) compatible with ABI PRISM (Perkin-Elmer, Norwalk, Conn.) capillary electrophoresis. DNA amplification was completed in MJ Research PTC-200 thermal cyclers (MJ Research, Watertown, MA). A 5 min denaturation step at 95°C was followed by 29 cycles of 20 s at 95°C, 30 s at 55°C and 30 s at 72°C. A final incubation at 72°C was extended for 1 hour to alleviate problems associated with addition of non-template nucleotide (dA) to the PCR products. PCR products were mixed with a GeneScan (Perkin-Elmer, Norwalk, CT) size standard and deionized formamide as directed by the manufacturer. Mixtures were run on denaturing polyacrylamide gels at 51°C using an ABI 3100 Capillary Sequencer. Output was analyzed using ABI PRISM, Gene-Scan Analysis Software and Genotyper DNA Fragment Analysis Software (Perkin-Elmer, Norwalk, CT) to identify alleles.

Statistical analysis of microsatellite allele frequencies

The microsatellite allele frequencies were examined using *Arlequin* (available at http://anthropologie.unige.ch/arlequin/), developed by Excoffier and colleagues (Excoffier *et al.*, 2005). Each microsatellite locus was tested separately for significant departure from Hardy-Weinberg Equilibrium, using a Markov-chain algorithm (Guo and Thompson, 1992). Multiple comparisons were corrected using the Bonferroni and Šidák correction method by the formula 1-(1-0.05) ^ (1/n), where n is the number of independent comparisons (Abdi, 2007). We used *Arlequin* to calculate pair-wise F_{ST} values (Excoffier *et al.*, 2005), and 10,000 permutations were used to determine the significance of F_{ST} distance. We used the Neighbor-joining algorithm implemented in *neighbor*, a part of the *Phylip* software package (Felsenstein, 2005) to calculate the unrooted tree based

on the matrix of pair-wise F_{ST} distances. The cladogram was drawn using the *drawtree* programme, also provided in *Phylip*. As an estimate of gene flow, the number of migrants per population per generation (*Nm*) was calculated for F_{ST} according to the equation, $Nm = (1-F_{ST})/ 4F_{ST}$ (Slatkin, 1995). A Bayesian clustering analysis was applied based on the 13 microsatellite markers on chromosomes X, 2 and 3. Using *structure* software (Pritchard *et al.*, 2000), individuals that share the same alleles (unique to that group) are placed into groups/clusters termed as (*K*), chosen in advance. This model calculates the probabilities of each individual for each subgroup, within which Hardy-Weinberg (H-W) equilibrium and linkage equilibrium are met. These probabilities are used to infer the membership of each individual at their most probable subgroup, and these are referred to as *membership coefficients* which sum to 1. The probability distribution of an individual X in a putative population K (P(X/K)) was also plotted. Based on allele diversity, individuals with unique alleles are grouped together into assumed population (*K*) which is pre-determined. The *K* value with the maximum posterior probability (P(X/K)) is retained and assumed to be the most probable number of clusters in that putative population.

Results

A total of 603 *An. gambiae s. l.* mosquitoes were collected in this study from the seven localities. Of these, 113 (18.7%) were *An. gambiae s. s.* and 490 (81.2%) were *An. arabiensis.* DNA from 386 (288 *An. arabiensis* and 98 *An. gambiae s. s.*) mosquitoes gave good amplification and these samples were screened for the 13 microsatellite loci.

Genotype frequencies

The result of Guo's Exact Hardy-Weinberg test (Guo and Thompson, 1992) are shown in Table 1. There was substantial departure from H-W expectations: 22 out of 78 tests in *An. arabiensis* (28%) and 10 out of 39 tests in *An. gambiae s. s.* (25.6%) were significant ($P < 0.00044$). Further inspection in *An. arabiensis* populations revealed that 9 out of 21 tests (42%) involving only X-chromosome loci significantly deviated from H-W expectations and no test out of 9 tests (0%) in *An. gambiae s. s.* significantly deviated from H-W expectations (Table 1) suggesting subdivision or admixture within *An. arabiensis* populations in the Kilombero valley.

Linkage Disequilibrium

The non-random association between polymorphism at different loci is measured by the degree of linkage disequilibrium. Linkage disequilibrium was determined (Slatkin, 1995) and 4.6% of the overall pairwise comparisons between *An. arabiensis* populations were statistically significant ($P < 0.00033$), (Figure 2). In *An. gambiae s. s*. however, there was 0% overall pairwise comparison observed to be significant ($P < 0.00033$). It has been established that linkage disequilibrium is predicted to approach zero for an ideal population, in the absence of forces such as genetic drift, population mixing, mutation and natural selection (Ohta, 1982). We therefore hypothesize that

Table 1: P-values indicating statistical significance (P < 0.00044) of deviations from Hardy – Weinberg expectations for 13 microsatellite loci in populations of *An. arabiensis* and *An. gambiae s. s.* along the Kilombero/Ulanga valley, Tanzania. Ag - Stands for *An. gambiae s. s*. and Aa stands for *An. arabiensis*. Significant test at P ≤ 0.00044 are in bold.

	An. arabiensis							*An gambiae s. s.*		
Chromosome/Locus	Ilonga (Aa)	Malinyi (Aa)	Lupiro (Aa)	Ukindu (Aa)	Mikerege mbe (Aa)	Mkamba (Aa)	Kaliua (Aa)	Ilonga (Ag)	Lupiro (Ag)	Mkamba (Ag)
X AGXH100	0.02719	**0.00000**	0.01362	**0.00000**	**0.00000**	1.00000	0.00198	0.52185	0.02420	0.12993
AGXH25	0.03480	**0.00000**	**0.00001**	**0.00000**	**0.00000**	**0.00014**	**0.00000**	0.05385	0.05672	0.78633
AGXH71	0.00171	0.01108	0.25532	0.01049	0.00982	0.00270	0.01200	0.00057	0.23734	0.16610
2 AG2H85	0.05530	0.14529	0.37553	**0.00006**	**0.00014**	1.00000	**0.00029**	0.94075	0.00507	0.28175
AG2H164	**0.00006**	0.07883	**0.00000**	0.00586	0.01234	0.00857	0.28081	**0.00009**	**0.00000**	**0.00000**
AG2H175	0.79302	0.22363	0.54858	0.30838	0.42998	0.36502	1.00000	**0.00000**	**0.00000**	**0.00000**
AG2H197	0.08331	0.02250	0.31518	**0.00006**	**0.00000**	0.25521	0.83194	0.00430	0.48666	0.51077
AG2H675	1.00000	0.48918	0.01798	0.17940	0.46848	0.52015	0.54126	0.50763	0.30074	0.02176
3 AG3H127	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.00078	0.27888	**0.00003**
AG3H249	0.16835	0.07418	0.06044	0.22735	0.00259	0.31076	0.23614	0.67873	0.05164	0.19956
AG3H311	N/A	N/A	1.00000	N/A	N/A	N/A	N/A	**0.00000**	**0.00000**	**0.00000**
AG3H811	**0.00004**	**0.00000**	0.02007	**0.00000**	**0.00021**	**0.00010**	0.51587	0.28928	0.47032	0.01804
AG3H93	1.00000	0.30044	0.42682	**0.00000**	0.19691	0.02498	0.00926	0.04169	0.97865	0.13654

the 4.6% linkage disequilibrium observed for *An. arabiensis* is indicative of population subdivision (Nei and Li, 1973).

Population structure

The difference in allele frequencies was studied using F_{ST} estimates as a measure of differentiation between the two species and between populations, which is based on the assumption that mutations occur equally to all alleles. Our results are presented in Table 2. *Anopheles arabiensis* was genetically distant from *An. gambiae s. s.* (F_{ST} ranging from 0.21-0.27) (Figure 3a and Table 2). The level of genetic divergence between *An. gambiae* populations was low (F_{ST} ranging from 0.003 to 0.01) whereas the level of genetic divergence between *An. arabiensis* populations was higher (F_{ST} ranging from 0.006 to 0.1). The product of effective population size N_e and the migration rates m was used to estimate the amount of gene flow within each species based on the observed F_{ST} values. Thus there is a low amount of gene flow between the two species (N_em, ranging from 0.7 to 0.9; see Table 3). These values are in line with other reported studies (Lanzaro *et al.*, 1998). The estimates of gene flow among *An. arabiensis* populations indicate that there is a limited number of migrants between some populations (N_em ranging from 1.9 to 45; see Table 3), as would be expected in reproductively isolated populations.

The relatively large genetic differentiation between *An. arabiensis* populations within the valley can further be visualized by the unrooted neighbor-joining tree, based on the matrix of pairwise F_{ST} values (Figure 3a). Populations of *An. arabiensis* fall into

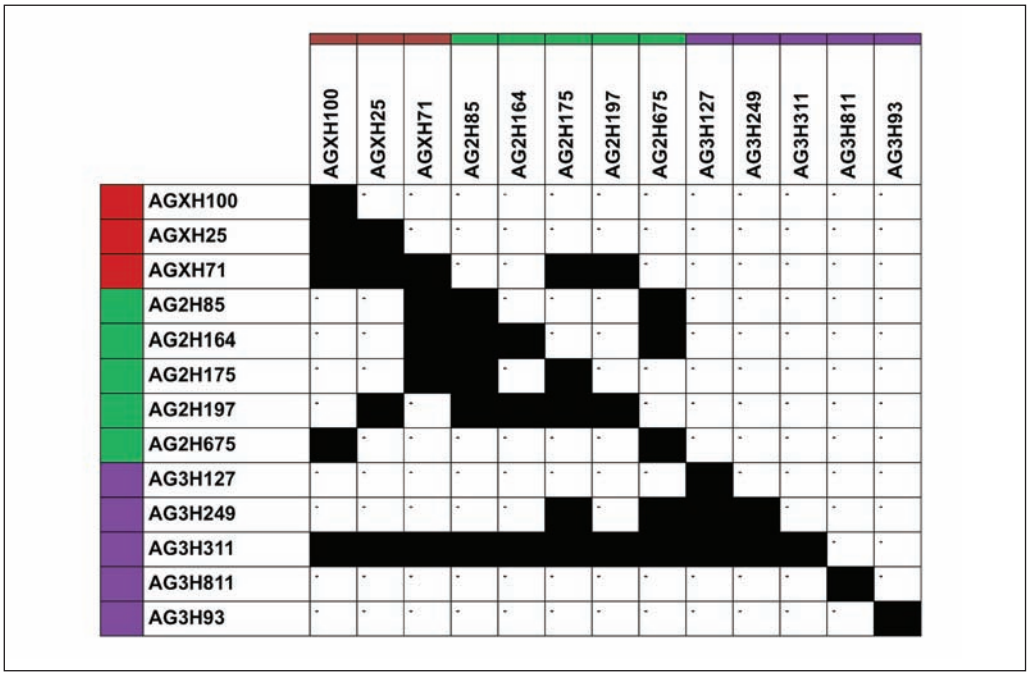

Figure 2: Pairwise linkage disequilibrium for 13 microsatellite loci for An. gambiae s.s. (above diagonal) and An. arabiensis (below diagonal). Black boxes with "+" indicates statistical significant at linkage disequilibrium (P < 0.00033). The "–" indicates a no significant linkage disequilibrium (P < 0.00033).

two distinct clusters, with samples from Mkamba and Ilonga forming the first cluster (cluster I) and all the other populations the second cluster (Cluster II), (Figure 3b).

Bayesian clustering analysis was applied for all populations within and outside the Kilombero Valley. This approach is advantageous in that it does not require a prior population classification, but instead it estimates the shared population ancestry based on observed genotypes, under the assumption of the presence of a H-W Equilibrium and linkage equilibrium within each cluster (Friedlaender *et al.*, 2008). Thus, based on the assumption that alleles occur in more than one population, individuals with unique alleles from all populations are placed into *K* clusters (chosen in advance), i.e. corresponding to the number of postulated ancestral populations. As shown in Fig. 4, solutions for hypothetical *An. arabiensis* population clusters from *K*= 4 and *K*=5 showed a similar range of likelihood and the highest likelihood was observed at *K*= 3. Thus the most likely number of clusters (K) is three. This corresponds to three distinct genetic clusters: (1) The *An. gambiae s. s.* (2) The *An. arabiensis* cluster I (Mkamba, Mikeregembe and Ilonga) and (3) *An. arabiensis* cluster II (all other sites).

Discussion

In this study we used 13 microsatellite loci to screen *An. gambiae s. s.* and *An. arabiensis* populations from the Kilombero valley, Southern Tanzania. Both species (*An. gambiae s. s.* and *An. arabiensis*) occur in the Kilombero valley with *An. arabiensis*

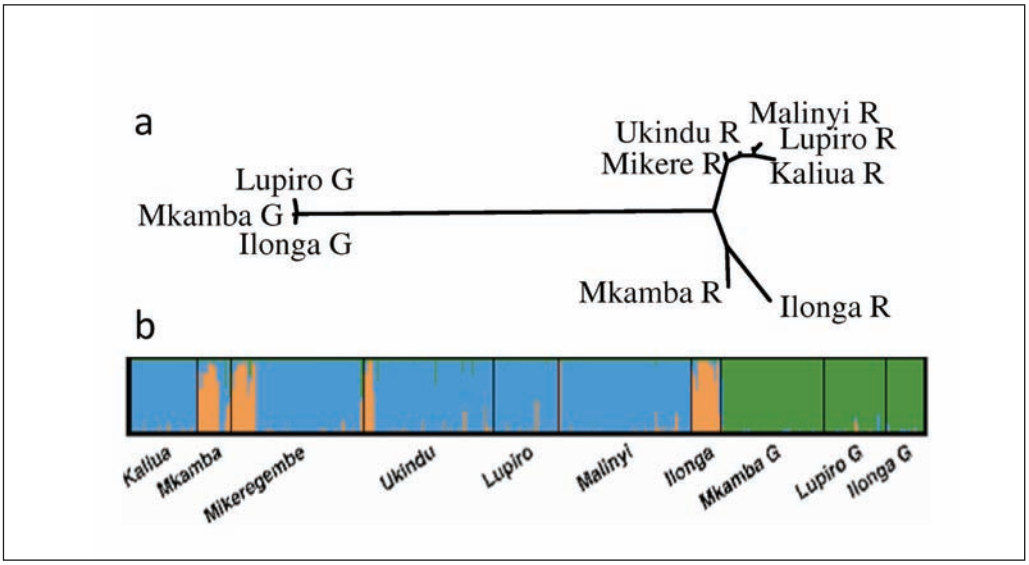

Figure 3: (a) Unrooted neighbour-joining cladogram for *An. arabiensis* and *An. gambiae* populations based on F_{ST} values. "GS" and "R" in each site indicate *An. gambiae s. s.* and *An. arabiensis* respectively. (b) Results from an individual level clustering analysis. The vertical bar indicates proportionate assignment of an individual to each cluster. "M" indicates *An. gambiae s. s*. The blue and green color denotes *An. arabiensis* and *An. gambiae s. s*. populations respectively and the pink color indicates an *An. arabiensis* subgroup.

more widespread. The *An. gambiae s. s.* appear to be a single genetic population whereas *An. arabiensis* appear to occur as two discrete populations that occur in sympatry at some sites (for example, Mkamba, Ilonga and Mikeregembe) across the Kilombero valley of southern Tanzania (6650 km^2). The higher genetic differentiation among *An. arabiensis* populations than among *An. gambiae* populations is evidenced by (i) the genome-wide signature of departures from H-W Equilibrium and high linkage disequilibrium between loci, (ii) higher F_{ST} estimates between *An. arabiensis* than between *An. gambiae s.s.* populations and (iii) higher levels of gene flow between *An. gambiae s.s.* than *An. arabiensis* populations (iv) Bayesian probability distribution at *K* =3 presented for *An. arabiensis* populations. This clearly indicates that the gene pool of *An. arabiensis* populations across the Kilombero valley is not homogeneous. In contrast, *An. gambiae s. s.* showed low levels of genetic differentiation with high level of gene flow across populations, indicative of a homogeneous gene pool.

The signature of population substructure observed in Ilonga, Mikeregembe and Mkamba *An. arabiensis* populations may be a consequence of several factors that need further investigations. Cytogenetic evidence suggests that *An. arabiensis* is an ancient species from which other members of the complex descended and inhabited East Africa more than 6,000 yrs ago (Ayala and Coluzzi, 2005), indicative of a possibility for population structuring (Kaeuffer *et al.*, 2007). Consequently, the Kilombero valley is surrounded by ancient Eastern Arc mountain ranges (e.g., the Udzungwa and Mathenge mountains) providing stable wet climatic conditions (Griffths, 1993), estimated to have existed for over 30 million yrs and this is where the two sampling sites (Ilonga and Mkamba, Figure 1) are located. Therefore, such climatic stability provided by the Eastern Arc mountains and the environmental heterogeneity maintained within it, may be among the major forces driving the genetic structure of *An. arabiensis* populations

Table 2: Pairwise estimates of genetic divergence (F_{ST}) between *An. gambiae* and *An. arabiensis* in the Kilombero valley. The total numbers of mosquitoes screened in each location are in brackets. Underlined values indicate inter-species comparison between *An. gambiae* and *An. arabiensis*. The non-underlined values indicate intra-species comparisons, i.e. within *An. gambiae* and *An. arabiensis*. Values in italics are statistically significant. Significance levels at ¥P < 0.05, $P< 0.01 and *P<0.001. 'Ag' stands for *An. gambiae s. s.* and 'Aa' stands for *An. arabiensis*

	Ilonga (Aa)	Malinyi (Aa)	Lupiro (Aa)	Ukindu (Aa)	Mikeregembe (Aa)	Mkamba (Aa)	Kaliua (Aa)	Ilonga (Ag)	Lupiro (Ag)
Ilonga (15)	-	-	-	-	-	-	-	-	-
Malinyi (64)	*0.1031**	-	-	-	-	-	-	-	-
Lupiro (32)	*0.0998**	*0.0055*	-	-	-	-	-	-	-
Ukindu (64)	*0.0837**	*0.0084 ´*	*0.0157 $*	-	-	-	-	-	-
Mikeregembe (64)	*0.0731**	*0.0205**	*0.0241**	*0.0129 $*	-	-	-	-	-
Mkamba (17)	0.0143	*0.0391**	*0.0374**	*0.0250 $*	*0.0273 $*	-	-	-	-
Kaliua (32)	*0.1191**	*0.0125 ´*	*0.0181$*	*0.0127 ´*	*0.0265**	*0.0479**	-	-	-
Ilonga-Ag (18)	*0.2471**	*0.2611**	*0.2393**	*0.2378**	*0.2453**	*0.2193**	*0.2645**	-	-
Lupiro-Ag (30)	*0.2508**	*0.2625**	*0.2410**	*0.2384**	*0.2443**	*0.2273**	*0.2661**	0.0109	-
Mkamba-Ag (50)	*0.2393**	*0.2578**	*0.2340**	*0.2333**	*0.2399**	*0.2164**	*0.2569**	0.0029	0.0071´

inhabiting Mkamba and Ilonga, as opposed to *An. arabiensis* populations outside and along the rest of the Kilombero valley, such as Kaliua, Ukindu and Malinyi. It is important to note that Mikeregembe is a fishing camp (Charlwood, 2000), thus it is plausible that a subpopulation of *An. arabiensis* existing in Mkamba and Ilonga may have been introduced in Mikeregembe by fishermen from Mkamba (20 kms) who regularly visit the camp for fishing activities. These mountains also create unequal climatic conditions across the Kilombero valley which correlate very much with the abundance of the two malaria vector species (Charlwood *et al.*, 2000; Ng'habi *et al.*, 2008). For example, (i) *An. gambiae s.s* are commonly found in close proximity with forested Eastern Arc mountain areas where climate is stable and annual rainfall is higher than in the rest of the valley (> 2000 mm/yr), (ii) *An. arabiensis* are commonly found in riverine areas where climate is less stable and annual rainfall is low (<1200 mm/yr) (Charlwood *et al.*, 2000). Therefore it is plausible that *An. arabiensis* populations inhabiting sites along the Eastern Arc mountains are subjected to higher degree of environmental heterogeneity typical to these mountains, promoting genetic variation and emergence of new adaptive genotypes (Moreno *et al.*, 2007).

Based on the difference in allele frequency from F_{ST} estimates, the level of genetic divergence between *An. gambiae s. s.* populations is lower (mean F_{ST} =0.006) than that between *An. arabiensis* populations (mean F_{ST} = 0.066). The degree of divergence between *An. arabiensis* populations (mean F_{ST}= 0.066) is even greater than the reported divergence between the well-known Mopti and Savannah forms of *An. gambiae s.s.* in West Africa (mean F_{ST}=0.035), (Wondji *et al.*, 2002). From these observations it can be hypothesized that the *An. arabiensis* subpopulations are maintained by some degree of reproductive isolation mechanisms. This hypothesis can also be supported by the absence of hybrids in sympatric *An. arabiensis* populations (e.g. in Mkamba, Mikeregembe and Ilonga; Figure 3b).

Table 3: Estimated number of migrants between *An. gambiae s. l.* populations within and outside Kilombero valley (Kaliua). 'Ag' stands for *An. gambiae s. s.* and 'Aa' stands for *An. arabiensis*.

Villages	Ilonga (Aa)	Malinyi (Aa)	Lupiro (Aa)	Ukindu (Aa)	Mikeregembe (Aa)	Mkamba (Aa)	Kaliua (Aa)	Ilonga (Ag)	Lupiro (Ag)
Ilonga (Ag)	0.8	0.7	0.8	0.8	0.7	0.9	0.7	-	-
Lupiro (Aa)	2.3	45	-	-	-	-	-	-	-
Lupiro (Ag)	0.8	0.7	0.8	0.8	0.7	0.9	0.7	22.5	-
Mkamba (Aa)	17.2	6.2	6.4	9.4	8.9	-	-	-	-
Mkamba (Ag)	0.8	0.7	0.8	0.8	0.7	0.9	0.7	85.9	34.6
Kaliua (Aa)	1.9	19.8	13.6	19.4	9.2	5	-	-	-

The F_{ST} estimate reported in this study (mean 0.066) is higher than the reported F_{ST} estimate for *An. arabiensis* from west, south and east Africa (0.035-0.038) where extensive *An. arabiensis* population differentiation was observed (Besansky *et al.*, 1997; Donnelly and Townson, 2000). The F_{ST} estimate observed in this study (0.066 - 0.100) is in line with the largest reported *An. arabiensis* F_{ST} estimate (0.080-0.215). This is a comparison between mainland and island *An. arabiensis* populations (Simard *et al.*, 1999), where gene flow is restricted by ocean. The extensive *An. arabiensis* genetic differentiation observed in this study was neither a result of physical barrier nor geographic distance, consistent with reports from other studies (Besansky *et al.*, 1997; Nyanjom *et al.*, 2003). It can be deduced that other factors than physical barrier or geographical distance may be responsible for genetic differentiation in *An. arabiensis* (Besansky *et al.*, 1997). In this study, highest genetic differentiation in *An. arabiensis* was recorded for markers located on chromosome X: 42% of tests involving markers on chromosome X deviated from Hardy-Weinberg equilibrium, in contrast to 0% in *An. gambiae s.s*. This does not only suggest the population to be structured, but also supports the hypothesis developed by Stump and colleagues that sex-liked differentiation is characterized by some degree of reproductive isolation mechanisms controlled by genes located in genomic regions within X chromosome (Stump *et al.*, 2005).

The observed genetic *An. arabiensis* subgroups existing along the Kilombero valley may be of great significance to malaria epidemiology. The genetic structure of vector populations responsible for the higher malaria transmissions has not been identified yet; thus if this population substructure is ignored, degrees of medically important phenotypes in *An. arabiensis* species, such as vectorial capacity, host preference and insecticide resistance could be obfuscated. Thus, further investigations are needed to thoroughly understand the epidemiological significance of the observed population substructures.

Conclusions

We provide evidence that *An. arabiensis* in East Africa has a complex genetic structure with distinct populations occurring in sympatry, apparently maintained by some degree

of reproductive isolation mechanisms. We therefore hypothesize that ecological differences rather than a physical barrier or geographical distance are responsible for the observed *An. arabiensis* population substructure. We recommend that more investigation is needed not only to clearly establish the specific mechanisms underlying genetic differentiation in *An. arabiensis* populations, but also to identify the number of discrete malaria vector populations sustaining transmission along the Kilombero valley, important for guiding the implementation of available and future control strategies. Proposed new genetic control strategies such as sterile insect technique (SIT) and genetically modified mosquitoes (GM), requires a homogeneous population structure. Thus the observed level of genetic substructure is a challenge in the implementation of these approaches (Benedict and Robinson, 2003; Knols *et al.*, 2003). Therefore, as opposed to *An. gambiae s. s*. whose population structure is homogenous, this approach will face more challenge in *An. arabiensis* due to population substructuring. Since *An. arabiensis* populations appear to be maintained by some degree of reproductive isolation mechanism, multiple genetic modifications of *An. arabiensis* may be needed if genetic control is to be successful. Furthermore, the observed *An. arabiensis* population substructure may have different malaria transmission intensity, as populations with distinct genetic composition may have different vectorial capacity (Petrarca and Beier, 1992). Further investigation of the association between the observed subpopulations and their susceptibility to malaria parasite infection, would be useful to explore if and how these genotypes could be influencing malaria transmission intensity along the Kilombero valley. We have therefore shown that there may still be another level of genetic subdivision within the *An. gambiae* complex, suggesting the possibility of population expansion (Donnelly *et al.*, 2001), with potential implication not only in understanding the evolutionary process of this complex but also for the application of vector control approaches. We recommend further study to investigate the spatial and temporal distribution of the observed genetic variation and population substructure in both *An. gambiae* and *An. arabiensis* along the Kilombero valley.

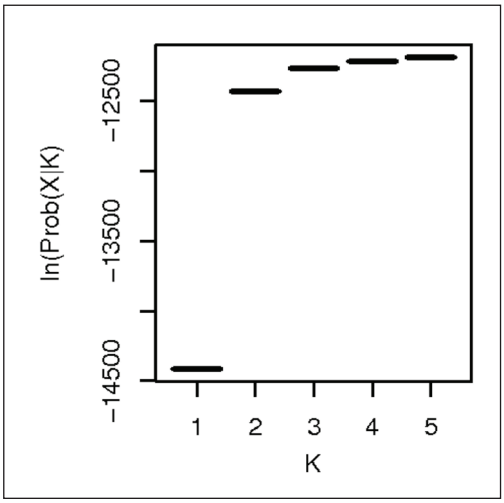

Figure 4: Bayesian clustering analysis using structure suggesting the possibility of structured population in An. arabiensis. The high numbers (probabilities) on the Y-axis corresponding to the pre-defined cluster (K) supports the possibility of a subpopulation at that cluster (K-value).

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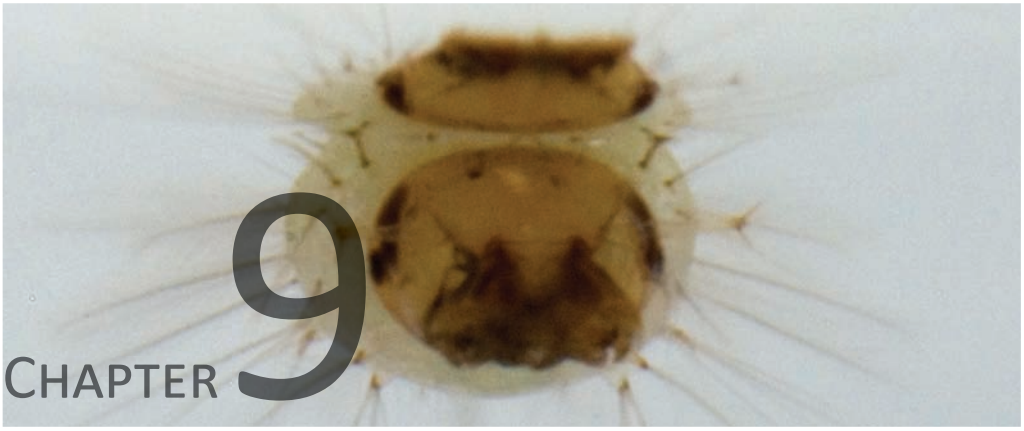

TIBOR BUKOVINSZKY

Clarification of anomalies in the application of a 2La molecular karyotyping method for the malaria vector *Anopheles gambiae*

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> **Abstract**

Chromosomal inversions have been considered to be potentially important barriers to gene flow in many groups of animals through their effect on recombination suppression in heterokaryotypic individuals. Inversions can also enhance local adaptation in different groups of organisms and may often represent species-specific differences among closely related taxa. We conducted a study to characterize the 2La inversion karyotypes of An. gambiae sensu stricto mosquitoes sampled from the Kilombero Valley AA using a newly designed PCR assay. We frequently encountered a (687 bp) fragment which was only present in the Kilombero Valley populations. Laboratory crossing between An. gambiae s. s. from Njage (Tanzania) and Kisumu (Western Kenya) populations resulted in F1 offspring carrying the observed fragment. Karyotype analysis did not indicate differences in 2La region chromosome morphology between individuals carrying either of the two PCR fragments, i.e. the 207 bp fragment, or the 687 bp fragment. The observed insertion/deletion polymorphism within the region amplified by the 2La PCR diagnostic test may confound the interpretation of this assay and should be well considered in order to maintain an acceptable level of reliability in studies using this assay to describe the distribution and frequency of the 2La inversion among natural populations of An. gambiae s. s.

Introduction

The *Anopheles gambiae* complex consists of seven closely related species, including two of the most important vectors of malaria in Africa, *An. gambiae s. s.* Giles and *An. arabiensis* Patton. Chromosomal rearrangements in the form of paracentric inversions are common in these species and have been studied extensively in this complex (Coluzzi *et al.*, 1985; Coluzzi *et al.*, 2002; della Torre *et al.*, 2002; Toure *et al.*, 1998). Various 2La chromosome inversions play a role in the subdivision of *An. gambiae s.s.* populations from West and Central Africa (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985; della Torre *et al.*, 2002; Toure *et al.*, 1998) and the seven recognised species within the complex can be distinguished by fixed chromosomal arrangements (Coluzzi *et al.*, 2002; Toure *et al.*, 1998). *Anopheles arabiensis* Patton and *An. merus* Dönitz are monomorphic for the 2La arrangement, whereas *An. bwambae* White, *An. melas* Giles and *An. quadriannulatus* Theobald (A & B) are fixed for the alternative arrangement $2L^{+a}$. *An. gambiae s.s.* Giles is the only complex member in which the 2La inversion is polymorphic having $2L^{+a}$, $2L^{a}/L^{+a}$ and $2L^{a}$ arrangements (Coluzzi *et al.*, 2002; Toure *et al.*, 1998). In *An. gambiae s.s.*, it is believed that this chromosome inversion provides adaptation to arid conditions (Coluzzi *et al.*, 2002; Powell *et al.*, 1999). Specifically the spatial distribution of the mosquitoes with different types of 2La inversions is strongly associated with particular habitats. For example the wild type phenotypes ($2L^{+a}$) are associated with wetter climate while the inverted phenotypes ($2L^{a}$) are common in dry climates (Brooke *et al.*, 2002), and its frequency within a population changes in response to seasonal fluctuations in rainfall (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985; Coluzzi *et al.*, 1979). Similarly, the 2La inversions have been linked to microclimatic differences that impact mosquito feeding and resting behaviour. For example, 2La inversion phenotypes have commonly been found resting indoors where there is reduced humidity saturation (Coluzzi *et al.*, 1979). Such behavioural heterogeneity may have serious epidemiological impact and may influence the outcome of malaria vector control programmes. For example, the indoor residual spraying (IRS) strategy will not uniformly impact the *An. gambiae s.s.* population (Coluzzi *et al.*, 1977) as this approach will miss the subpopulation that rests outdoors.

The 2La chromosomal inversion polymorphism is also associated with susceptibility to *Plasmodium* in some 2La phenotypes (Petrarca, Beier, 1992; Vernick, Collins, 1989), and LRIM1, a major anti-*Plasmodium* gene located within the breakpoints of the 2La arrangement (Osta *et al.*, 2004), has been shown to be variable for adaptive alleles in *An. gambiae* (Slotman *et al.*, 2007). Since the 2La inversion is associated with important phenotypes in *An. gambiae*, its frequency and distribution in natural populations is of major interest.

A PCR-based assay to determine 2La karyotypes has been developed and extensively validated under field conditions (Sharakhov *et al.*, 2006; White *et al.*, 2007). The PCR utilises three primers that produce two products that distinguish 2La karyotypes. A 207 bp fragment is produced if the mosquito carries a standard (un-inverted) arrangement, a 492 bp fragment if it carries the 2La inversion and in heterozygotes the PCR results in two fragments (207/492). In this study we attempted to characterize 2La inversion karyotypes in *An. gambiae s.s*. populations from the Kilombero Valley of Tanzania, whose population genetic structure has not been previously described. Malaria transmission within this area occurs at some of the

highest intensities ever described (Smith *et al.*, 1993; Smith *et al.*, 1998), and there is thus strong motivation to increase knowledge of the demography, ecology and genetic structure of resident vector populations in order to generate biological insights that could strengthen current and future control strategies. In the course of these studies we observed a PCR band of 687 bp which was reported by Obbard et al (Obbard *et al.*, 2007) from individuals collected from Kilombero valley (Fig. 1). To examine the origin and diagnostic relevance of this fragment we performed polytene chromosome analyses, crossing experiments and sequence analysis.

Materials and Methods

Mosquito collection

Mosquitoes were collected from seven localities within the Kilombero Valley (Tanzania), from January - May 2007: Ilonga, Lupiro, Malinyi, Mikeregembe, Mkamba, Taweta and Ukindu (Fig. 1). In each village, CDC light traps were set in different houses for three consecutive nights. Every morning, traps were retrieved and mosquitoes identified morphologically. Mosquitoes identified as *An. gambiae s.l.* were individually preserved in tubes with silica gel for species-diagnostic PCR (Scott *et al.*, 1993).

We also analysed 30 male and 79 female *An. gambiae s.s* from a colony (Njage strain) originally established from females collected in Njage village (Huho *et al.*, 2007; Ng'habi *et al.*, 2008) about 70 kms away from the Ifakara research Institute (Fig. 1). In addition, we analyzed thirty *An. gambiae s. s.* samples from each of the following colonies maintained at Davis, CA: Kisumu (Western Kenya), Banambani (Mali) and Loum (Cameroon).

Crossing experiments

Crosses were conducted by placing 100 virgin males with 100 virgin females of the Kisumu and Njage colonies together in mating cages. Crosses were done *en masse* (100 females + 100 males), rather than single pair matings. Crosses were conducted in both directions (i.e. Cross 1: Kisumu females x Njage males, Cross 2: Njage females x Kisumu

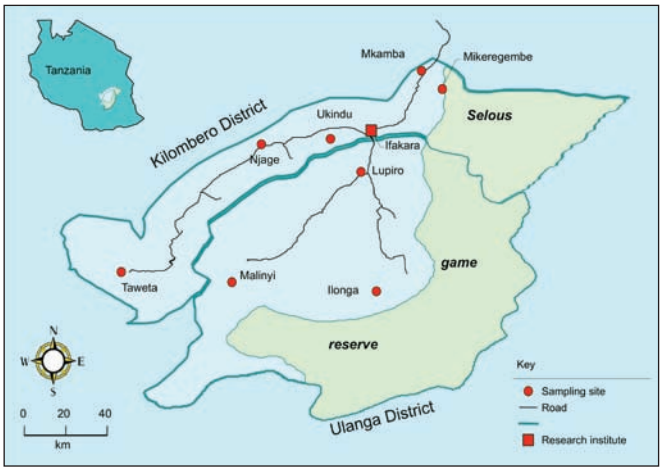

Figure 1: Map of Kilombero/Ulanga District, Tanzania showing mosquito collection sites and origin (Njage) of the laboratory colony *An. gambiae s. s.* used in this study.

males). The offspring obtained from the two crosses were reared in separate trays to the adult stage. Larvae were fed with fish food Tetramin® and maintained at 27°C. Forty eight male and female F_1 offspring from each cross were taken for molecular analysis of the 2La inversion karyotype and visual karyotyping by microscopy.

Molecular Methods

Individual mosquitoes were ground in a TissueLyser® for high-throughput disruption of biological samples. DNA purification was carried out using a Qiagen BioSprint 96 workstation following the protocol for DNA extraction from animal tissues as supplied by the manufacturer. Molecular species diagnostic of field-collected mosquitoes was performed as described by Fanello *et al.* (Fanello *et al.*, 2002). Samples from laboratory colonies (F_0) and F_1 progeny of the crosses were subjected to PCR procedures as described by Favia *et al.* (Favia *et al.*, 2001) to determine molecular forms. All specimens were also screened using the PCR assay for the 2La inversion (White *et al.*, 2007). This PCR produces a 207 bp fragment for the $2La^+$ arrangement and a 492 bp fragment for the 2La arrangement. PCR products were visualized in 1.5% agarose gels.

Several 687 bp fragments resulting from the 2La diagnostic PCR of Njage strain samples were purified using a Qiagen PCR purification kit. Purified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen). Plasmid DNA of colonies containing a 687 bp insert was purified using a Mini-prep kit from Qiagen. Inserts were sequenced in both directions on an ABI 3100 Genetic Analyzer, using the M13 forward and reverse primer and ABI Big-Dye Terminator v3.1. Sequences were edited and aligned in the DNA-STAR© package from Lasergene. Sequences were compared to those deposited in the National Center for Biological Information (NCBI). All sequences were submitted to Genbank. Accession Numbers are provided in Table 1.

Table 1: Sequence and GenBank accession numbers for 687 bp and 207 bp PCR products generated by the White et al. (2008) *An. gambiae* 2La molecular karyotype diagnostic. Sequences from *An. gambiae* colonies established from mosquitoes collected from Njage village (Ifakara) and from field collected mosquitoes collected in the village of Lupiro, Kilombero Valley, Tanzania.

Sequence name	GenBank accession No.	Sequence name	GenBank accession No.
687 bp fragments		207 bp fragment	
Ifakara1f	EU805810	Ifakara19a	EU805818
Ifakara2j	EU805811	Ifakara19b	EU805819
Ifakara8k	EU805812	Ifakara19m	EU805820
Ifakara45a	EU805813	Ifakara60a	EU805821
Ifakara60b	EU805814	Ifakara60e	EU805822
Ifakara78b	EU805815	Ifakara74a	EU805823
Ifakara79a	EU805816	Ifakara77d	EU805824
Lupiro1a	EU805817	Lupiro1e	EU805825

Polytene chromosome analysis

This analysis was done for colony mosquitoes only. Twenty nine hours following blood feeding, 79 female mosquitoes from Njage strain had their ovaries removed and preserved in Carnoy's solution for karyotyping following procedures described by Della Torre *et al.* (della Torre, 1997). The same procedure was performed on thirty mosquitoes from the F_0 and F_1 progeny of the two populations crossed in this study.

Results

A total of 603 field-collected *An. gambiae s.l.* mosquitoes were analysed in this study. Of these, 113 (18.7%) were *An. gambiae s. s.* and 490 (81.2%) were *An. arabiensis.* As expected, all *An. gambiae s.s.* belonged to the S molecular form (the M molecular form has never been found in East Africa (Coluzzi *et al.*, 1985; Toure *et al.*, 1998)). Both *An. gambiae s. s*. and *An. arabiensis* were found in all villages except Ukindu (only *An. arabiensis*). However, the relative frequency of the two species varied substantially between villages (Table 2).

We subjected each *An. gambiae s.s.* specimen to the 2La molecular karyotyping assay of White *et al.* (White *et al.*, 2007). We observed the 687 bp fragment from the 2La PCR in 12.4% of field-collected specimens (Table 2, Fig. 2). This fragment (Figure 3) was not present in the Loum (Cameroon), Banambani (Mali) or Kisumu (Kenya) colonies. However, in the Njage colony (Tanzania), it was found in 61.6% of mosquitoes heterozygous for the 2La arrangement ($2L^{a}/2L^{+a}$) and 60% of mosquitoes homozygous for the standard arrangement ($2L^{+a}/2L^{+a}$), based on polytene chromosome analysis (Table 2). Additionally, specimens that were homozygous for the 687 bp, or heterozygous for

Table 2: Locations of collection sites and origin of laboratory colonies of *An. gambiae s.l.* mosquitoes used in this study. Total number of *An. gambiae s.s.* (Ag) and *An. arabiensis* (Aa.) collected from each locality; molecular form and chromosome 2La arrangement, as determined using the PCR diagnostic of White et al., (2007) are provided. Chi-square P= values for observed vs expected, HWE for 2La karyotype frequencies are provided in the far right column.

Country	Locality	coordinates (Lat; Long)	Source	*N*		Molecular form	2L arrangements and 2L PCR genotypes					
				Ag	*Aa*		$2L^{+a}/2L^{+a}$		$2La/2L^{+a}$		2La/2La	
							207/207	207/687	207/492	687/492	492/492	HWE: χ^2; P values
Cameroon	Loum	-4.100; 11.500	Colony	30	0	M	0	0	16	0	14	*P* = 0.6
Mali	Banambani	-8.050; 12.800	Colony	30	0	M	1	0	12	0	17	*P* = 0.9
Kenya	Kisumu	-0.583; 34.466	Colony	37	0	S	6	0	22	0	9	*P* = 0.9
Tanzania	Njage	-8.133; 36.683	Colony	79	0	S	2	3	23	37	14	*P* < 0.001
Tanzania	Lupiro	-8.377 36.667	Field	45	101	S	10	1	7	8	19	*P* < 0.001
Tanzania	Mkamba	-8.033; 37.767	Field	51	23	S	18	0	1	2	30	*P* < 0.001
Tanzania	Mikeregembe	-8.036; 37.967	Field	1	138	S	1	0	0	0	0	-
Tanzania	Ukindu	-8.277; 36.667	Field	0	76	N/A	0	0	0	0	0	-
Tanzania	Malinyi	-8.933; 36.133	Field	1	122	S	1	0	0	0	0	-
Tanzania	Ilonga	-9.067; 36.855	Field	15	15	S	3	0	3	2	7	*P* = 0.5

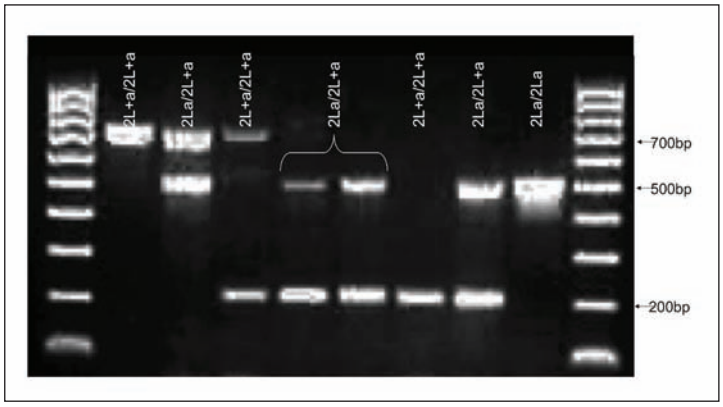

Figure 2: Observed PCR banding patterns from both field and colony Anopheles gambiae s. s. mosquitoes from Tanzania and their corresponding polytene chromosome arrangements as visualized using microscopy (the label of each lane stands for the polytene chromosomal arrangement).

the 687 bp and 207 bp fragment were homozygous for the 2La standard arrangement, $2L^{+a}/2L^{+a}$ (Fig. 2, Table 2). Specimens that had both the 687 bp and 492 bp fragments were heterozygotes, $2L^{a}/2L^{+a}$ (Fig. 2, Table 2). The 687 bp fragment was not observed in mosquitoes homozygous for the inverted arrangement, $2L^{a}/2L^{a}$ (Fig. 2, Table 2). No single individual out of the 79 mosquitoes from the Njage colony was found to have all three PCR fragments together, indicating that the 687 bp fragment segregates with $2L^{+a}$. With the exception of the collection from the village of Ilonga, all populations collected from Kilombero valley showed significant departure from Hardy-Weinberg equilibrium (Table 2).

Crossing experiments

To examine whether the 687 bp fragment followed a Mendelian pattern of inheritance, we crossed the Njage and Kisumu strains (both S forms) in both directions. A total of 192 F_1 progeny from the two crosses were analyzed. Based on PCR analysis, the frequencies of chromosome 2La arrangements in the progeny F_1 mosquitoes are presented in Table 3. The 687 bp gene fragment was present in at least some offspring possessing karyotypes in which it would be expected ($2L^{+a}/2L^{+a}$ and $2L^{a}/2L^{+a}$). We observed no 687/687 homozygotes as expected, since this "allele" is absent from the Kisumu colony. The results of the crossing experiments further confirm that the 687 bp fragment is not a PCR artifact.

Table 3: Distribution of chromosome 2 left arm arrangements in F1 progeny from crosses of the Kisumu and Njage strains in the laboratory.

Cross	$2L^{+a}/2L^{+a}$ 207/207		$2L^{+a}/2L^{+a}$ 207/207		$2L^{+a}/2L^{+a}$ 207/687		$2L^{+a}/2L^{+a}$ 207/687		$2L^{+a}/2L^{+a}$ 687/687		$2L^{+a}/2L^{+a}$ 687/687		$2La/2L^{+a}$ 207/492		$2La/2L^{+a}$ 207/492		$2La/2L^{+a}$ 687/492		$2La/2L^{+a}$ 687/492		2La/2La 492/492		2La/2La 492/492		TOTAL
	F		M		F		M		F		M		F		M		F		M		F		M		
NjageX Kisumu*	5		7		5		0		0		0		15		21		5		2		18		17		95*
Kisumu X Njage	6		7		14		9		0		0		28		27		0		2		0		3		96

*A total of 96 mosquitoes were sampled in both directions. One mosquito DNA did not amplify.

ÔMÕand ÔFÕstands for male and female respectively.

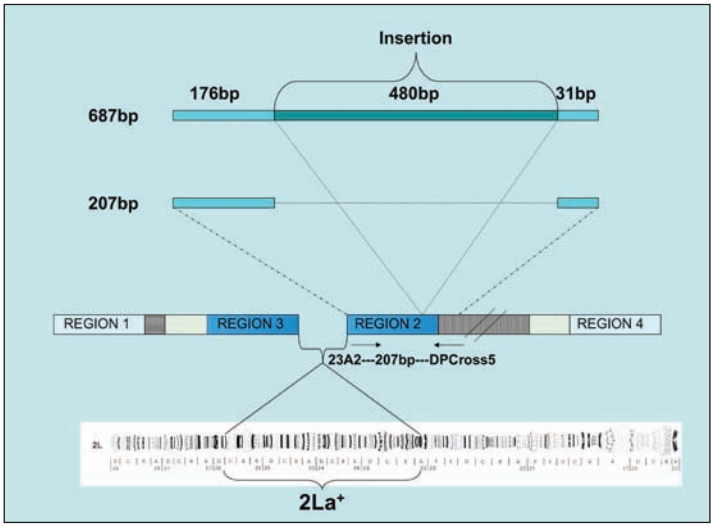

Figure 3: Illustration of the PCR diagnostic for the 2La+ gene arrangement showing the position of the 207 bp fragment produced by the 23A2 and DPCross5 primers and 480 bp insertion we commonly observed in mosquitoes from Tanzania. Modified after White et al. (2007).

Sequence Analysis

Clones containing the 687 bp fragment from eight (laboratory and field) individuals and clones with the 207 bp fragment from eight (field and laboratory) individuals were sequenced. GenBank accession numbers are provided in Table 1. A total of thirteen polymorphic sites were observed in both groups, two in the 207 bp group and 11 in the 687 bp group. Sequence analyses showed that the 687 bp fragment is comprised of the 207 bp fragment containing an insertion of 480 bp (Fig. 3). A blast search of this 480 bp insertion against the *An. gambiae* genome (Holt *et al.*, 2002) indicates that it contains three sequences that are present at least 70-80 times in the genome. That is, the insertion is comprised of repetitive DNA sequences. A comparison of the 687 bp fragment to sequences submitted to GenBank showed that one of the 687 bp fragments we sequenced, showed 100% similarity with a putative 2La chromosomal inversion-assay fragment (Mbt8_2L+) observed by Obbard *et al.* (Obbard *et al.*, 2007) using the diagnostic 2La PCR from White *et al* (White *et al.*, 2007) in samples from Mbita (Kenya). Anomalous bands of four different sizes in samples from Western Kenya and Mount Cameroon were reported by Obbard et al., as well as one reported by Slotman et al. (Slotman *et al.*, 2007) in a Cameroonian sample. The Kilombero fragment, however, was similar to one of the fragments found in Western Kenya (Mbita). Furthermore, the sharing of insertion sequence between Mbita and Kilombero may suggest that this phenomenon is not localised, thus further studies are needed to envisage this phenomenon.

Discussion and conclusions

The 2La inversion in *An. gambiae* is linked to phenotypes that are important to malaria transmission. These include drought tolerance, endophily (Petrarca, Beier, 1992) and susceptibility to *Plasmodium* infection (Blandin *et al.*, 2004; Osta *et al.*, 2004; Slotman *et al.*, 2007). Studies aimed at describing the distribution of the 2La inversion among natural populations are therefore of great interest. The PCR-based method developed

by White et al. (White *et al.*, 2007) to facilitate the determination of the 2La karyotype is a significant contribution to *An. gambiae* population biology. The method developed by White *et al*. (White *et al.*, 2007) utilizes PCR primers based on sequences around the 2La breakpoints provided by Sharakhov *et al.* (Sharakhov *et al.*, 2006). The application of this assay is confounded by polymorphism in the region amplified by this PCR-based method, resulting in the production of fragments inconsistent with those described in the original paper (Obbard *et al.*, 2007; Slotman *et al.*, 2007). We encountered such a polymorphism that produced an "atypical' fragment of 687 bp which occurred at varying frequencies in *An. gambiae s. s.* populations at several sites in the Kilombero Valley of Tanzania Table 2.

The occurrence of an apparently high degree of insertion/deletion polymorphism within the sequence amplified by the White *et al.* PCR diagnostic (White *et al.*, 2007) may confound its application. We confirm that this fragment can be reliably detected through the robust 2La inversion karyotyping techniques previously developed for *An. gambiae s.s.* mosquitoes (White *et al.*, 2007). Our results indicate that the 687 bp fragment is not an artifact, but a result of insertion/deletion polymorphism within the region amplified by the 2La PCR diagnostic. Therefore, the amplification of these unexpected fragments may confound the interpretation of this assay and we recommend that they should be well considered in order to maintain an acceptable level of reliability in studies aimed at describing the distribution and frequency of the 2La inversion among natural populations of *An. gambiae s. s.*

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KIJA R. NG'HABI

Summarising discussion

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The research presented in this thesis centered on answering important questions that may affect the outcome of genetic control programmes of African malaria mosquitoes. Research focused on how environmental conditions experienced during larval development influence An. gambiae male mating success. It was also attempted to simulate a near-natural environment in which the laboratory-generated results could be verified. Besides, the genetic factors that mediate gene flow within malaria mosquito populations were researched and are presented here. A number of objectives were listed in the introductory chapter and the findings are discussed in more detail in each respective chapter, specifically in the context of genetic vector control approaches like the Sterile Insect Technique (SIT) or genetically-modified mosquitoes (GM).

Effect of larval density and food on mating competitiveness

For mosquitoes, the amount of food ingested by larvae determines their adult body size and teneral reserves. Well provisioned larvae develop faster and emerge as large adults while deprived larvae have their developmental time lengthened and emerge as small adults. Like-wise, densely crowded larvae have their development slowed and emerge as small adults. These effects in turn influence long-term survival and fecundity in females, which are important predictors of reproductive success. The findings reported in this thesis indicate that larval crowding affects male *An. gambiae s.s.* mating performance. When allowed to compete in a swarm, males from a less crowded larval environment were 11 times more likely to be the first to acquire a female mate than males originating from a densely crowded environment. These males were also 4 times more likely to acquire the first female mate than males from an environment with intermediate densities. The three crowding conditions (low-medium-high) affected neither the abundance of teneral reserves nor the body size of emerging male *An. gambiae s.s.* It can be deduced that the mating performance observed in males reared in less crowded larval conditions, could not be explained by body size or teneral reserves (Chapter 2). When mosquito larvae are crowded, they may release chemicals (growth retardants) which inhibit the growth of their conspecifics (Roberts, 1998). These chemicals tend to increase with increasing larval density (Moore and Whitacre, 1972; Roberts, 1998). Although we did not assay for the presence of these chemicals in our treatments, this phenomenon has been reported in other mosquito species, although not to our knowledge for *An. gambiae*. However, these chemical retardants can be removed by daily changing of rearing water (Roberts, 1998). Therefore, given the absence of food limitation in the three crowding treatments and that rearing water was not changed daily in my experiment, I suggest that crowding chemicals could account for the observed poorer mating performance for the males reared under densely crowded conditions as larvae.

Findings reported in this thesis also showed that, by varying larval nutrition, three distinct *An. gambiae s.s.* male phenotypes could be generated. The body size, amount of teneral reserves and survival of emerged males were affected by the amount of food they received during larval development. Better nourished larvae (high food treatment) resulted in bigger males which were 18% and 9% bigger than those in the low (food-deprived) and medium food treatments, respectively (Chapter 3). When allowed to compete for female mates in a swarm, males from the medium food treatment group were 6 times and 2 times more competitive than males from the better nourished group and food-deprived males, respectively. Therefore, it could not be established whether body size or energetic reserves were important factors in determining male mating competitiveness. Although this observation agrees with the explanation that male body size does not influence male mating success (Charlwood *et al.*, 2002), it contrasts with reports from other studies (Yuval *et al.*, 1994; Yuval *et al.*, 1993). It was further observed that males and females that were similar in size were more likely to mate than males and females that were not similar in size. Thus, males from the medium food treatment were closest in size to the females made available to them during the mating experiment (Chapter 3). This finding also complements the findings reported in Chapter 2, where less crowded males accrued more mating with females from a less crowded environment. The coupling of a male and female that are close in body size may be more likely because their genitalia are similar in size and

may be corresponding well to allow courtship (Arnqvist, 1997). It may also be that females can recognise the wing beat frequencies of males with body sizes similar to them as potential mates (Gibson and Russell, 2006; Pennetier *et al.*, 2010). However these findings deserve further investigation by allowing males of different sizes to compete for females of varying sizes in which the body size and wing beat frequencies of coupling specimens is compared.

Is there an advantage to being the first to obtain a female mate in natural mosquito swarms in the context of genetic control? The ultimate way to deliver the desired traits (i.e. refractoriness or induced sterility) in a natural target population is through mating between released males and wild females. In natural mosquito swarms, the male to female ratio is highly skewed, ranging from 10:1 to 600:1 (Charlwood and Jones, 1980). In such a highly competitive environment, it is extremely important for a released male to obtain a female at the earliest opportunity, and as many females as possible because there is no guarantee for another female to arrive, given the limited swarming period which lasts for only 15-20 mins (Charlwood and Jones, 1979; Diabate *et al.*, 2003; Marchand, 1984). Swarming males are highly exposed to natural enemies and predators, thus winning a female mate at an earliest opportunity is advantageous as a released male can introduce the desired trait before being attacked (Subramanian, 2002). Alternatively, it is uncertain whether laboratory-reared males will survive long enough in the harsh field environment to acquire more mates, thus obtaining a female mate at an earliest opportunity will ensure gene transfer before males dies. Therefore if laboratory-reared males can better adapt and out-compete wild males for female mates, then it is most likely that SIT / GM traits can successfully be introduced in field target population.

In conclusion, the findings reported in this thesis show that environmental factors experienced during early larval development can affect the mating behaviour of adult male *An. gambiae s.s.* mosquitoes. The observation that crowding and food availability have an impact on male life history and can be manipulated under laboratory conditions has direct implications for improving male mating success of males designated for release in genetic control programmes. It is important to note that these findings are based on laboratory-maintained strains, thus the next step is to validate these findings in a semi-field system population. Specifically, experimental manipulation of these factors using laboratory-maintained males using improved protocol and subsequently allowing them to compete with wild males for wild females in a semi-field system should be a priority for further research to assess the suitability of these factors to enhance male mating success.

Physiological and genetic status of laboratory, semi-field and natural populations of male Anopheles mosquitoes

Laboratory-derived experimental results provide the major source of our knowledge of insect ecology and behaviour. It is obvious that males designated for release in genetic control programmes have to be colonised first, genetically-modified / sterilised and then mass-reared (for both GM and SIT) in the laboratory. This necessitates the need to understand the biological and behavioural differences between laboratory and wild mosquitoes. It is important that physiological and behavioural parameters of

laboratory-reared males match as closely as possible with those of wild mosquitoes for the proposed technology to be successful. Therefore it is important that any significant differences in the physiological and genetic status of laboratory and field mosquitoes are identified because they have an effect on the mosquito fitness. Their impact on mating success needs to be assessed and procedures to minimise or eliminate them implemented.

This thesis has shown that there is a significant difference in energetic reserves (carbohydrates, lipid and glycogen) between laboratory-reared and wild *An. gambiae s.s* males. Laboratory-reared male *An. gambiae s.s.* have higher abundance of sugars than their wild conspecifics ($Median_{LAB}$=8.01 μg, $Median_{FIELD}$=0.00 μg). Similarly, glycogen content was higher in laboratory-reared males, being on average three times larger than the amount found in wild males ($Median_{LAB}$ =15.26μg, $Median_{FIELD}$= 4.21μg). This observation was expected given that laboratory-maintained male mosquitoes have *ad libitum* access to sugar water. In contrast, lipid content in wild *An. gambiae s.s.* males was more than twice that of laboratory-reared males ($Median_{LAB}$ =4.6 μg, $Median_{FIELD}$= 9.6 μg) (Chapter 4). This observation was not expected and it was suggested that, due to limitations in the availability of sugar sources, wild males probably do not sugar-feed to repletion. Alternatively, free-living mosquitoes may sugar-feed less during the night than laboratory males, resulting in lower detectable carbohydrate reserves when they were sampled in the early morning hours. It was also observed that wild males were 17% bigger in body size than laboratory-reared males. This observation led to the hypothesis that larger body size of field-collected males may have influenced their larger lipid stores, because body size has a linear relationship with the energetic reserves (Briegel, 1990). It can be deduced that laboratory maintenance has an impact on the physiology of mosquitoes and a rearing regime to match their size and energetic reserves with that observed in wild males may affect their survival and competitiveness after release.

Furthermore, research presented in this thesis indicates that laboratory-maintained males represent less genetic variation than populations of wild males (Chapter 7) which is in agreement with findings from other studies (Matthews and Craig, 1987; Norris *et al.*, 2001). It was found that males colonized in a semi-field system have higher genetic variation than laboratory-reared males. However, semi-field system males maintained lower genetic variation than their reference field population. The laboratory-reared males also experienced more inbreeding depression than semi-field system males, which is indicative of repeated mating between closely related individuals. It was suggested that, contrary to laboratory cage colonies, environmental heterogeneity in semi-field systems provides more selection pressures for individual mosquitoes which helps to maintain the genetic variation of the entire SFS population at a higher level. Although the maintenance of genetic variation by semi-field colonisation fills an important scientific gap, there is an immediate need to determine whether and how the maintained genetic variation is related to semi-field population fitness. Studies to link the maintained genetic variation with fitness components such as survival, fecundity and mating competitiveness is important to evaluate and implement new control strategies (Benedict *et al.*, 2009). Also, the assessment of whether the maintained genetic variation in semi-field populations may counter-balance fitness costs imposed during male sterilisation requires attention.

With regard to these findings, what can we conclude about the success of laboratory-reared against wild male mosquitoes when competing against each other in natural swarms? Greater lipid content has an advantage for the long time reproductive success as it is associated with long-term survival (Van Handel, 1984) and insect flight muscle performance (Haunerlan, 1997). Although swarming activity has been associated with high carbohydrate reserves (Briegel *et al.*, 2001; Yuval *et al.*, 1994), lipid reserves are the preferred fuel for sustained flight in insects (Haunerlan, 1997). Therefore, it is important that laboratory-reared SIT / GM males have their lipid content matched to those of a wild population upon release. This is because their performance in the field will depend on a number of factors including flight performance (for dispersal and swarming), carbohydrate levels and longevity. Thus if the physiological status of laboratory-reared males is close to that of males in the field, the performance of released males will be competitive.

Furthermore, it has been reported that a change in genetic variation may affect the relative abundance of certain behaviour-influencing genes and thus the phenotypic frequencies of the mosquitoes in the population with respect to their behaviour. For example, a genetic change in the screwworm fly *Cochliomyia hominivorax*, affected the relative abundance of an enzyme related to energy flow during flight, resulting in the higher frequency of flies with reduced flight activity (Bush and Neck, 1976). In addition, low genetic variation and higher inbreeding depression are associated with lower fitness, e.g. in *Drosophila melanogaster* (Reed and Frankham, 2003; Reed *et al.*, 2003). The ultimate male reproductive success in a particular swarm depends on its ability to obtain a female mate during a particular swarming event and the number of swarming events it can participate in during its lifespan. Therefore, higher lipid content for field males is advantageous in providing long-term survival to participate and sustain flight in several swarming events. Similarly, the maintained higher genetic variation and low inbreeding depression in semi-field populations may be advantageous as will ensure that, the mating ability of semi-field system reared males is maintained as close as possible to that of field males and thus maximise the chances of obtaining female mates in a swarming event during field releases.

Therefore, if maintenance of genetic variation within semi-field populations improves mating competitive ability and minimises the observed physiological differences between laboratory and field males, then semi-field systems provide ideal conditions to maintain mosquito populations that are aimed for both research and release purposes in control programmes (Ferguson *et al.*, 2008). The observation that genetic variation is maintained by semi-field system maintenance, is a major step towards improving rearing protocols for mosquitoes destined for release due to loss of genetic variation when colonised in standard laboratory conditions (Clayton, 2006; Knols *et al.*, 2007). However, it remains unknown whether the maintained genetic variation in semi-field systems will improve mating competitiveness of semi-field system males versus field-collected males, and whether it will help to minimise the observed energetic reserve differences between laboratory and field males (Huho *et al.*, 2007). Further research is needed to measure other fitness components and pave the way for implementation of the proposed new malaria control approaches.

Transitioning bench work to field application

The release of transgenic mosquitoes into field populations requires, in addition to the developed technology, prior proven efficacy in the laboratory (i.e. using laboratory strains and cage conditions) and subsequent evaluation inside enclosed semi-field environments. Such enclosed environments can help to maximise the potential to evaluate new technologies, and at the same time help in minimising possible risks and side effects (Ferguson *et al.*, 2008; Knols *et al.*, 2003). Because any release is irreversible (Engels, 1992), studies have recommended that GMM should be evaluated in a controlled semi-field system prior to field release to assess possible risks and side effects (Knols *et al.*, 2007; Knols and Louis, 2006). However, other arguments have been made that sterile transgenic males (GM) can be released at first place, to allow reversibility and evaluation of mating competitiveness of genetically altered sterile males (Benedict and Robinson, 2003). The use of an enclosed semi-field system outweighs the first release of sterile GM males in that, in addition to the fact that the fate of genetic manipulation can be monitored and evaluated at close range, the efficacy of drive systems used can easily and repeatedly be evaluated, which cannot be monitored for released sterile insects (Knols *et al.*, 2007; Knols and Louis, 2006). In such a controlled environment all the unforeseen irreversible risks and side effects can be monitored and managed accordingly. Hitherto it was not known if such enclosed semi-natural environments can sustain a mosquito population for multiple generations (Ferguson *et al.*, 2008; Knols *et al.*, 2002) to allow continued monitoring and evaluation. This thesis reports the successful establishment of a self-replicating mosquito population for some twenty four generations within the enclosed semi-natural environment (Chapter 5). Additionally, the semi-field population maintained higher genetic variation than mosquito strains maintained as standard laboratory colonies (Chapter 7). Furthermore, it is assumed that semi-field specimens will exhibit similar behavioural properties to those of the wild reference population. However, more behavioural studies are needed to confirm this.

Therefore, the semi-field population may be useful in several ways in the context of genetic control programs. It can be used as a source of material for research purposes to generate insights that are close to those of their field reference population. Also, in genetic control programmes, semi-field populations may provide source material for up-scaled production of males designated for release. Furthermore, the SIT and GM mode of action requires the ability to rear large numbers of target vectors under laboratory conditions, however not all important target vectors can be reared in the laboratory. For example *An. darlingi* Roots, an important vector of malaria in Latin America cannot be colonised in the laboratory because males are incapable of mating in a restricted space (Lima *et al.*, 2004). Therefore, simulated semi-field systems may be ideal for colonising such species, and make material available for research and control purposes. This tool provides the opportunity to thorough exploitation of vectors life-cycle and identifications of important ecological and biological vulnerabilities necessary for developing new and strengthening available control intervention such that, successful control of malaria and other mosquito-borne diseases can be maintained in a long-term.

Although the semi-field environment is expected to mimic the natural environment, there are other factors that could not be introduced in the system which are important selection pressures in natural populations. For example, human hosts were not introduced in the system and there was not any control intervention likely to

be encountered by mosquitoes under field conditions (e.g. bednets). There was also no interaction between *An. arabiensis* and other mosquito species, such as inter-species competition for food in larval habitats. Most important, the population was maintained parasite-free, and away from their natural predators, these both being important selection pressures within field populations (Ferguson *et al.*, 2003; Subramanian, 2002). Albeit with such an incomplete natural mosquito ecosystem within semi-field systems, we suggest that semi-field mosquito populations will not be fully similar to a natural population but nevertheless more representative of a field- than laboratory colony.

The development of a self-replicating mosquito population alone is not enough. Effective and robust tools need to be developed to effectively evaluate mosquito mating behaviour and ecology within a semi-field system. Mating success has traditionally been assessed through visual observation of copulations in mating swarms (Charlwood and Jones, 1980; Clements, 1992), although this does not apply in all circumstances as some species deploy different mating strategies (Dao *et al.*, 2008). Research presented in this thesis reports a new PCR-based method that can be used to determine the insemination status of females as a means to assess male mating success (Chapter 6). This method is advantageous over the traditional way of assessing mating in that visual observation or microscopic dissection of female spermatheca is tedious, labour-intensive and requires fresh specimens (Chapter 6). Using PCR, *An. gambiae s.l.* females collected during entomological surveys can be screened to assess their insemination status. In addition to PCR, other developed techniques that can readily be used to evaluate mosquito and other insects behaviour in semi field system includes the use of stable isotopes (Helinski *et al.*, 2008; Hood *et al.*, 2006). For example by labeling males with stable isotopes, their mating success can be evaluated by mass spectrometric examination of females (Helinski *et al.*, 2007). It is recommended that these developments are used to further the evaluation of the ecology, biology and efficacy of genetic manipulation so that these approaches can be advocated as malaria vector control tools (Clayton, 2006; Knols and Louis, 2006).

Genetic determinants of gene flow within target mosquito populations

The genetic structure of a target mosquito population has to be clearly understood before releases are to commence. The issue to be explored is whether the target population belongs to one homogeneous gene pool or is a complex of species within the same population which might interfere with the introduction and spread of the desired trait (della Torre *et al.*, 2002; Lanzaro *et al.*, 1998; Lanzaro and Tripet, 2003). Whilst characterising the genetic structure of sympatric *An. gambiae s.s.* and *An. arabiensis* populations in the Kilombero valley, research reported in this thesis showed that *An. arabiensis*, which was originally regarded a single taxonomic unit (Ayala and Coluzzi, 2005; Coluzzi *et al.*, 1985), may occur as a complex of more than one species that is maintained by some degree of reproductive isolation (Chapter 8). This observation is new and is not in line with observations reported by studies conducted in East Africa which concluded the absence of extensive genetic differentiation among continental *An. arabiensis* populations (Donnelly *et al.*, 1999; Donnelly *et al.*, 2002; Kamau *et al.*, 1999; Kent *et al.*, 2007). However such observation would normally be expected to occur on island populations due to wide stretches of the ocean that act as barriers to gene flow (Simard *et al.*, 1999). The introduction of sterility or spreading of genes in a target population requires free interbreeding between individuals carrying the gene and the target population. Any population substructure may pose a challenge and

may undermine the effectiveness of the intervention. Therefore, characterisation of field populations should be prioritised even in malaria vector populations traditionally considered to consist of single taxonomic entities, by using modern molecular genetic technologies (Lanzaro and Tripet, 2003; Zheng *et al.*, 1996). The observed *An. arabiensis* population substructure in the Kilombero valley indicates that multiple strain genetic manipulation may be needed for successful introduction and spread of desirable traits. *Anopheles gambiae s.s.* in the Kilombero valley however, was represented by a population which seems to share a homogenous gene pool, suggesting that no multiple-strain genetic manipulation/sterilisation may be required for successful implementation of genetic control approaches.

This thesis also evaluated other factors that have been known to promote population substructure. Chromosomal inversion is an important factor responsible for population structure among *An. gambiae s.s.* in West Africa (Coluzzi *et al.*, 1985; della Torre *et al.*, 2002). Employing a new PCR method to determine inversion polymorphism, this thesis reported no differences in inversion frequencies, although it came up with a novel observation that the method itself presented some anomalies unique to *An. gambiae s. s.* populations in the Kilombero valley (Chapter 9). This observation provides further evidence that the *An. gambiae s. s*. population shares a homogeneous gene pool. However, additional studies are needed to substantiate the observed anomalies in the Kilombero valley population, considering that this has not been observed in other *An. gambiae s. s.* populations (White *et al.*, 2007). Non-natural processes also interrupt gene flow by preventing the mating between individuals from different populations. This includes induced male sterilisation which depending on the radiation condition, renders males to have a reduced mating competitiveness when compared to their wild counter parts. When radiation-induced male sterility at higher dose is used, it imposes fitness costs which compromise mating ability (Helinski *et al.*, 2008; Helinski and Knols, 2008). However, semi sterilisation dose results in higher mating competitiveness if irradiation was performed using larger cages (Helinski and Knols, 2008). Therefore male semi-sterilisation doses can be used to avoid adverse effect of irradiation prior to release in order to maximise male performance in the field.

Integrated vector management (IVM) and genetic control

It is believed that SIT and GM area-wide approaches that focus on reducing mosquito population size can have enormous potential impact on disease transmission in varying settings. However previous successful control with SIT suggests that these approaches may not be successful by themselves but need to be integrated with other control methods (Dyck *et al.*, 2005). Like other approaches there are other instances where SIT and GM may not have obvious potential value for example in areas where there are more than one dominant vector species. Thus the optimal use of SIT and GM would be within an IVM programme (Beier *et al.*, 2008; WHO, 2008). Despite difficulties in effective implementation of IVM, other control approaches within the framework are facing challenges (Beier *et al.*, 2008). Emerging artemisinin resistance threatens the lifetime of ACT combination therapies (Maude *et al.*, 2009). In addition, the emergence of pyrethroid resistance among major malaria vectors could compromise the long-term success of insecticide-treated bednets (ITNs) in reducing malaria transmission (Pinto *et al.*, 2007; Santolamazza *et al.*, 2008). Therefore, the SIT and GM area-wide genetic control approaches will provide additional tools and are worth consideration within the IVM framework. Historically, SIT has proven successful in controlling some agricultural

pests as part of area-wide integrated pest management programmes (Townson, 2009) and has proven to be not only environmentally safe, but also a cost-effective strategy to eradicate / suppress target populations and can even protect re-invasion in already controlled areas. Although field application of SIT and GM for mosquito control is yet to commence due to biological, political and ethical challenges, these approaches are expected to have high impact in the ongoing battle against malaria and other mosquito-borne diseases (Howell and Knols, 2009; Knols *et al.*, 2007). If issues hindering field implementation such as mating competitiveness are resolved, SIT and GM would be potential additional mosquito control tools within IVM. I recommend that ongoing efforts to develop these approaches should be prioritised and given all possible support if the long-term and sustainable malaria and other mosquito-borne diseases eradication goals are to be achieved (Águas *et al.*, 2008).

Conclusions and recommendations

Environmental factors experienced during early mosquito larval development influence male mating behaviour and mating success. Consequently, these factors can be manipulated in the laboratory and be incorporated in mass-rearing protocols of mosquitoes designated for release in genetic control programmes. Semi-field systems provide suitable ecological conditions for propagation of malaria vector populations that sustain higher levels of genetic variation than laboratory-reared specimens. GM approaches can now be evaluated in these settings, to attain maximum impact and minimise unforeseen risks prior to field implementation. The genetic structure of field populations needs to be characterised in advance, prior to mass releases, in order to identify possible barriers to gene flow. I recommend that support and priority should be given to all ongoing efforts to explore these approaches so that they become socially, economically and environmentally acceptable vector control approaches as IVM components, if the long-term goal to eradicate malaria in Africa is to be achieved.

Research findings of this thesis should improve the components of SIT and GM to develop genetic approaches feasible for mosquito control. Figure 1 illustrates some aspects of genetic control that the research outcomes reported here can contribute to improve rearing protocols for SIT and GM approaches. When the target population is identified and its genetic structure characterised then blood-fed females from this population can be used to establish a founding population in a semi-field system. In the semi-field system, developed laboratory-confined technology can be evaluated as a first step toward field implementation. For example studies to better understand the biological potentials / risks of GM can be executed to pave the way for field implementation. The semi-field system also can be a source of mosquitoes for release purposes, e.g. eggs can be collected for up-scaling, genetic manipulation (GM) and sterilisation (SIT). During the mass-rearing process, an improved protocol has to be devised. This includes less crowding with sufficient amount of food (e.g. 0.2 mg per larvae per day) available (without over feeding) and daily changing of rearing water to avoid the negative impact of crowding chemicals on larval growth (see Chapters 2 & 3). If an efficient sex separation mechanism is available males can then be released back in their original population if ethical and legal challenges are resolved. However, there are a number of questions that need to be answered. As mosquitoes are not going to be maintained for more than one generation, mass-rearing in the laboratory during up-scaling is not expected to cause more impact on the genetic variation. Likewise, it still needs to be verified whether the physiological differences between laboratory

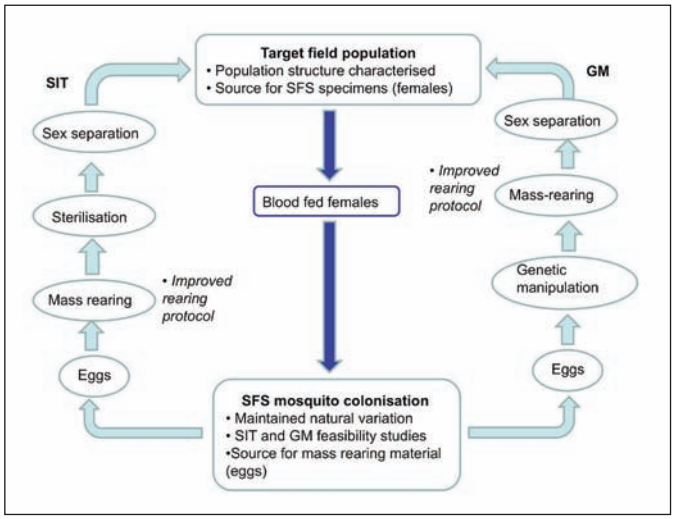

Figure 1: A schematic diagram illustrating suggestions to improve components of genetic control. Abbreviations, SIT- Sterile Insect Technique; GM- Genetically Modified Mosquitoes; SFS- Semi Field System.

and field males is a result of change in genetic variation or in fact is a result of different larval development condition. This will require comparison of lipid reserves of emerging males from laboratory and those from the semi-field system.

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> Summary

Introduction

Ongoing efforts to control malaria and other vector-borne diseases using contemporary vector control approaches have resulted in commendable successes. However, the emergence of drug and insecticide-resistant parasite and vector strains, respectively, may reverse the benefits already achieved. Thus, development of new approaches to supplement existing control methods has been the forefront option to build on the achieved successes. There are two genetic control approaches proposed, the Sterile Insect Technique (SIT) and genetically modified (GM) mosquitoes. These approaches require the release of genetically altered males to induce sterility within a wild vector population to reduce the size of the vector population or introduce parasite-refractoriness through gene constructs that spread within such populations. The concept of genetic control is not new and successful attempts were made during the last century, particularly with SIT. Despite successes there were also failures and the unsuccessful trials were a result of several factors which were generally related to insufficient knowledge of male mating biology.

Current advances in molecular biology have fuelled interest in genetic control strategies for malaria and other mosquito-borne diseases, some of which are now approaching field implementation. With regard to past experiences, it is clear that a key issue in the success of these approaches is the better understanding of the mating behaviour of the target mosquito species. The mating behaviour of malaria mosquitoes is a complex process as it is suggested to occur in male aggregations (swarms), which are space (marker) and time (dusk) dependent. This makes it difficult not only to locate the swarms but also to understand the factors that govern mating processes such as male-male recognition, male-female recognition and ultimately mating success. Our understanding of the important factors that mediate male mating success in a mosquito swarm is still insufficient to predict the outcome of new genetic control strategies. The research presented in this thesis focused on investigating factors that determine male mating success in natural mosquito swarms besides genetic factors that mediate gene flow within field populations. Given the limited knowledge on the biology of male mosquitoes, we initially focused on investigating ecological and behavioural factors that have been reported to determine reproductive success of female mosquitoes such as body size, survival and energetic reserves. The study of how these parameters determine male mating success in nature is difficult, and was therefore primarily conducted in the laboratory. However, it was also attempted to develop a novel research tool by mimicking natural environmental conditions in an enclosed semi-field system. The successful establishment of a self-replicating malaria vector population in such a system is reported here. Also, genetic factors that are responsible for determining gene flow in a field population were determined.

Effect of crowding and food abundance on mating competitiveness (Chapter 2 &3)

It was observed that the number of larvae and the amount of food available during larval development influenced adult *An. gambiae s. s.* mating behaviour. Mal*e An. gambiae s. s.* that developed in a low density environment could more readily acquire female mates in a mating swarm than males that developed in a medium or a high larval density environment. Males that developed under low-density conditions were 3 times more competitive than males that developed under medium-density conditions, and 11 times more competitive than males developed under high-density conditions (Chapter 2). Similarly, when *An. gambiae s. s.* larvae were given large amounts of food they emerged as larger adults with more energetic reserves. However, large males were not 'the best man' to win the mating competition. In a food regime where larvae were given small, medium and large amounts of food (chapter 3), it was observed that medium-sized males were 6 times more successful at acquiring female mates than larger males, and were 2 times more successful than small sized males. Being the first male to mate is advantageous because there is a limited number of female mates during a swarming session and also a better opportunity for a male to reproduce before unexpected death occurs, e.g. because of predation. It is concluded that larval crowding and food allocation have strong effects on the mating competitiveness of adult male *An. gambiae s.s.* Thus manipulation of larval crowding and food allocation during mass-rearing could provide a simple technique for boosting the competitiveness of sterile or transgenic male mosquitoes; a prerequisite for successful SIT and GM application.

Physiological and genetic status of laboratory, semi-field and natural populations of male Anopheles mosquitoes (Chapter 4 & 7)

It was observed that body size and lipid reserves of wild males were substantially greater than those of males reared under standard laboratory conditions (chapter 4). We cautioned that the observed energetic limitations of insects maintained under laboratory conditions could underestimate their resilience in the wild after release, as lipid reserves are known to be responsible for flight performance in insects. This may directly affect new vector-borne disease control programmes based on genetic control of mosquitoes. Contrary to standard laboratory colonisation which results in loss of genetic variation, colonisation of mosquitoes in a semi-field system indicated that a substantial amount of genetic variation is maintained (chapter 7). The semi-field population experienced a lower inbreeding depression than a laboratory-maintained colony. It remains to be shown that the maintained genetic variation is correlated with male mosquito fitness.

From laboratory studies to field application (Chapter 5 & 6)

Most of the findings related to the development of genetic control approaches are laboratory-based. However, the feasibility and the fate of genetic manipulation need to be evaluated for the technology to reach field application. This thesis has laid down grounds necessary for transitioning laboratory research to field research. It was shown that a self-replicating mosquito population can be maintained in an enclosed semi-field

system where the behaviour and ecological parameters of mosquito life–history can be continuously monitored and evaluated. Because any field release is irreversible, a semi-field mosquito population can be an intermediate testing ground to evaluate the feasibility of the developed technology prior to open field application. This development also has the potential to evaluate available control interventions and improve their usage and long-term control success in varying settings. It was also shown that male mating success can reliably be evaluated in the semi-field system using a newly designed PCR-based method (chapter 6). The method detects the presence or absence of male sperm in the spermatheca of female mosquitoes. This will be a valuable tool in research on male mating biology.

Genetic determinants of gene flow within target mosquito populations (Chapter 8 &9)

After semi-field evaluation of the new genetic control approach, a thorough understanding of the genetic structure of the target population is a prerequisite before any release is to commence. It has been reported that chromosomal inversion, mutation, laboratory colonisation and induced sterility are genetic factors that may prevent free mating between individuals from two populations. Thus a field population may result into several genetic populations that do not hybridise. Consequently, laboratory colonisation and induced sterility may result in males with poor mating success that thus are unsuccessful in the field. In chapter 8 it is reported that *An. arabiensis* along the Kilombero valley in Tanzania, which was formerly regarded as one population, is a structured population that seem to be maintained by some degree of reproductive isolation mechanisms. Thus characterisation of the genetic structure of any target population is important to ensure successful implementation of newly proposed approaches.

Integrated vector management (IVM) and genetic control

Integrated Vector Management (IVM) is a global strategic framework which seeks to improve the efficacy, cost-effectiveness, ecological soundness and sustainability of disease vector control. IVM promotes the better use of more environmentally friendly vector control methods. The main goal of IVM programmes is to reduce vector populations and disease transmission by using all available and effective measures that contribute to a durable and integrated control strategy. Although the new approaches to control malaria, such as sterile insect technique (SIT) and genetically modified mosquito release (GM) are promising, they are still investigated under laboratory conditions. Issues regarding their suitability for malaria vector control and environmental still need to be assessed. Thus research priority and financial support need to be provided so that these approaches can be investigated for their contribution to vector control and help curb malaria, as components of an IVM framework.

Conclusions and recommendations

Environmental factors experienced during early mosquito larval development influence male mating behaviour and mating success. These environmental factors can be manipulated and incorporated in mass-rearing protocols in the laboratory to improve the quality of males designated for release in SIT or GM mosquito control

programmes. Enclosed semi-field systems provide suitable ecological conditions for the propagation and maintenance of genetic diversity of mosquito populations. This tool provides a readier and easier source of near-natural vector populations. Within this tool vector behaviour can be monitored and evaluated at greater precision than in natural settings. It is also expected that more realistic insights can be gained than from studies on mosquitoes maintained in laboratory conditions. I recommend that (i) semi-field system (SFS) should be used to improve available control interventions in order to attain longer-term control success, (ii) newly proposed genetic control approaches can be evaluated within these systems to maximise potential impacts while minimising possible risks prior to field implementations, (iii) the genetic structure of field populations needs to be characterised in advance, prior to field implementations of genetic control approaches in order to identify possible barriers to gene flow. Finally, I recommend that support and priority should be given to all ongoing efforts to explore these new approaches so that they become socially, economically and environmentally acceptable vector control approaches as IVM components, if the long-term goal to eradicate malaria in Africa is to be achieved.

> Curriculum vitae

Kija Richard Ndyai Ng'habi1 was born in 1976 in Maswa district inTanzania. He grew up and attained Certificate of Primary Education at Longalonhiga primary school in Meatu district. Then he joined Bihawana Secondary School in Dodoma region and attained a Certificate of Secondary Education Examination (CSEE) in 1995. He then joined Mkwawa High school ('A' level) in Iringa and attained an Advanced Certificate of Secondary Education Examination (ACSEE) in 1998. The successful completion of the 'A' level earned him a chance to join the faculty of Science at the University of Dar es Salaam in 2000, and obtained his BSc degree (Wildlife ecology and management, zoology major) in 2003. In September, 2003 he joined the Ifakara Health Institute after being recruited from the University of Dar es Salaam as a research intern. During his 3-month internship, he conducted research as part of a team that discovered that a soil-born fungus can actually infect and kill the malaria mosquito Anopheles gambiae before it can become infectious to people. These results represented the first demonstration of a potential bio-control agent for adult malaria vector control. In 2004, he began his MSc studies at the University of Dar es Salaam which focused on the mating behaviour of male African malaria vectors and its implication to the success of vector control strategies based on the mass-release of genetically-modified (GM) or sterile male (SIT) mosquitoes. After completion of his MSc studies in 2006, he secured an International Atomic Energy Agency Fellowship to spend seven months in an internationally renowned malaria vector genetics laboratory at the University of California in Davis (USA), under the supervision of Prof. Gregory Lanzaro. At UC Davis, he received basic training in mosquito population genetics, their relevance to estimating gene flow in African malaria vectors and implications for vector control techniques such as SIT and GM vector control approaches. After finishing his fellowship, he decided to combine his interests in field-based vector ecology and population genetics by returning to the IHI to pursue PhD studies on the ecological and behavioural determinants of gene flow in African malaria vectors. Working as a research scientist at the IHI, he pursued his PhD (Sandwich programme) at the Wageningen University under Drs. Bart Knols, Gerry Killeen and Heather Ferguson supervision. He secured a Caroline McGillavry fellowship funded by The Royal Netherlands Academy of Arts and Sciences (KNAW) to support his PhD studies. His PhD work has combined different research approaches from laboratory to field, and allowed him to acquire multidisciplinary knowledge in areas such as animal behaviour, population dynamics and population genetics. He has been using these skills to investigate the relative contribution of genetics and environmental factors to malaria vector fitness and behaviour. After his PhD thesis defence, Kija Ng'habi will return to IHI to continue his research on vector control and help improve malaria control.

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> List of publications

List of peer-reviewed publications

Cator, L., **Ng'habi, K.R.,** Hoy, R., Harrington, L. (2010). Sizing up a mate: variation in production and response to acoustic signals in *Anopheles gambiae,* Vector ecology

Ng'habi, K. R., Meneses, C. R., Slotman, M., Knols, G. J. B., Ferguson, H. M., Lanzaro, G. C. (2008). Clarification of anomalies in the application of a 2La molecular karyotyping method for *Anopheles gambiae*, *Parasites and Vectors* 1:45

Ferguson, H. M., **K. R. Ng'habi,** T. Walder, D. Kadungula, S. J. Moore, I. Lyimo, T. Russell J. Kihonda , H. Mshinda, G. F. Killeen, B. G. J. Knols (2008). Establishment of a large-scale Semi-field System for the experimental study of African malaria vector ecology and control in Tanzania, *Malaria Journal 7: 158*

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Scholte, E. J. **Ng'habi, K. R.,** Kihonda, J., Takken, W., Paaijmans, K., Abdulla, S., Killeen, G., Knols, B. J. G. (2005) An Entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* 308: 1641-1642.

Papers submitted

Ng'habi K.R., Mwasheshi, D., Knols, G.J.B., Ferguson H.M (2010) Establishment of a self-propagating population of the African malaria vector *An. arabiensis* under semi-field conditions. Malaria Journal

Papers to be submitted

Ng'habi, K. R., Lee, Y., Knols, G .J. B., Ferguson, H. M, Lanzaro, G. C. (2010). Population genetic structure of sympatric malaria vectors *Anopheles gambiae s.l* of the Kilombero valley Tanzania.

Ng'habi, K. R., Lee, Y., Knols, G .J. B., Ferguson, H. M, Lanzaro, G. C. The impact of laboratory cage versus semi-natural colonization on the genetic variation of *Anopheles arabiensis* mosquitoes

Papers presented at conferences/workshops

Ng'habi, K. R., Huho, B. J., Nkwengulila, G., Knols, B. G. J., Killeen, G., and Ferguson, H. M. Effect of larval crowding on Mating competitiveness of *Anopheles gambiae* mosquitoes The Fourth MIM Pan-African Malaria Conference, Nov. 13 - 18, 2005, Yaoundé, Cameroon. (Oral)

Ng'habi, K. R., Huho, B. J., Nkwengulila, G., Knols, B. G. J., Killeen, G., and Ferguson, H. M. Sexual selection in mosquito swarms: may the best man lose? The ASTMH 55th Annual Meeting. November, 2006, Atlanta, Georgia, USA (Oral)

Ng'habi, K. R., Holton, A. Knols, B. G. J., Lanzaro, G. A New Robust Diagnostic Polymerase Chain Reaction (PCR) for determining the mating status of female *Anopheles gambiae* mosquitoes. The ASTMH 56th Annual Meeting, November 2007, Philadelphia, USA. (Oral)

Ng'habi K.R., Mwasheshi, D., Knols, G.J.B., Ferguson H.M A self sustaining population for studying ecology and behaviour of African malaria vectors. The Annual Entomological society of Netherlands, Wageningen, January 2009 (Oral)

Ng'habi, K. R., Lee, Y., Knols, G .J. B., Ferguson, H. M, Lanzaro, G. C. Population genetic structure of sympatric malaria vectors *Anopheles gambiae s.l* of the Kilombero valley Tanzania. The 5th International SOVE congress, Belek-Atanlya-Turkey, October 2009 (Oral)

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PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Determinants of mosquitoes mating competitiveness (2007)

Writing of project proposal (4.5 ECTS)

- Behavioural, ecological, and genetic determinants of gene flow in the African malaria mosquito *Anopheles gambiae* (2007)

Post-graduate courses (1.5 ECTS)

- Multivariate analysis (2008)

Laboratory training and working visits (4.5 ECTS)

- Population genetic (for two years, six months each year); University of California, Davis (2007 & 2009)

Competence strengthening / skills courses (6 ECTS)

- Technique for writing and presenting a scientific paper (2008)
- Project and time management (2008)
- Workshop on scientific publishing (2008)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- Scientific publishing workshop (2008)
- PE&RC Weekend (2008)
- PE&RC Day (2008)

Discussion groups / local seminars / other scientific meetings (9 ECTS)

- Local seminars and discussion groups; Ifakara Health Institute (2007-2009)
- PhD Students discussion group & mosquito control lunch group; Entomology, Wageningen (2008)
- The Netherlands Entomological society (NEV); the Netherlands; including presentation (2008)
- Vector biology group; Entomology Department, University of California (2008 & 2009)

International symposia, workshops and conferences (9 ECTS)

- American Society of Tropical Medicine and Hygiene (2007)
- The Fifth MIM Pan-African Malaria Conference; Nairobi, Kenya (2009)
- The 5th International SOVE congress; Antalya, Turkey (2009)

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