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Studies on the development and survival of anopheles gambiae sensu stricto at various temperatures and relative humidities

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Studies on the development and survival of *Anopheles gambiae sensu stricto* at various temperatures and relative humidities

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Abstract

Anopheles gambiae sensu stricto is the most efficient malaria vector in Africa. Recent advances in mapping the distribution of this vector have exploited the relationship between climatic factors and vector parameters such as growth, survival and reproduction. This work was designed to investigate the effect of temperature and humidity on the development and survival of the vector and to test the use of recently developed tools in describing its distribution. The development rate and survival of the aquatic stages of the vector were investigated at 16 constant temperatures. Adults were produced between 16°-34 °C with a peak development rate at 28°C and peak number of adults at 22-26 °C. Larvae survived for less than 7days at 10°, 12°, 38°, and 40°C but for more than 5 weeks, at 14-18 °C without any development of adults. Laboratory models accurately predicted development times at natural breeding sites in The Gambia suggesting the applicability of the models to field situations. The survival and mortality rates of adult *An. gambiae s.s.* were monitored at combinations of temperatures from 0-45°C at 5° intervals and 40%, 60%, 80% and 100% relative humidity. Survival was highest at 15-25°C and 60-100% relative humidity. The temperature - larva development relation was used to produce a distribution map across Africa while climatic data from sites at which chromosomal forms of the insect have been found were used to map the distribution of the forms across West Africa. Climate is an important determinant of insect distribution and the use of climate and vector parameters in describing or predicting vector and disease distribution will provide a cheaper and less labour intensive tool than traditional methods.

Key words: *Anopheles gambiae*, climate, Africa, survivorship, development rate, chromosomal forms, mapping, malaria, mosquito ecology.

Dedication

To mum

For a job well done.

Declaration and Copyright

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M. N. Bayoh

Acknowledgement

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Abbreviations used

°C	Degrees centigrade
BIDS	Bath Information and Data Services
CFCs	Chloroflurocarbons
cm	centimetre
DNA	Deoxyribonucleic acid
GLM	General Linear Model
Hr(s)	Hour(s)
IPCC	United Nations Intergovernmental Panel on Climate Change
IQR	Inter-quartile range
GIS	Geographical Information Systems
hsps	Heat-shock proteins
KOH	Potassium hydroxide
L	Litre
LSHTM	London School of Hygiene and Tropical Medicine
MARA/ARMA	Mapping Malaria Risk in Africa / Atlas du Risque de la Malaria en Afrique
Min(s)	Minute(s)
ml	Millilitre
MRC	Medical Research Council, The Gambia
P	Precipitation
PCR	Polymerase chain reaction
PE	Potential Evapotranspiration
RH	Relative humidity
SCT	Spatial characterisation tool
SD	Saturation deficit
SVP	Saturation vapour pressure
VP	Vapour pressure
RBM	Roll back malaria
SCT	Spatial characterisation tool
T_{max}	Maximum temperature
T_{min}	Minimum temperature
WHO	World Health Organisation

Chapter 1

Introduction

This document is the outcome of 3 years of research into the effect of changes in temperature and humidity on the rate of development and survival of one of the main malaria vectors in Africa, *Anopheles gambiae sensu stricto*. It is hoped that data presented here will be utilised to improve current models on the effect of climate change on malaria transmission and the distribution of the disease.

The work has been presented in eight chapters. This chapter gives a brief outline of the research objectives, a review of the consequences of climate change on insect vectors and disease transmission, the vector *An. gambiae*, modelling malaria transmission, and a brief justification of the study. The second chapter contains a detailed account of the general methodology employed, including the equipment and materials used. My findings on how temperature affects development rate and survival of immature stages of *An. gambiae s.s.* under laboratory conditions are presented in Chapters 3 and 4 respectively. The laboratory data on development and survival of the immature stages are compared with field studies in Chapter 5, while Chapter 6 is an account of the effect of temperature and relative humidity on adult mosquito survival. The seventh chapter consists of predictive models describing the distribution of chromosomal forms of *An. gambiae s.s.* based on climatic factors and the final chapter is a summary of the conclusions drawn from the entire study and suggestions for further research.

Background information

There is now a consensus that the earth is experiencing a rise in surface temperature leading to a global climate change (Houghton *et al.*, 2001). The predictions are that several environmental (biotic and abiotic) and social factors including human diseases will be influenced by the rate and extent of this change. Insect populations, including those of vectors of many diseases, are very responsive to changes in climatic conditions (Buxton, 1933; Andrewartha & Birch, 1954; Sutherst *et al.*, 1995). The geographical spread of a disease and the seasonal occurrence of some epidemics seem to be directly related to the alteration in the numbers of insects transmitting the disease (Buxton, 1933). Mosquitoes transmit malaria and any change in the production, survival and ecology of these insects where malaria parasites are present will have significant

consequences on disease transmission. Being ectothermic, these insects survive only within certain limits of temperature and humidity. The development of the malaria parasites in the mosquito is also critically affected by ambient temperature. In certain situations, rainfall can also be critical since mosquitoes require water surfaces to breed. Changes in temperature or rainfall will directly affect the distribution and survival of mosquitoes. The main focus of research now is investigating the direction and extent by which these changes will influence mosquito development and survival and ultimately the global transmission and distribution of malaria.

Research objectives

The main aim of the study was to determine the effect of changes in temperature and relative humidity on the development and survival of the major African malaria vector: *An. gambiae s.s.*

The primary objectives include, to:

- Determine the effect of temperature on development rates and survival of the immature stages of *An. gambiae s.s.*
- Investigate development and survival rates of the insects at their natural breeding sites in the field and compare these to laboratory data
- Determine the combined effects of temperature and relative humidity on adult survival
- Test the use of climatic factors in predicting the occurrence and geographical distribution of the different chromosomal forms of *An. gambiae s.s.*

Literature review

Global climate change and human health

It has long been recognised that climate plays a major role in the distribution of insects (Andrewartha & Birch, 1954). Climate can act directly on insect populations themselves by affecting survival, longevity, reproduction and life cycle or indirectly by affecting the other components of the ecosystems they inhabit (Sutherst *et al.*, 1995).

Human activities have in recent times increased the emission of the greenhouse gases: carbon dioxide, methane, the chloroflurocarbons (CFCs) and nitrous oxide, which are implicated in the rise in global temperature, a phenomenon often described as 'global warming'. This human induced climate change is effected by several activities such as

the burning of fossil fuels (coal, oil and gas), removal of vegetation, decomposition of animal and domestic wastes, and of plant materials in rice paddies or wetlands; the use of refrigerators and air conditioners, and nylon production. Carbon dioxide is by far the most important gas since its production is associated with the global industrialisation and modernisation of recent times. The cutting down of forest further exacerbates the concentration of this gas in the atmosphere.

As the earth radiates the energy it has absorbed from the sun back towards outer space, greenhouse gases trap some of the heat. This natural phenomenon known as the greenhouse effect helps to keep the planet warm. But as a result of the over-production of the green house gases, more heat than necessary is trapped causing excessive warming; the enhanced greenhouse effect. Although projections of regional climate change are poor, and the extent of change uncertain, the United Nations Intergovernmental Panel on Climate Change (IPCC) reported that the mean surface temperature of the earth would increase by 1.4 -5.8°C over the next century (Houghton *et al.*, 2001).

The two main components of climate change, temperature and precipitation affect atmospheric moisture and the extent, depth and flow of surface water. Also of great significance are localised but widespread cumulative effects such as water storage, drainage or irrigation, vegetation clearing or re-growth, and changes in human and animal population densities and movements. It has been suggested that climate change will shift the composition and geographic distributions of many ecosystems (forests, deserts, and aquatic systems) and lead to a change in the global hydrological cycle with impacts on regional water resources (Tegart *et al.*, 1990; Houghton *et al.*, 1996). This will in turn affect biodiversity as individual species respond to the changed climatic conditions. Thus by altering local weather patterns, climate change can have significant effects on the growth, development and survival of many organisms in natural ecosystems.

There is rising concern over the potential impact of a global climate change on human health especially in the developed world where it has been identified as a priority for research and action in the next century (Kovats *et al.*, 1999). The consequences of global warming on human health range from direct effects of heat-related illnesses, exposure to air pollutants which is said to be generally higher during heatwaves

(USEPA, 1989) and infectious disease transmission and also the socio-economic problems that ensue as a result of ecological and demographic changes. Malaria, transmitted by mosquitoes, visceral leishmaniasis, transmitted by sandflies, and tick borne encephalitis are among the infectious diseases that may be affected by climate change. Ecological changes may also occur as a consequence of the flooding of rivers or drought with effects on water borne diseases; diseases related to poor hygiene, and human deprivation.

Insects and Temperature

Most insects are poikilothermic and in consequence, their metabolic processes are governed by environmental temperature. In particular, development is markedly affected by temperature. Low temperatures are shown to reduce growth rates and so development takes longer than at higher temperatures (Atkinson, 1994; Partridge *et al.*, 1994; Robinson & Partridge, 2001). The final adult size is usually found to be larger as a result of development at low temperatures. This may result simply from the longer developmental period allowing more food to be ingested, but if the efficiency of the assimilation of food is also temperature dependent, then this too could be a contributing factor. This phenomenon is well described in *Drosophila* where the sizes of the thorax, wings and abdomen are all larger in adults when development occurred at lower temperatures (French *et al.*, 1998).

The small size of insects allows them to exploit a wide range of microenvironments but often they are confronted with adverse climatic conditions. Many insects have adopted mechanisms for survival and reproduction in such conditions. Some species of insects respond to environmental conditions by the inclusion of a resistant phase in their life cycle, this may be aestivation, diapause or a state of quiescence. The midge, *Polypedilum vanderplancii*, inhabits temporary tropical pools in West Africa. During the dry season, the pools dry up and the organism survives the combined effects of desiccation and high temperature in the form of a resistant larval stage that can withstand high temperatures by a reduction in tissue water and a corresponding reduction in metabolism (Hinton, 1960). Adult beetles of the family Coccinellidae are able to survive unfavourable environmental conditions by entering a state of dormancy ranging from simple quiescence to diapause. The induction of diapause was found to be regulated mainly by photoperiod (Iperti, 1999). In the bug, *Deois flavopicta*, low

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temperature was found to accelerate diapause while availability of moisture determined postdiapause hatching of the eggs (Pires *et al.*, 2000).

Some species of insects are capable of regulating their body temperature by behavioural as well as physiological mechanisms as a means of counteracting adverse thermal conditions. By postural adjustments, insects orientate their body to increase the area exposed to sunlight in order to maximise radiative heat gain (e.g. dragonflies; May, 1979; Heinrich & Casey, 1978), while others stilt, drop or straddle to gain or lose heat from a surface (e.g. tiger beetles, Dreisig, 1990; grasshoppers, Anderson *et al.*, 1979). Insects may seek and occupy areas of thermal heterogeneity within their environment in response to high or low ambient temperatures as in grasshoppers (Anderson *et al.*, 1979), migratory locusts (Uvarov, 1977) and desert beetles (Cloudsley-Thompson, 2001). Bees produce heat by shivering before or during flight (Heinrich, 1975), and some are known to maintain thoracic temperatures 15°C above air temperature (May & Casey, 1983). Grasshoppers (Prange, 1996) and bees (Roberts & Harrison) are known to lose heat by evaporative cooling. Generally such behavioural and physiological mechanisms are available only to larger bodies insect species (see Heinrich, 1993). In smaller bodied species such as mosquitoes the avoidance of adverse thermal conditions, or the choice of favourable conditions results from behavioural selections of a thermal range of preference (May, 1979).

Insects have different thermal limits. Sub-zero temperatures are withstood by some species by super-cooling and preventing their bodies from freezing (Clements, 1963). Other species accumulate significant quantities of cryoprotectant polyols (Storey and Storey, 1991), whilst in other species the accumulation of lipids and a reduction in activity (David *et al.*, 1983) has been shown to be important. High temperatures may cause insect death by the destruction of proteins and enzyme activity (Somero, 1995) and the wax content of the cuticle leading to physiological disorders or desiccation (Roti Roti, 1982; Hochachka & Somero, 1984; Hepburn, 1985; Bowler, 1987). Insects that survive at high temperatures probably do so by producing heat shock proteins (hsps) which confer thermotolerance (Alahiotis & Stephanou, 1982; Bergh & Arking, 1984; Stephanou *et al.*, 1983; Berger, 1984; Benedict *et al.*, 1991; Feder *et al.*, 1997). In response to thermal stress, allopatric populations of *Drosophila* have been shown to posses behavioural, morphological and physiological heterogeneities that appear to be

adaptive to the thermal profiles of their respective environments (Sorensen *et al.* 2001; Dahlgaard *et al.*, 2001)

Mosquitoes and temperature

Mosquitoes, being insects, are subject to the same constraints of temperature, and Trpis (1972) has clearly shown that normal functioning can only occur within a limited temperature range. Leeson (1939) reported that the aquatic stages of tropical anophelines failed to develop or breed below 16°C. Insect sex ratio can also be modified by temperature. Anderson and Horsfall (1965) reported that under natural conditions for development of *Aedes stimulans* (5-20°C) 50% of the population is limited to being female since half of the individuals are homozygous for female characters. The other half has the potential to develop into males or females for they are heterozygous for sex. The heterozygous component expresses itself entirely as males when reared below 23°C and wholly females when reared above 28°C. At temperatures intermediate between 23°C and 28°C various intersexes are produced, but the higher the temperature, the more severely are the male characters suppressed. The development and survival of insects is critically dependent on temperature. As temperature increases, growth rates for mosquitoes generally increase and larval duration decreases (Brust, 1967; Hagstrum & Workman, 1971; Lyimo & Takken, 1993; Lassiter *et al.*, 1995; Tun-Lin *et al.*, 2000) leading to increased adult turnover.

The tracheal system of respiration and the generally small size and large surface area of insects make them very susceptible to desiccation. The amount of moisture in the air affects the activity and longevity of most terrestrial insects. Mosquitoes tend to be more active and survive longer at higher humidities compared to lower humidities (Gill, 1921; Mayne, 1930; Leeson, 1939; Siddons, 1944; Dow & Gerrish, 1970), although very high humidities, especially at high temperatures, may not always be favourable (Thomson, 1938; Pal, 1943; Platt *et al.*, 1957). Because humidity can depend on temperature (Unwin & Corbet, 1991), the effects of the two are usually inter-related and quite difficult to separate in the natural environment of the insect.

Some mosquitoes respond to adverse conditions by overwintering either as eggs, larvae or adults. Mogi (1996) investigated the overwintering strategies for several mosquito species in Japan and reported that some *Anopheles* and *Culex spp.* diapause as adults while most *Aedes spp.* do so as eggs or larvae. The tree breeding temperate mosquito

Wyeomyia smithii overwinters as a larval diapause that is initiated and maintained by short days and terminated by long days (Smith and Brust, 1971). The appropriate sites for aestivation of fertilised female mosquitoes are dark and cool such as dwelling huts, animal sheds, caves, disused water wells, thatched roofs and uninhabited houses. The female becomes immobile and does not blood feed, her oviposition activity is completely suspended during this time and resumed as soon as conditions become favourable again. In the semi-arid parts of sub-Saharan Africa, these methods were reported to be responsible for the disappearance of active adult malaria vectors during the dry season and their reappearance as soon as the rains resumed (Omer and Cloudsley-Thompson, 1970). It has been suggested that *An. arabiensis* probably survive the dry season in the sub-Saharan Sahel and northern Savannah regions of West Africa in a physiologically altered state, rather than extinction or severe bottlenecks. The few individual survivors or immigrants from other locations recolonise the site in the subsequent rainy season (Taylor *et al.*, 1993). The speed with which biting females reappear with the onset of the rains and the almost immediate resurgence of malaria suggest that aestivating fertilised females may be involved. However recent investigations in a dry savannah zone of east Africa failed to show any evidence of aestivation among the 3 main malaria vectors *An. funestus, An. gambiae* and *An. arabiensis* (Charlwood, *et al.*, 2000). Other work has pointed to the possibility of egg aestivation in the dry season. Beier and others (Beier *et al.*, 1990) recovered viable *An. gambiae* and *An. arabiensis* eggs from dry soil at known breeding sites in western Kenya and suggested that the resistant eggs may represent a significant short term survival strategy for the species. However, the authors could not determine whether the eggs recovered were present in the soil before or after drying up, and they did not report the moisture content of the soil samples collected. A similar study recently reported by Minakawa and other (Minakawa *et al.*, 2001) implicated the presence of one viable egg out of a total of 124 dry soil samples investigated as a dry season survival strategy for *An. gambiae*. Much more research is required to substantiate the notion that *An. gambiae* survives dry conditions as eggs. In the meantime, the most plausible explanation lies in the close association of this species with man. This association can ensure year round presence of breeding sites around homes, farms or river systems and the probable sequestering of the adults in shaded homes or cultivated vegetation. Behavioural mechanisms such as acclimatisation or relocation to more favourable microclimatic environments have been projected for the response of mosquitoes to unsuitable conditions (Mellanby, 1954; Haufe & Burgess, 1956). Endophilic species

would not have problems with regulating body temperature as they are mostly protected by the stable conditions inside houses. Activity at night would also imply that these insects would be resting in places away from the harsh conditions such as among vegetation or other shade providing materials. It is necessary that in the overall estimation of global climate change and insect vector models, such subtle microclimatic adaptations be given due consideration. (Lindsay & Birley, 1996).

Mapping insect distributions

Mapping the distribution of insects has in recent times taken the spotlight in many disciplines of biology and medicine. Like all maps, these distribution maps provide a clear and instant image of the situation being described. In the past, distribution maps took the form of point or range maps. Point maps consisted of dots indicating sites where the organisms were found and range maps were produced by interpolating between these dots (White, 1989). Point maps give little or no information about adjacent sites and to provide a broader picture, extensive investigations demanding large quantities of limited resources are required. The range maps rely on expert opinion and are based on the use of sparse data usually to interpolate large areas. Due to these deficiencies new approaches have been developed for mapping insect distribution.

Insects usually occur within specific ranges of certain climatic or environmental factors. The beauty of our knowledge of the relationship between climate and insects is that if we can mathematically describe the climate envelope in which an insect survives we can use the derived equations to map its distribution over large areas. There have been two main approaches in describing the relationship between climate and insect distributions. In the empirical or statistical method, the specific climate factors to which the insect is adapted are determined experimentally and projected on maps without specifying any underlying biological mechanisms, while in the process-based or physiological method the biological responses of the insect to climate are also incorporated. Biological based responses to climate allow process models to address growth in current conditions and those that may occur in the future. Both methods have been possible because of the marked improvement in spatial characterisation tools and computation. For instance, the availability of the Geographical Information Systems (GIS) software, coupled with use of satellite imagery of potential breeding sites (Hayes *et al.*, 1985) and adult abundance (Thomson *et al.*, 1996) has made mapping mosquito distributions easier and more accurate. These tools have also made possible the

generation of maps predicting insect distributions with climate change (Beerling *et al.*, 1995). Lindsay and colleagues used these basic principles to map the distribution of *An. gambiae s.s.* and *An. arabiensis* across Africa with very high precision (Lindsay *et al.*, 1998). It should be pointed out that within the climate space the distribution of a species is likely to be further constrained by such factors as vegetation, soil type, and competition as well effects of biotic factors associated with the insect or on a smaller scale density-independent factor such as microclimate. However the maps present the situation at the macro level that forms the basis for further investigations or actions.

Anopheles gambiae complex

An. gambiae is a complex of at least six sibling species that show varying degrees of behavioural and ecological differences (Davidson, 1964; White, 1974; Coluzzi, 1984; White, 1985). Three of the sibling species are adapted to freshwater breeding sites: *An. gambiae* Giles *sensu stricto* predominates in humid areas and is generally highly anthropophilic; *An. arabiensis* Patton extends into drier Savanna areas, is more zoophilic and exophilic; and *An. quadriannulatus* Theobald is zoophilic, comprising allopatric taxa in Ethiopia and southern Africa (Hunt *et al.*, 1998). Two saltwater tolerant species, *An. melas* Theobald of West Africa and *An. merus* Donitz of East Africa, are generally more exophagic and zoophilic and thus less efficient vectors than *An. gambiae s.s.* The sixth species, *An. bwambae* White, occurs around hot springs in Uganda (Gillies & DeMeillon, 1968). *An. melas* and *An. merus* have distinct geographical distributions while sympatry between them and the others or among the others is of common occurrence (White, 1974; Bryan *et al.*, 1982; White, 1985; Lindsay *et al.*, 1998; Coetzee *et al.*, 2000)

Various degrees of genetic incompatibility, mainly expressed as hybrid male sterility, have been shown to exist between all six species of the complex. Studies of the inversions which occur on the polytene chromosomes in the ovarian nurse cells of adult females as well as on the salivary glands of 4th stage larvae has largely confirmed these differences (Coluzzi, 1984). Intraspecific polymorphism in these paracentric inversions has been shown among most of the sibling species with *An. gambiae s.s.* and *An. arabiensis* having the most (Coluzzi *et al.*, 1979; Bryan *et al.*, 1982; Petrarca *et al.*, 1983; Coluzzi *et al.*, 1985; Bryan *et al.*, 1987; Touré *et al.*, 1994; Touré *et al.*, 1998) Within *An. gambiae s.s.* the different forms have been named as Forest, Bamako, Bissau, Mopti and Savannah chromosomal forms and it has been reported that the frequency of their inversions vary with changes in environmental conditions suggesting

some sort of climatic adaptation (Coluzzi *et al.*, 1979; Thomson *et al.*, 1997). Molecular forms of *An. gambiae s.s.* have been proposed (Favia *et al.*, 1997) but these did not conform with the defined chromosomal types (Della Torre *et al.*, 2001; Gentile *et al.*, 2001).

Life cycle of An. gambiae

The adult female *Anopheles* requires at least one blood meal before she can oviposit. After a blood meal, she must find a suitable site for laying her developed eggs. Eggs are deposited singly on the surface of water at various breeding sites including shallow sun-lit pools, borrow pits, drains, car tracks, hoof prints near water holes, ricefields, irrigation canals, pools left behind by receding rivers, and rainwater collecting in natural depressions (Gillies & DeMeillon, 1968). The choice of oviposition site for mosquitoes in general seems to be related to chemical cues given off from the breeding sites (Takken & Knols, 1999; McCall & Eaton, 2001). The eggs hatch into larvae, which remain at the surface of the water where they are adapted to feed. The larvae undergo three successive moults during their development and change into pupae after the fourth instar stage. The pupa does not feed and it breathes from the water surface by means of respiratory trumpets. The adults later emerge from the pupae and disperse.

An. gambiae larva feeding behaviour

Larvae of *An. gambiae* spend most of their time lying horizontally near the surface of the water where they feed and obtain atmospheric oxygen. Hydrophobic organic matter that accumulates at the surface film of water bodies form microlayers (Maki & Hermansson, 1994) which consist of a wide range of particles including living and dead matter as well as dissolved organic particles. The micro-organisms proliferate in the surface microlayers by feeding on this accumulated organic matter. Larvae are known to ingest a wide variety of dissolved and particulate organic matter from within and just below these surface microlayers (Walker & Merritt, 1993) and the micro-organisms found there (Laird, 1988; Merritt *et al.*, 1992a; Maki & Hermansson, 1994). Larval feeding involves a complex pattern of behaviour including the generation of water currents by rapid movements of the lateral palatal brushes which deliver water containing particulate food to the preoral cavity (Merritt *et al.*, 1992b) from where they are ingested.

An. gambiae adult feeding behaviour

Both male and female mosquitoes feed on plant nectar and honey dew which principally consists of fructose, glucose, sucrose and dextrin (Wykes, 1952; Auclair, 1963). Males have mouth parts that enable them to seep fluids while females can probe flowers and pierce skin. Plant juices thus provide an important source of energy during most of the adult life of both sexes (Clements, 1992). Females in addition take a blood meal mainly to produce eggs. They locate their host and feed mainly at night with biting activity peaking towards midnight (Gillies, 1957; Maxwell *et al.*, 1998). Host location and selection is generally mediated by carbon dioxide or other host-specific chemicals (Gillies, 1980; Costantini *et al.*, 1998; Takken & Knols, 1999). During the course of blood feeding, an infected mosquito injects malaria parasites from her salivary glands to the feeding site and thus infects the host.

Climate change and malaria distribution

Just how climate change may affect human health has been the subject of many studies and reviews (WHO, 1990; Bradley, 1993; Patz *et al.*, 1996; Martens, 1998). Some of these studies have focused on vector borne diseases, particularly malaria (Martens *et al.*, 1994; Lindsay & Birley, 1996; Lindsay & Martens 1998). The present distribution of this debilitating disease is mainly restricted to the tropics and sub-tropics. It is projected that global warming may substantially change this scenario by increasing the area at higher latitudes and altitudes suitable for transmission (Sutherst, 1993; Martens, 1998; Lindsay & Martens, 1998). Rogers and Randolph (2000) used a two-step statistical approach based on current global distribution and reported insignificant changes from current distributions even with the most extreme climate change scenarios. This puts into question the substitution of statistical methodologies for biological information especially in a system that is mostly driven by such biological components. This further highlights the need to improve our knowledge on how climatic factors interact with all vector and parasite attributes, and where possible, human factors that are important in disease transmission.

Lines and colleagues, in a study in Tanzania, found no relationship between a very small rise in seasonal temperature (about 0.5°C) and the doubling of infection probabilities of mosquitoes in the coastal lowlands (Lines *et al.*, 1991). Given that temperatures at their study sites are usually well above the threshold for development of both the vector and the parasite, the lack of an effect of temperature is not surprising.

Increases in temperature of this order under global warming are therefore unlikely to greatly affect the epidemiology of malaria in areas where it is currently endemic but may, nonetheless, affect areas adjacent to endemic regions that are currently protected by insufficient heat accumulation.

Following extensive clearance of the rain-forests of the Usambara mountains in Tanzania, and the movement of a large number of infective immigrants from the surrounding lowlands, Matola and others reported the presence of sporozoite positive *An. gambiae* and *An. funestus* mosquitoes. This indicated the spread of malaria into a region previously malaria free (Matola *et al.*, 1987). In addition, there was an increase in average temperature from 12.8-15.6°C to 17.7-20.8°C after the vegetation clearance. However recent work by B▶dker and colleagues (B▶dker *et. al.*, 2000) implicated changes in the use and efficacy of anti-malarial drugs as the more important reasons for the changes in malaria pattern in the Usambara than change in climate. Apart from increasing temperature, forest clearing also facilitates the breeding of some mosquito species by giving way to sunlight penetration of previously shaded pools of water. Loevinsohn (1994) reported an increase in malaria incidence in Rwanda between 1984 to 1988 associated with increased temperature. In Pakistan, Bouma and colleagues related prolonged periods of falciparum malaria transmission to the unusually high late season temperatures in past decades which were associated with the El Nino-Southern Oscillation weather phenomenon (Bouma *et al.*, 1994).

Estimating mosquito survival and mortality

The main causes of mortality in mosquitoes include competition for food and space, adverse climatic conditions, parasitism and predation (Service, 1993). The impact of these may vary with the life stage of the insect. Mortality rates are often described by the use of life tables (Lansdowne & Hacker, 1975; Gomez *et al.*, 1977; Reisen & Mahmood, 1980). Life tables provide a summary of either age-specific or time-specific mortality operating within a population. Service(1993) suggests that because laboratory conditions cannot perfectly mimic field situations, mortality estimates based on laboratory data are of limited value. However, the probability of survival in the laboratory where food and space is abundant and climatic conditions are favourable gives an estimate of the potential of the insect in nature and this could be of use in generalisations. Also, field conditions cannot be easily controlled and controlled

laboratory studies are the best way possible to estimate the cumulative effect of all the various factors in the field on insect mortality.

Field estimates of mortality of the immature stages of mosquitoes often require the estimation of the duration of each instar stage and the construction of an age distribution curve. After larval sampling, the different instars are sorted and counted and each total is divided by the duration of the specific instar. The values are then plotted against age in days. A smooth line through the points represents the age-specific age distribution, which is equivalent to the time-specific survivorship curve. The assumption made is that the population is in a steady state where the number of additions cancel out the number of deaths. For age-specific life tables, the series of samples are taken at different times and the numbers in each age class determined. The differences between successive estimates represent the numbers lost from the population, which is only valid where there is no overlap of generations. In the laboratory these factors can all be controlled since you introduce a cohort of 1st instars and observe the daily number of losses till the very last individual.

Adult survival rate is the most important factor in determining the stability of any mosquito population. It affects total egg output and also the potential of mosquitoes to be vectors of disease organisms. Females that become infected when taking a blood meal must survive through the incubation period of the parasite before they can transmit the disease. The probability of survival of the vector is thus critical in the transmission cycle. It is therefore of great significance that the survival and mortality of adult mosquitoes be thoroughly investigated or described.

Direct measurements of mosquito survival and mortality rates in nature are difficult to obtain. Some investigators have made use of data on the proportions of females that have laid one or more batches of eggs or that have not yet laid eggs. This ratio and knowledge of the duration of the first gonotrophic cycle can be used to estimate daily survival rate (Davidson, 1954). This is applicable on the assumption that mortality rates are independent of age and the population is stable. Others have estimated the daily survival rates from the results of mark-release-recapture experiments (see Service, 1993). If the ages of the captured mosquitoes are correctly established, an estimation of survival rates can be made. A direct method for establishing the physiological age of individuals has been based on the observation that irreversible changes occur in the

internal reproductive organs of female mosquitoes at every oviposition (Detinova, 1962). During each gonotrophic cycle, there is formation of bead-like dilatations in most ovarioles, resulting from either the distension of certain ovarian membranes by the developing oocyte or from the residue of resorbed follicle. The changes that occur during and after follicular development are used to determine the age of the individual (Tyndale-Biscoe & Hughes, 1968; Wall *et al.*, 1991; Hoc & Wilkes, 1995). It has been shown in many mosquito species including *An. gambiae* (Gillies & Wilkes, 1965) that a 1:1 relationship exists between the number of gonotrophic cycles completed and the largest number of dilatations in any ovariole. Using mark-recapture experiments, they were able to correlate the reproductive history of recaptured *An. gambiae* females with their chronological age. However, recent investigations have reported significant weaknesses in this approach and there is need for improved methods. Other methods of age grading exploit somatic changes such as the accumulation of fluorescent pigments in the eyes (Mail *et al.*, 1983; Moon & Krafsur, 1995) and formation of cuticular bands (Tyndale-Biscoe & Kitching, 1974); and cuticular degradation such as mandibular wear (Butterfield, 1996) or wing abrasion (Jackson, 1946). Each of these methods has inherent weaknesses (see Hayes & Wall, 1999) and consequently, many workers have used a combination of methods e.g. ovarian development and wing fray for tsetse flies *Glossina* spp. (Woodhouse *et al.*, 1993) and sheep blowfly *Lucilia sericata* (Hayes *et al.*, 1998).

Modern concepts of the epidemiology of malaria are based largely on the model developed by Macdonald (Macdonald, 1952). Using published data on mosquito survival in the laboratory and in the field, he pointed out that the intensity of the environmental hazards from which female mosquitoes die in nature is the same for all adult age classes. Thus considering that death rate should not change with age; he assumed an exponential model of mortality, basing the mathematical treatment of survival on the factor p, the probability of the mosquito surviving through one day. Estimates of p, have been employed in models of mosquito population dynamics (Miller *et al.*, 1973), vectorial capacity (Garrett-Jones, 1964b) climate change and malaria transmission (Lindsay & Birley, 1996; Martens, 1998) and in the assessment of control measures (Molineaux & Gramiccia, 1980). However, re-analysing Gillies and Wilkes' survival data, Clements and Paterson (Clements & Paterson, 1981), pointed out that there was tendency for female mortality rate to increase with age over the adult life span as a whole, thus finding the simple exponential model less satisfactory. Gillies and

Wilkes observed with three species of *Anopheles*, that female mortality rates increased during the later gonotrophic cycles but stated that in *An. gambiae* and *An. funestus* the mortality rates remained constant up to and including the age at which most malaria transmission occurs. This may be the reason why recent contributors to the model of malaria epidemiology have continued to use the exponential model of survival in calculating longevity factors for the vector species.

Vectorial capacity and modelling malaria transmission

Concern that malaria may emerge or re-emerge in different parts of the world due to climate change has prompted interest in modelling or elucidating how this may possibly happen (Bradley, 1993; Sutherst *et al.*, 1995; Lindsay & Birley, 1996; Craig *et al.*, 1999; Martens *et al.*, 1999; Kovats *et al.*, 2001). However, some of the basic insect parameters which form the basis of process-based models are lacking or poorly derived (Hay *et al.*, 2000).

The prevalence or incidence of malaria in a given locality is determined by the vectorial capacity of the anopheline species transmitting the disease. Vectorial capacity is defined as the daily number of infective inoculations inflicted by a mosquito per person, the infection originating from a single case. It is represented mathematically as

$$C = ma^2 p^n \, / -\ln(p)$$

where *ma*; is the number of bites per person per day (person-biting rate), *a*; the number of meals taken on humans per mosquito (person biting habit), *p*; the daily survival probability of the mosquito and *n*; the length of parasite development in the vector (Garrett-Jones, 1964a).

Vectorial capacity is thus governed by the abundance of the vector (*m*), its tendency to bite humans (*ma*), and the expectation of infective life, which is a function of the daily survival rate of the mosquito (*p*) and the rate at which the parasite develops in the mosquito (*n*). Vector abundance is dependent on the rate at which adult mosquitoes are produced from their breeding sites and is determined by the gonotrophic cycle (and also the rate of blood digestion), the availability of suitable breeding sites and the rate of development of the aquatic stages. In the wild, tropical mosquitoes generally feed at regular intervals of 2-5 days. Gillies reported that female *An. gambiae* and *An. funestus* fed after every 3 days at temperatures below 25 °C while at higher temperatures the interval was shortened to 2 days (Gillies, 1953). A change in temperature will have a

direct impact on the malaria parasites whilst both temperature and precipitation will affect transmission directly through effects on the developmental cycle, adult production and survival rates as well as feeding behaviour of the mosquito..

In general, the relationship between a developmental cycle and temperature is represented by

$$n = f / (T - g)$$

where n can be either the duration of the sporogonic cycle or the gonotrophic cycle, f is the thermal sum in degree days, g a threshold below which development ceases and T is the ambient temperature (see Lindsay and Birley, 1996).

Many mathematical models employed to identify areas at greatest risk from malaria epidemics in order to improve the direction and control of disease outbreaks make use of the vectorial capacity equations and components. These models are however fundamentally flawed since data describing the relationships between climate and the ability of mosquitoes to transmit disease are inadequate, making assumptions, which have not been tested in the field. Also, most models developed for predicting the effect of temperature increase on vectorial capacity are based on data obtained from European vectors e.g. *An. maculipennis* or from cited graphs with no consideration to the scatter reported in the original data (Jepson *et al.*, 1947). Another assumption made is that the daily probability of survival of the insect is constant throughout its life span and that relative abundance is constant at each site, which is unlikely to be true (Martens *et al.*, 1994; Lindsay & Birley, 1996)

Also, relative humidity has not been adequately accounted for in these models. The assumption made is that relative humidity remains at a favourable level and does not change with change in precipitation (Martens, 1998). These shortfalls are mainly because of the limited nature of available data. It is thus important that the bionomics of the vector be investigated at varying temperatures and humidities to provide such vital information.

Rationale

There are an estimated 300-500 million cases of malaria each year (WHO, 1994). The vast majority of the estimated 1.5-2.7 million annual deaths from the disease occur mainly in Africa and among children under 5 years of age (WHO, 1996). The World

Health Organisation (WHO) has launched the Roll Back Malaria (RBM)[*] programme to focus the world's attention and support on renewed efforts to beat this disease of the young and poor. It also aims to develop the health systems of endemic countries as a major strategy for controlling malaria and to build new means of tackling global health concerns. Among the other goals of the project is the mapping of malaria regions and of medical facilities to better direct health resources. They plan to integrate national malaria information from participating countries with regional information to produce a comprehensive national malaria control map, as part of the international mapping of the disease. This should allow a better estimation of the burden of malaria and the population at risk, thus providing more reliable and area-specific information for national and international effort towards malaria control.

Despite the increasing awareness of climate change and its consequences on human health, the need for a comprehensive quantitative evaluation of the impact on vector borne diseases is still present. With about 50 species of mosquitoes acting as vectors of malaria it is not feasible to infer climatic requirements for persistence of the disease on a global scale with any degree of confidence without a detailed analysis of the requirements of each vector species. In addition, more information is needed about the feeding behaviour of the mosquitoes and the fact that mosquitoes may find suitable microclimates when ambient temperatures rise.

This study will consider the most important vector species in the transmission of malaria in the worst affected region, *An. gambiae s.s.* Useful vector parameters, which are currently inadequate, will be produced to improve our knowledge of how the vector may respond to climate change. Also, tools for current and predictive mapping of the vector and malaria will be exploited for its usefulness to the RBM and other disease control planners.

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[*] Web address for RBM : http://www.who.int/rbm

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Chapter 2

Methods for investigating the effect of temperature and humidity on the development and survival of *Anopheles gambiae sensu stricto*.

Summary

A large colony of *An. gambiae s.s.* was successfully maintained in the laboratory to provide a regular source of insect stages for the study of the effect of temperature and humidity on the development, survival and reproduction of the vector. Colony insects were reared in a regular and reproducible fashion to ensure that individuals emerging were of similar size and physiological condition. The studies also required the provision of controlled environmental conditions. This was made possible by the use of programmable growth chambers that provided controlled temperatures and potassium hydroxide solution in small environmental chambers that controlled the amount of moisture. These methods form the basis of the laboratory experiments reported in this thesis.

Introduction

Anopheles gambiae is the most efficient malaria vector in the world (Service, 2000). This can be partly attributed to the vector being extremely anthropophilic, exophagic as well as endophagic and its ability to adapt to changing environmental condition especially man-induced changes. Adaptation of the vector to anthropogenic changes in the environment has been manifested in the way the vector colonises different types of larval habitats such as small puddles, borrow pits, hoof prints and rice fields and also in its behavioural responses that ensure survival in and around homes (Gillies & de Meillon, 1968). Mosquitoes are cultured and maintained in laboratories for a range of purposes including vector species identification (Davidson, 1964), behavioural studies (Pates *et al.*, 2001), insecticide susceptibility and resistance (Kolaczinski & Curtis, 2001) and genetic studies (Norris *et al.*, 2000). For whatever reason mosquitoes are kept, the breeding procedure must be such that each batch of insects produced for experimental purposes are of similar size or physiological state. It is thus important that rearing procedures are kept constant, especially the type and amount of feed and the rearing temperature and humidity.

Where insects are to be observed under controlled environmental conditions, the system must be designed to make sure that it is reproducible. The conditions under investigation should be regularly monitored to verify that it has not changed from the prescribed conditions. There is thus a need to use improved equipment that should be regularly tested as well as using monitoring devices.

The control of humidity in small closed environments is of fundamental importance to ecologists and physiologists studying small terrestrial animals, especially insects. Owing to the tracheal system of respiration, insects in general are particularly susceptible to desiccation. The amount of moisture in the air is an important factor that affects the survival and reproduction of insects (Collier & Smith, 2000; Gillespie *et al.*, 2000; Russell *et al.*, 2001) and must therefore be kept under careful control in insectaries. Various mosquito species have adapted to a range of climatic conditions and some are very sensitive to changes in certain microclimatic factors of their habitat. Even closely related species such as among the *An. gambiae* complex may show different adaptations to dry environments (Lindsay *et al.*, 1998).

The relative humidity at a particular temperature is the ratio of actual water vapour present to that necessary for saturation of the air at that temperature. Since more water is required to saturate warmer air (Capinera *et al.*, 1981), equivalent relative humidities at different temperatures will have different vapour pressures. Williams and Brochu (1969), suggested the use of vapour pressure deficit, also known as saturation deficit, which is the difference between the saturation vapour pressure and the actual vapour pressure at the existing temperature. Relative humidity reflects the amount of moisture in the air while saturation deficit reflects the rate of evaporation. It is easier to relate insect responses to dryness than to wetness.

In this chapter, I will describe how *An. gambiae s.s.*, the subject of this study was maintained in the laboratory for use in the experiments described in this thesis, how the environment was controlled, maintained and monitored, and explain the design of individual experiments on the effect of these factors on vector development and survival.

Methods

Maintenance of a colony of *Anopheles gambiae s.s.* in the laboratory.

To provide a constant supply of various stages of *An. gambiae s.s.*, a colony was started at the insectaries of the School of Biological and Biomedical Sciences, University of Durham in November 1998. This colony has been maintained at $26^{o}C \pm 1^{o}C$ and 80% humidity $\pm$ 10% and 12:12 light: dark regime on defibrinated horse blood, glucose solution and fish food. All laboratory experiments reported in this document used insects obtained from this colony. Here a detailed description of how the colony was maintained and its performance are given.

Colony history

The strain of *An. gambiae s.s.* maintained at Durham was obtained from the London School of Hygiene and Tropical Medicine (LSHTM) in November 1998. This strain, the 16CSS, was originally from Lagos and has been colonised at LSHTM since 1951. Eggs were obtained from the insectaries at the LSHTM on wet No.1 Whatman filter papers, which were wrapped in cotton wool. These were then taken to Durham in sealed plastic sachets and were the source of the first set of adult *An. gambiae s.s.* of the mosquito colony at the insectaries of the School of Biological and Biomedical Sciences. The specific maintenance and handling procedures for each of the life stages of the insect are presented below.

Maintenance of the various life stages of An. gambiae s.s.

Adult maintenance and blood feeding

Adult *An. gambiae s.s.* were held in cages made of cube shaped wire frames (45cm x 45cm x 45cm) with sides covered in mosquito net of mesh size $1mm^{2}$. A sleeve made of the same net and 20cm long, attached to a 15cm diameter opening at the front of the cage was used for accessing the cage (Plate 1). Cages were kept well stocked with 100-120 adult mosquitoes of both sexes by periodic addition of newly emerged adults. A piece of sponge 5cm x 6cm x2cm in a petri dish of diameter 9cm and height 1.5cm soaked in water was placed on the top of each cage and the entire set up covered with a plastic bag. The plastic bag reduced the amount of evaporation from the petri dish and by this way kept the cage environment humid. Water was added to the humidity dish every day.

Plate 1. Adult mosquito holding cage

After emergence, adults were fed continuously on 10% glucose solution made from D-Glucose monohydrate (BDH laboratory Supplies, England) from a sugar fountain placed in each cage. The fountain consisted of a tube (2.5cm in diameter and 7.5cm deep) with its sides (inside and outside walls) lined with absorbent surgical lint 2cm wide and 32cm long. Another tube 3.5cm x 5cm was used to hold the fountain (Plate 2). The fountain filled with glucose solution was inserted in an inverted position into the holding tube. More sugar was added to the lint so that the holder was full. The lint absorbs the sugar and the insects feed directly from it. The glucose solution was changed daily after draining the lint with distilled water to remove any sticky sugar. The lint was replaced weekly to prevent the growth of fungus and fermentation of the sugar.

Plate 2. Sugar fountain: continuous source of food for adult mosquitoes

Female mosquitoes were offered a blood meal twice a week in order that they would produce viable eggs. They were fed on defibrinated horse blood (Oxoid, England) using the Hemotek Membrane Feeding System (Discovery Workshops, Accrington, UK). The system consists of a power unit, heating device, feeder, feeding membranes and membrane fasteners. The feeder consisted of a blood heating device and a small blood holding pot 3.5cm diameter and 0.5cm depth. About 4ml of blood was placed in the holding pot on each feeding. The feeding membrane, made of processed collagen is placed over the top of the blood holding pot and an elastic band used to keep the membrane firmly secured around the sides of the pot. The base of the pot was screwed into the heating device, which obtains heat from the power unit. During feeding, the feeder was placed on top of the cage with the membrane in contact with the cage netting. (Plate 3) Towards the later stages of the study parafilm (Parafilm American National Can, Chicago, USA) was used instead of collagen membrane. A piece of parafilm was stretched out thinly and placed over the top of the blood and fastened to the holder by the elastic band. Both membranes were equally effective. The mosquitoes were attracted to the membranes, possibly by the heat generated from the power unit and allowed to feed for about 15 minutes. Sometimes newly emerged females were attracted to the feeder by lightly blowing exhaled air near the feeder. Once they got close to the feeder, they easily located the blood meal.

Plate 3. Artificial blood feeding of mosquitoes using Hemotek membrane feeders

Egg collection and hatching

Eggcups made of white polystyrene, 9cm in diameter and 6cm deep (Insulpak, UK) were used to collect eggs. After the females have received a blood meal, an eggcup containing about 150ml of aged tap water (i.e. tap water kept in bowls for at least 48hrs) was placed in each cage for egg deposition. The eggcup was lined with Whatman No.1 filter paper by simply placing the filter paper over the top of one cup and using the bottom of another cup, gently pressing the paper down making sure that it slotted smoothly around into the base cup. The filter paper prevents the eggs from sticking to the surface of the cup and provides a resting site for ovipositing females.

On oviposition, usually 2-3 days after a blood meal, the eggcups were removed from the cage and placed on a shelf. The bowls were covered with cardboard to preclude oviposition by stray females and to ensure high hatching success.

Larvae rearing procedures

The newly hatched larvae were gently poured from the eggcups into polythene bowls (13cm deep and 30cm wide) containing 2L of the larval rearing medium. The medium consisted of a bacterial soup and few drops of Liquifry No2 for Live Bearers (Interpet Ltd, Surrey, England). The bacterial soup was prepared by placing 5 whole grass plants in 4 litres of aged tap water for 24 hours at 26°C. The delay allowed the water to equilibrate with the insectary temperature and the bacteria to grow from the decaying grass. The bacteria and Liquifry served as immediate food for the newly hatched first instar larvae. The larval bowls were covered by a piece of netting with 12 curtain hangers or beads evenly attached along the edges (Plate 4). The weights helped to maintain the cover in place and avoid slipping, a precaution for preventing hatched adults from flying out into the room or stray gravid females from ovipositing.

Larvae were fed daily on Tropical Fish Food (Tetramin®, Germany). The fish food was ground into a fine powder and dispensed through a mesh fitted on the top of a universal tube. The tube was lightly tapped once at three different sites over the rearing medium. This allowed a shower of approximately 15mg of food particles to fall evenly over the surface of the water. The first feeding was carried out on the third day of hatching and then continued once daily until all larvae hatched into pupae. If the feed does not spread out quickly on the first shake, then it is likely that scum will build up on the surface,

which must be removed to prevent suffocation of larvae. Scum was cleared by dragging a piece of tissue paper over the water surface from one end of the bowl to the other.

Plate 4. Larva rearing bowl and cover

Pupal collection and treatment

Upon pupation, the pupae were transferred to plastic cups (5cm diameter and 6cm deep) containing 80ml aged tap water and placed in the adult holding cages under an emergence funnel. This was a cone shaped flexi-metal funnel with bottom and top diameters 15cm and 2.5cm respectively and height 21cm. It was placed over the pupal container in the cage and it allowed emerged adults to fly out from the top, but prevented them from flying back in. It also prevented gravid females from ovipositing in the pupal cups. Adults that emerged within the larval bowls were transferred to adult holding cages using aspirators.

Materials used to study mosquito development rates and survival at constant and fluctuating temperatures

Growth chambers and temperature loggers

Programmable growth chambers, LMS cooled incubators (S. H. Scientific, Kent, UK) with dual temperature and illumination were used in studies on immature development and adult survival at temperatures ranging from 0 to 45°C. The internal dimensions of the chambers were 45cm x 30cm x 100cm and were large enough to accommodate six experimental bowls or three environmental chambers. Temperature of the rearing medium and ambient temperature in case of adult survival were monitored using Tiny Talk II data loggers (Gemini, UK) which were accurate to ±0.5 °C.

Experimental bowls, cages and environmental chambers

The bowls used in studying the immature stages of *An. gambiae s.s.* were clear plastic
bowls 15cm x 10cm x 8cm. These were labelled on their sides using self-adhesive
masking tapes (Guilbert Nice Day, UK) and placed on the shelves of the growth
chambers (Plate 5).

Plate 5. Set up for experiment on the immature stages of *An. gambiae s.s.* in a growth
chamber.

The test cages were of a similar design to the colony cages but smaller ($15cm^3$). Frames
were covered with netting of mesh size $1mm^2$ and a sleeve attached to one side. Cages
were placed inside environmental chambers, which were then placed on shelves in the
growth chambers (Plate 6). The environmental chambers were rectangular glass
chambers (39cm x 20cm x 22cm) with a close fit lid and a handle for lifting the lid.
Adhesive tape (Elephant tape, Guilbert Nice Day, UK) was used to seal the edges of
the lid, making the chamber airtight. Each chamber held a maximum of two
experimental cages. Lids were opened briefly daily for observation of the cage contents
and also to allow air to circulate.

Plate 6. Set up for adult *An. gambiae s.s.* studies in environmental chambers inside growth chambers.

Controlling relative humidity in closed environments

This experiment was designed to validate the use of various grades of potassium hydroxide solution to control relative humidity in small enclosures. This was necessary for the study of the survival of adult *An. gambiae s.s.* at various relative humidities and different temperatures in small environmental chambers.

Preparation and use of Potassium hydroxide solutions

In this study, 40, 60, 80 and 100% humidities were required at constant temperatures ranging from 0°- 45°C at 5° intervals. Various concentrations of potassium hydroxide were made by dissolving different quantities of potassium hydroxide pellets (BDH laboratory Supplies, England) in distilled water in a fume cupboard. The solution was mixed with a metal stirrer in 1L heat resistant bottles (Pyrex®) and left to cool at room temperature. Different concentrations ranging from 1:10 to 12:10 were made up and 300ml poured into a plastic bowl and lowered into an environmental chamber. An experimental cage containing a sugar fountain was placed on top of the bowl. The edges of the environmental chambers were sealed with tape and then placed in different

growth chambers producing different temperatures. Skye DataHog2 (Skye Instruments Ltd., Powys, UK) and Tinytalk humidity loggers were both used to measure the relative humidities produced by the different dilutions of potassium hydroxide solution at different temperatures. Readings from the Skye Instrument loggers were downloaded each day using the DataHog2 Communications Software (Skye Instruments Ltd) 9600-Baud Version. The mode of the first 24hr readings was calculated for each set up and the loggers stopped when they produced readings that were different from the mode by 10 units. This coincided with the length of time in which the actual humidity produced by the solution was ±10% of the required experimental humidity at the specific temperature.

Results

Observations of life processes and stages of *An. gambiae s.s.* under insectary conditions

On emergence, adult females took their first blood meal in 2-5 days. The ratio of male:female was approximately 1:1. A full adult holding cage consisted of 200 adult mosquitoes and between 20-40 females per cage fed at each feeding time. The longer the feeding interval, the greater the numbers that fed. Five percent of adults including blood fed females drowned in either the egg bowls or on the sugar fountain every week. Between 500-3000 eggs were laid per egg bowl and 60% of these hatched. The duration of blood meal digestion and oviposition of eggs, hatching of eggs, larval and pupal life was observed for five feeding regimes. The results are summarised in Table 2.1. A minimum of 11 days was required from blood feeding to adult emergence.

Table 2.1 Duration (days) of various processes in the growth of *An. gambiae s.s.* mosquitoes under insectary conditions at 26°C

	Minimum	Maximum	Mean	Std. Deviation
Oviposition	2.0	3.0	2.2	0.00
Hatching	1.0	3.0	2.0	0.70
Larval life	7.0	8.0	7.4	0.55
Pupal life	1.0	1.0	1.0	0.00

When the colony was thriving, on average, 80% of hatched larvae developed into adults, 2 % perished during adult emergence, adult females survived for 8 weeks while the males lived for 5 weeks

The rates of development of the instar stages were recorded for five feeding cycles and are presented in Fig. 2.1. Larval stages were identified using the techniques suggested by the World Health Organisation (WHO, 1975). More time was spent in the first two instar stages than the last two.
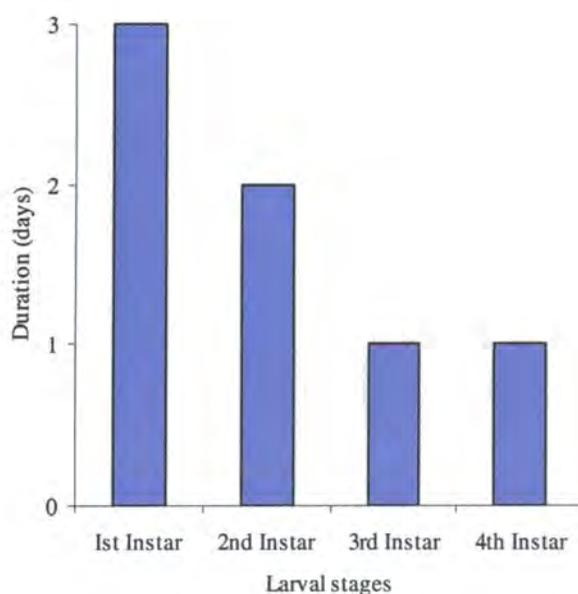
Fig. 2.1 Rate of development of larval stages of *An. gambiae s.s.* under insectary conditions.

When the colony was not doing well, most loses were at the larval stages and were mainly due to death of first instars as a result of contamination of the rearing medium. In such cases, blood-feeding frequencies were increased, experimentation was reduced and the stock population brought back to normal.

Performance of the growth chambers
The temperatures selected for the adult studies were accurate to $\pm 0.5^{\circ}$C. Read out from loggers indicated the maintenance of the required temperature with changes of 2-3°C occurring for less than 30mins after opening and closing of the doors of the growth chambers. In the larval studies, the temperature of the chamber was 2-3°C higher than the water temperature and thus the chamber temperature was always kept higher than

the required water temperature. Monitoring ensured that the water temperatures were kept within 1°C of the required temperature.

Humidity produced by different concentrations of potassium hydroxide at different temperatures

The solution, which provided the modal reading that coincided with the required humidity for each temperature, was used in the experiments. The amount of potassium hydroxide yielding the required humidity at each temperature is presented in Table 2.2. These values were mostly within 10% of the humidity from between 3 to 4 weeks. The solution was replaced whenever logger readings returned values above 10% of the required humidity. For 100% relative humidity, distilled water was used in place of potassium hydroxide solution.

Table 2.2 Concentrations of potassium hydroxide that provided various percentage humidities at different constant temperatures.

Temperature (°C)	Weight of KOH (grams)/100ml dH$_2$O		
	40% Humidity	60% Humidity	80% Humidity
0	100	60	40
5	100	60	40
10	80	50	30
15	80	50	25
20	80	45	20
25	80	40	20
30	80	40	20
35	70	40	10
40	70	40	10
45	70	40	10

Discussion

Status of the colony

The colony, which was established in 1998 from a few eggs, is now large, with between 10-12 adult cages, 10-15 larval bowls and 10-15 eggcups at any given time. It has been able to supply about 4,000 viable eggs within a 24hr period and 1,000 adult mosquitoes within 2 days. The temperature at which the colony was maintained has been described as optimum for adult and larval rearing (Armstrong & Bransby-Williams, 1961). The adults fed readily on defibrinated horse blood and produced adequate and viable eggs. Egg hatch rate was high and larval to adult development was within expected range. However, the colony suffered two near crashes and these both occurred during summer time. It is speculated that larval contamination through contaminated grass may have been responsible although changes in temperature of culture may also be involved.

Control of humidity

The result obtained from the potassium hydroxide investigation validated the use of the specified quantities of the substance to control humidity within the closed environmental chambers. Potassium hydroxide solution has long been used to control relative humidity in entomological experiments (Buxton, 1931). Potassium hydroxide is very hygroscopic and is suitable to provide humidities as low as 13% at room temperature (Solomon, 1951). Other compounds used to produce even lower humidities include sodium hydroxide and sulphuric acid. The widespread use of potassium hydroxide in entomological studies in growth chambers can be attributed to the fact that it absorbs carbon dioxide and thus reduces the frequency with which such chambers have to be opened for aeration. However chambers need to be opened regularly to avoid lack of oxygen. One important source of error when working with graded humidity solutions occurs through the absorption or release of moisture by materials enclosed within the chamber such as the sugar fountains or oviposition sites for the insects. This was been taken care of since the validation included the presence of all the materials required for the actual experiments excluding only the insects. Another source of error is the variation in ambient temperature. Air holds more water vapour at high than at low temperatures and solutions tend to give too low humidity when the ambient temperature is rising and vice versa. Thus accurate humidity control with graded solutions is only possible at constant temperatures. If temperature fluctuates, the temperature of the

solution will change more slowly than the air above it and this can cause deviations from the expected humidity. Only constant temperatures were investigated in this study.

Conclusions

The methods described in this chapter provided a system of reproducible studies, carefully tested with vigorous monitoring devices. Large numbers of insects of similar sizes and presumably similar physiological conditions were produced. A very high standard of cleanliness, careful regulation of larval feeding (type, frequency and quantity), regulation of numbers of larvae per tray, careful adult feeding and egg collection protocol was maintained in the insectaries. Where some individuals spent more time than normal during larval development, they were excluded from the population thus reducing the possibility of significant individual differences in vigour and other characteristics that may affect the outcome of experiments. The essential conditions of the mosquito's natural environment were recreated in the insectary including the maintenance and careful regulation of temperature and humidity conditions using air conditioners and the provision of a light and dark regime with timer control devices.

The environmental chambers and humidity reagents used provided experimental conditions that ensured comparisons between and within replicates while holding several factors constant. The monitor devices were properly looked after and adequately serviced and the monitoring procedures were standard for all experiments.

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Chapter 3

Effect of temperature on the development of the aquatic stages of *Anopheles gambiae sensu stricto*

Summary

Global warming may affect the pattern of vector-borne diseases yet the relationship between temperature and development has been inadequately described for many key vector species. This work describes the development of Africa's principal vector *Anopheles gambiae sensu stricto* at different temperatures. Development time from egg to adult was measured under controlled laboratory conditions at constant temperatures between 10-40°C. Rate of development from each immature stage to the next increased with increase in temperature up to an optimum of 28°C and then declined. The rate of larval development was greatest between 28-32°C, although the proportion of larvae that developed into adults was highest between 22-26°C. The lower limit for complete development to adult occurred between 16°-18°C, and the upper threshold was 32-34°C. Non-linear models were used to describe the relationship between developmental rate and temperature. These relationships provide parameters that are important for constructing process-based models of malaria transmission. The utility of these findings has been clearly demonstrated by showing that a map derived from the parameters suitable for the development of aquatic stages of *An. gambiae s.s.* is very similar to the best map of malaria risk currently available for Africa.

Introduction

Estimating the potential impact of climate change on malaria transmission has generated a great deal of interest due, in part, to the fear that this debilitating disease may emerge or re-emerge in many parts of the world (Bradley, 1993; Sutherst *et al.*, 1995; Lindsay & Birley, 1996; Craig *et al.*, 1999; Martens *et al.*, 1999; Kovats *et al.*, 2001). With the availability of detailed environmental data sets and increased computing power, modelling climate change and malaria transmission has never been easier. However, some of the basic insect data on which process-based models are constructed, such as vector survival and human-biting rate, are lacking or poorly derived (Hay *et al.*, 2000), raising concerns about the accuracy of future scenarios.

The rate at which new individuals are produced is one of the key factors that determine the growth rate of insect populations. This rate is critically dependent on the growth characteristics of the immature stages, which is governed by temperature where food is not limiting (Lassiter *et al.*, 1995). The influence of temperature on these stages has been studied in a number of different species of mosquitoes, including *Aedes quadrimaculatus* (Huffaker, 1944), *Ae. aegypti* (Bar-Zeev, 1958; Tun-Lin *et al.*, 2000), *Culex* and *Anopheles sp.* (Shelton, 1973), *Taeniorhynchites brevipalpis* (Trpis, 1972) and *Wyeomyia smithii* (Bradshaw, 1980), but not in detail in anophelines (Bradshaw, 1980). In general, within the limits of a lower development threshold and an upper lethal temperature, the aquatic stages of mosquitoes develop faster as temperature increases (Brust, 1967; Hagstrum & Workman, 1971; Lyimo, 1993). Usually, temperatures that produced the highest adult survival are lower than those that result in the fastest rate of development, where there is reduced survival and smaller adults. In nature, a prolonged aquatic life may result in increased chances of stranding due to drying up of breeding sites or a reduction in numbers from predation or disease pathogens (Speight *et al.*, 1999), although evidence for this is rare. The implication of a shortening of aquatic life is an increase in adult turnover with consequences for increased vector biting rates and disease transmission (Garrett-Jones, 1964).

Various models of development rate versus temperature relationships have been widely used to predict insect development times. Under temperature fluctuating regimes hourly rates can be accumulated and converted to daily rates and to development times. One approach is the use of the linear portion of the rate versus temperature curve along which the products of development time and the number of degrees above the threshold temperature are constant. Development time is calculated by adding up the number of thermal units (degree-hours or degree-days) contributed at each temperature. The degree-day approach is based on the work of several authors such as Baskerville & Emin (1969), Abrami (1972) and Sevacherian *et al.* (1977). It is very convenient as it requires minimal data for formulation and is easy to calculate and apply and is often accurate (Eckenrode & Chapman, 1972; AliNiazee, 1976). However, it is valid only over intermediate temperatures and the threshold temperatures below or above which development ceases are often determined by extrapolation. As a result, the number of degree-days required for complete development is often too low at temperatures near the lower threshold and too high at temperatures near or above the optimum (Howe, 1967). Several other models involving the use of exponential equations, second and

third degree polynomials, logistic equations, modified sigmoid equations have been developed and used with varying degrees of success. See Wagner *et al.* (1984) for references.

Despite the enormous medical importance of *Anopheles gambiae* mosquitoes, the relationship between temperature and their development is poorly understood. One of the few published references is the work of Jepson and others on *An. gambiae* mosquitoes from Mauritius (Jepson *et al.*, 1947). This work describes development at mean temperatures between 23-32 °C at 11, markedly different, natural-breeding sites. There were also large daily differences in temperature between sites, varying from 4-12°C. This is important since developmental rates of organisms depend both on the mean temperature and the frequency and magnitude of any fluctuations around the mean (Cossins & Bowler, 1987; Liu *et al.*, 1995). Lyimo and colleagues (Lyimo *et al.*, 1992) investigated the effect of temperature on *An. gambiae* development rate, also at a narrow temperature range (24-30°C). The aim of my study was to produce a mathematical expression for the relationship between *An. gambiae s.s* larval development rate and temperature over a wider temperature range (10-40 °C), investigate adult production at these temperatures and utilise the data in order to make better generalisations about malaria transmission over the African continent and future transmission scenarios.

Methods

The 16CSS strain of *An. gambiae s.s.* was used throughout the study. This strain, originally from Lagos, was first colonised at London School of Hygiene and Tropical Medicine and then at the University of Durham (see Chapter 2) where it has been maintained at 26°C (±1 °C), 80% relative humidity (±10%) and a 12:12hr light and dark regime. For investigating the effect of temperature on development of the insect, the immature stages were reared at constant temperatures ranging from 10-40°C (±1°C) under a 12:12hr light and dark regime in programmable growth chambers (LMS cooled incubators, S. H. Scientific). Water temperature was monitored using data loggers (Tiny Talk II, Gemini, with accuracy ±0.5 °C).

Egg development

A total of 200 female mosquitoes obtained from the stock colony were fed on defibrinated horse blood (OXOID, England) and the eggs produced used to investigate the effect of temperature on egg development. Sixty eggs, less than one day old, were added to plastic bowls measuring 15 x 10 x 8cm, lined with No.1 Whatman filter paper and containing 1L of 48hr old tap water. The filter paper prevents the eggs from adhering to the sides of the plastic bowl and avoids the risk of their drying out. Each bowl was housed in the environmental chambers at the respective temperatures. Hatched larvae were counted and removed daily until no further instars were seen. This procedure was repeated four times for each temperature (five in total).

Larva and pupa development

Thirty 2-day old larvae hatched from egg trays were dispensed into larval bowls containing 1L of aged tap water and 100ml of rearing medium. Larval bowls were the same as the egg bowls, but not lined with filter paper. The rearing medium was produced by placing five whole grass plants with soil attached to the roots in 4L of tap water for 48hrs. Bacteria produced from the grass served as starting food for the first instar larvae. Feeding with solid food commenced 24 hours after the hatched larvae were placed in the rearing medium. About 10mg of Tropical Fish Food (Tetramin[R], Germany), ground into a fine powder, was provided for each larval bowl on a daily basis. Any dead larvae or pupae were removed before the addition of feed each day and the rearing medium replaced every two days to prevent the formation of scum and accumulation of metabolites that might be toxic to the insects. Larvae were counted daily and categorised according to instar stages (WHO, 1975). Overall, 4,800 eggs and 2,400 1st instar larvae were used in this study.

Statistical analysis

Development time was recorded as the duration of development of 50% of individuals for the immature stages, and as mean emergence times for adults, in days. The rate of development was described as the sum of the inverse of the development times for each individual expressed as an average. The Kruskal Wallis non-parametric test in SPSS (Version 10.0 for Windows, SPSS Inc., Chicago) statistical package was used to compare between group (temperature regimes) and within group differences in mean development times and number of adults produced. The software TableCurve® 2D

version 4.0 for Windows was used to fit curves to the data for overall development rate and temperature and for rates of the instar stages and temperature. The automated Fill Sparse Grid surface-fit equations and the Renka 1 algorithm in TableCurve®3D (Version 3 for Windows, SPSS Inc., Chicago) was used to produce surfaces describing adult emergence times and number of adults emerging at different temperatures. The fill sparse grid procedure takes into account any incomplete elements of the independent variables. The output dimensions of the x and y grid were determined by the number of unique x and y data values and digitally enhanced non-parametric interpolations performed for all positions in the grid where data are missing. The Renka 1 algorithm is a standard triangulation process that yields interpolations and extrapolations of high order of smoothness while at the same time preserving the overall trends within the data through its global gradient computations (Renka, 1996).

Mapping

An environmental database for Africa, the spatial characterisation tool (SCT) (Corbett and O'Brien, 1997), operating within a geographical information system, (GIS arc/info Version 7.2; ESRI, Redlands, CA.) was employed in mapping malaria distribution in Africa using data produced from this study.

Results

There were no significant differences in adult emergence times and number of adults produced at the different replicates within each temperature regime and all five replicates were combined for further analysis. Mean emergence times as well as proportions developing into adults were different (p<0.001) between temperature regimes. The mean duration from egg to adult (in days) and the percentage of adults produced at the various constant temperatures are shown in Table 1. Less than 50% of pupae developed into adults at 30° and 32°C. Most deaths (>80%) at these temperatures occurred at the 4th instar and the pupa stages. The proportion of larvae becoming adults was similar between 20-28 °C (X^2 = 2.810, P = 0.590).

Table 3.1 Statistical analysis of development time of *An. gambiae s.s.* and proportion of adults produced at various temperature regimes.

Temperature (°C)	Time to adult (days)				No. adults produced		
	Mean (±95%CI)	X^2 *	P		%	X^2 *	P
16	none	-	-		none	-	-
18	23.3 (22.2-23.5)	1.427	0.839		42.0	1.841	0.765
20	20.4 (19.5-20.2)	7.139	0.129		70.0	8.476	0.076
22	17.5 (16.7-17.6)	4.571	0.334		76.0	1.175	0.882
24	13.5 (13.6-14.3)	3.592	0.464		78.7	2.424	0.658
26	11.5 (11.2-11.9)	5.817	0.213		72.7	0.128	0.998
28	9.8 (9.4-10.0)	4.858	0.302		66.0	7.010	0.135
30	10.0 (9.8-10.5)	5.918	0.205		27.3	1.073	0.899
32	10.2 (10.0-10.4)	2.274	0.685		34.7	4.538	0.338
34	none	-	-		none	-	-
Overall	-	755.328	<0.001			94.418	<0.001

*based on Kruskal Wallis Test, $n = 5$ for each temperature and $n = 8$ for all temperatures.
CI = Confidence interval, X^2 = Chi squared.

Overall, the rate of development increased with increasing temperature for each immature stage. However, as the stages advanced, the lower temperature threshold for development increased while the upper temperature threshold decreased (Fig. 3.1). The advanced stages were more sensitive to temperature than the egg or early instars. As the stages advance there is increased demand for nutrient intake and metabolic activities, which may lead to a pronounced effect of any environmental factors such as temperature that affect these processes. Adults emerged only at temperatures between 16 and 34°. There was no development of 1st instar larvae at 10, 12 and 40 °C. Larval development at 14° and 38° ceased at the 2nd instar stage with immediate death at 38° but prolonged life at 14°C. Similarly, development at 16° and 34° ceased at the 4th stage with prolonged duration of life at 16°C.
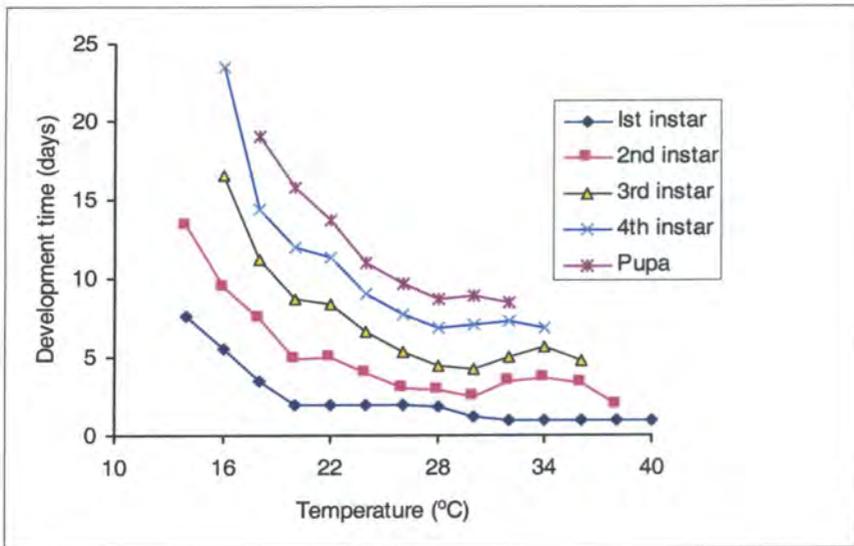
Fig. 3.1 Development times of instar stages of *An. gambiae s.s.* at different temperatures.

Smooth curves were fitted along data points for development times of each stage across the temperatures (Fig. 3.2) and all fitted the expression

$$D = a + b/(1 + (t/c)^d)$$

where D = development time, t = temperature, and a, b, c and d are coefficients for the fit which differ for each immature stage and are presented in Table 3.2. Thus the solution of the above expression will yield different values for each of the stages at the same temperature.
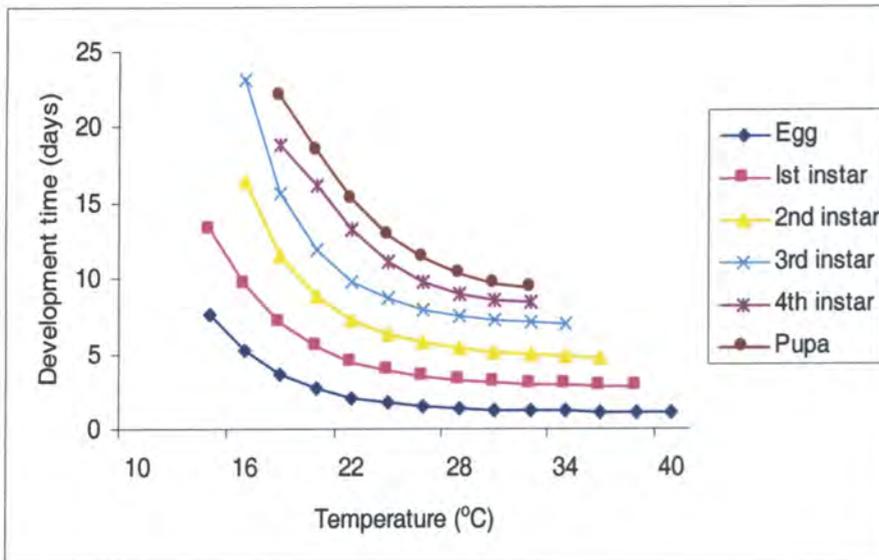
Fig. 3.2 Development times of instar stages of *An. gambiae s.s.* at different temperatures based on the equation $y = a+b/(1+(x/c)^d)$

Table 3.2 Coefficients of the curve fit $D = a + b/(1 + (t/c)^d)$, which describes the relationship between temperature and the stage specific development times of *An. gambiae s.s.* immature stages.

Stages	a	b	c	d	r^2	Fit SE	F-value
Egg	48.549	970.200	12.096	4.839	0.98	16.623	135.876
1st instar	131.322	988.156	14.134	5.425	0.97	28.816	114.707
2nd instar	210.666	7430.194	9.402	4.658	0.97	35.324	82.633
3rd instar	317.494	56199.178	7.038	5.174	0.98	43.781	96.438
4th instar	390.236	662.094	20.742	8.946	0.99	17.054	278.261
Pupa	410.863	991.391	19.759	6.827	0.99	22.046	234.515

SE = standard error

The optimal temperatures for adult development ranged from 28 to 32°C and for adult production from 22 to 26°C. The peaks for development and adult production relative to temperature were out of phase (Fig. 3.3).
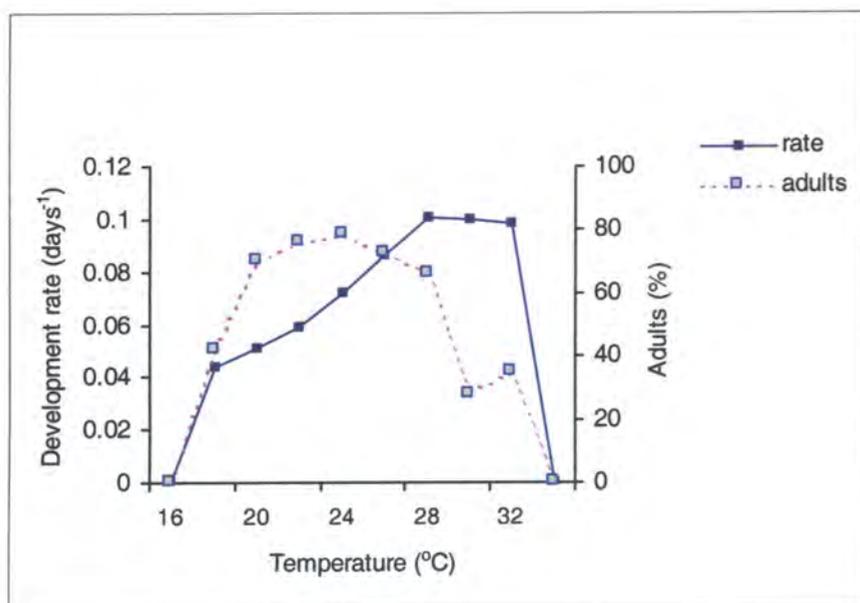
Fig. 3.3 Development rate of *An. gambiae s.s* and proportions developing to adults at different temperatures.

The relationship between overall development rate (R) and temperature (t) is best described by the following non-linear expression

$$R = a + bt + ce^{t} + de^{-t}$$

where a = -0.04993, b = 0.005138, c = -2.1393E-16, d = -281357.656 (goodness of fit, r^2 = 0.992 and F = 243.2) and e = exponential.

A linear degree-day model was obtained by substituting for temperature in the equation suggested by Craig and others (Craig, *et al.*, 1999) and overlaid with predicted rates from this study (Fig. 3.4). There was a close resemblance between the two models over the projected linear area depicted by our model, but not at the extremes.
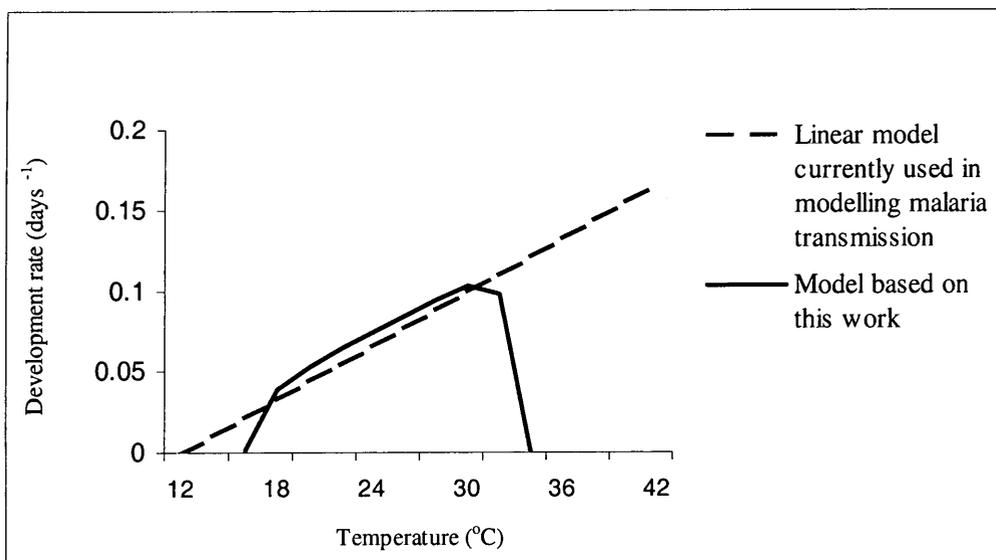
Fig. 3.4 Comparison of a linear degree-day model of relationship between temperature and development rate of *An. gambiae* mosquitoes (Craig, *et al.*, 1999) with the non-linear model produced from this study.

The relationship between temperature, adult emergence times and number of larvae surviving to adults is displayed on a 3D surface (Fig. 3.5). The period of adult emergence was narrow for all temperatures while the number of adults emerging peaked at the moderate temperatures. The different colours represent the number of adults produced in ascending order from no adults (red) to the highest number of adults (blue).
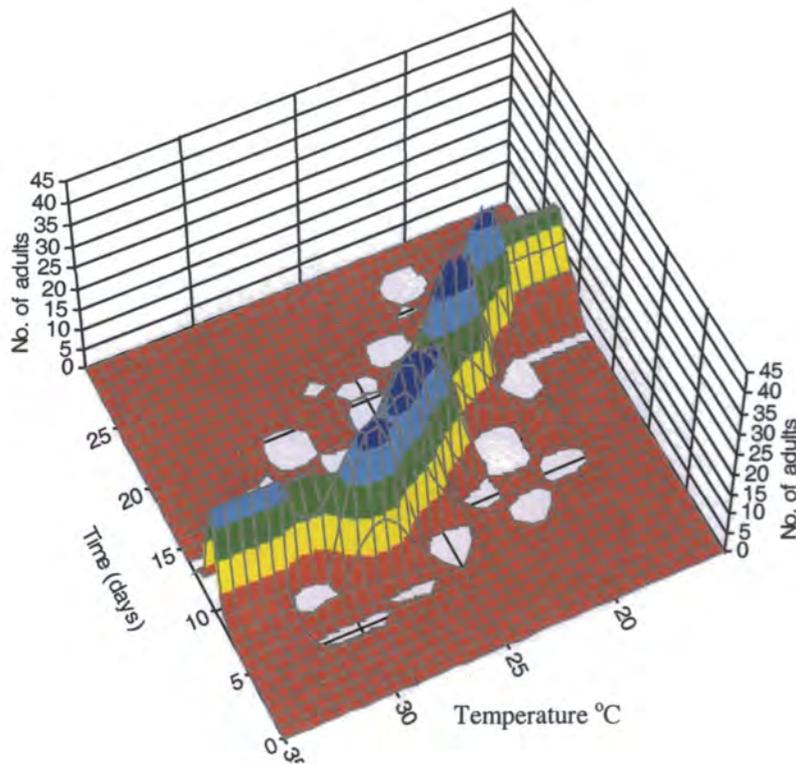
Fig. 3.5 A 3D view of the influence of temperature on adult *An. gambiae s.s* emergence times and numbers produced.

The climate suitability map for *An. gambiae s.s.* was based on temperatures between 15 and 35°C and rainfall higher than 350 mm during the wettest five months of the year. Since the sensitivity of the growth chambers was ±1 °C, we adjusted the temperature range for larval development to include this factor. Thus we used 15 °C as the lower limit and 35°C for the upper limit. The rainfall criteria employed were based on suggestions for stable malaria made by Craig and others, that 80mm rain for 5 months was sufficient but 60mm rain for 5months was not. (Craig *et al.*, 1999). The amount of rainfall necessary for stable malaria by taking the middle ground was taken to be above 350mm for 5 optimum months. The map generated by the temperature requirements for *An. gambiae s.s.* larval development where rainfall is not limiting approximately highlighted the areas known for stable malaria (Fig. 3.6A) . This map was very similar to the MARA/ARMA malaria distribution map (Fig. 3.6B).
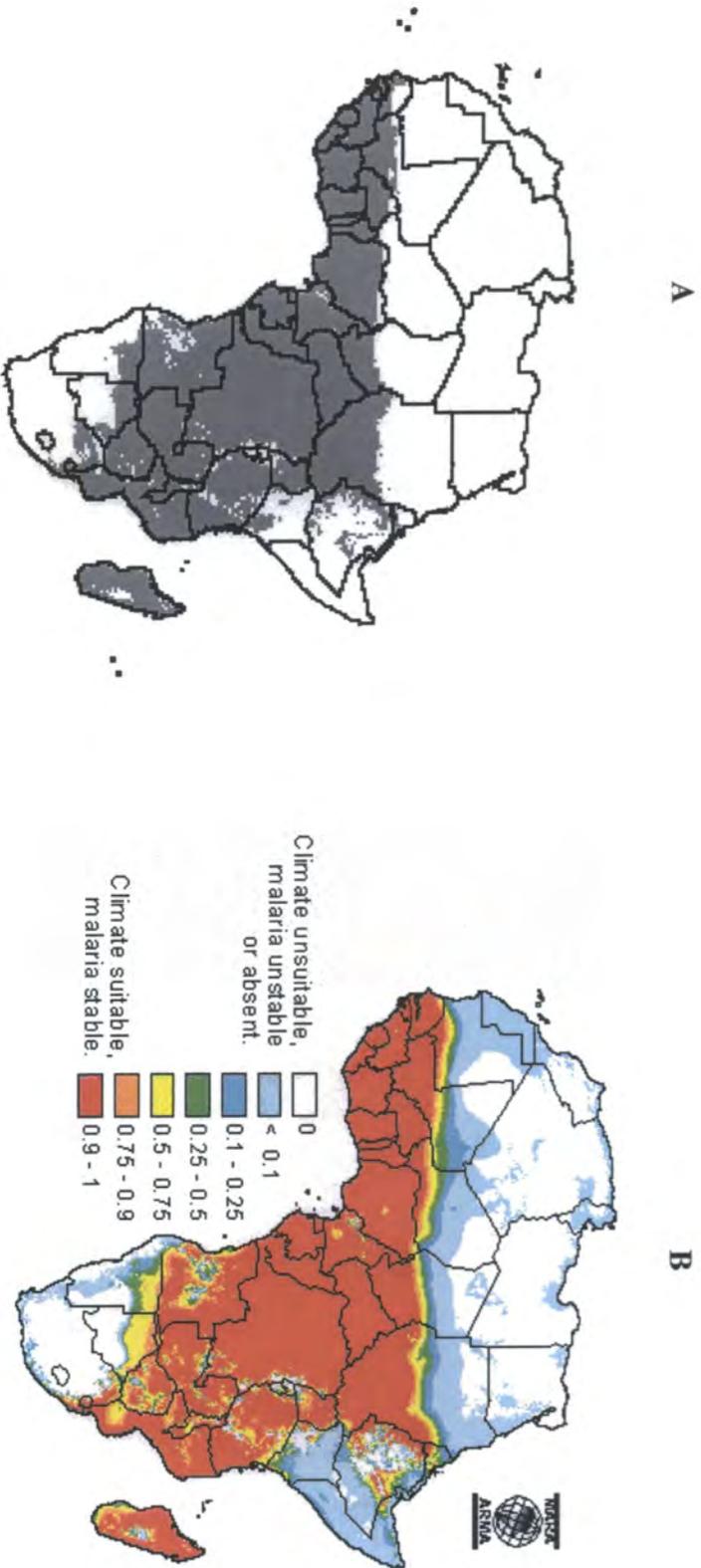
Fig. 3.6 Distribution map for malaria in Africa generated by **A**: the temperature restrictions shown by this study and **B**: the MARA/ARMA Project

Discussion

Insect development increases with temperature within a certain lower threshold and an upper limit. The overall relationship between development rate and temperature is non-linear and is made up of three parts. Firstly, there is a non-linear increase in development rate from zero development at a low temperature threshold, in this case 16°C. Secondly, there is a temperature where the rate begins to increase proportionally with temperature, between 22-28°C. Finally, after the optimum temperature is approached there is a rapid non-linear downward trend to zero development at an upper lethal temperature, in this instance 34°C. This trend has also been reported for other insects (Logan *et al.*, 1976; Briere & Pracros, 1998; Petavy *et al.*, 2001). The difference between the widely used linear model of development versus time and the polynomials used in this study was apparent when the curves were superimposed. The polynomials described here will be tested on data obtained from the natural habitat of the insect (Chapter 6). There is no upper bound for development in the linear model but in the non-linear polynomials upper and lower limits were imposed, reflecting better the relationship between temperature and development in which such extreme temperatures would not naturally support life.

Despite the fact that the duration of larval development reduced with increased temperature care must be taken when incorporating these relationships in malaria transmission models. As shown by this work, the proportion of larvae that develop into adults actually reduced with increase in temperature at high temperatures. Holometabolous insects, such as mosquitoes, must attain a certain critical mass during larval development before they can pupate (Clements, 1992). The potential attainable mass decreases with increasing temperature (Chambers & Klowden, 1990) and at higher temperatures, when development occurs quickly, many individuals may not accumulate sufficient mass. At these high temperatures most 4th instar larvae died during pupation or pupae failed to emerge into adults. The physiological explanation for why few adults emerged is unclear. One possibility could be that when developing at a rapid rate, the organism may be unable to keep up with the accompanying nutrient intake, metabolism or accumulation (Korochkina *et al.*, 1997) required for the complex physiological process in the change from 4th instar to pupa (Lassiter *et al.*, 1995), and as a result fails to develop. In field situations, such physiological activities may well occur when temperatures drop at sunset and the adults emerge in the early evening (Shute, 1956).

Inhibition of further development also occurred at low temperatures probably because of the low metabolic rate experienced and the inability of the aquatic stages to accumulate the required mass for moulting. When Jalil (1971) placed the four different larval stages of *Ae. triseriatus* at 6-8°C, he observed that although they were all active, moulting did not take place. However they did moult and complete their development when returned to 25 °C. This indicates that cold temperatures act as an inhibitor by which larvae are held back at a moulting barrier. However, larvae exposed to 38 °C did not complete development when they were transferred to 25 °C implying a possible cumulative injurious effect at the high temperature, which prevents further development.

This work suggests that development rate-temperature models alone are not sufficient for estimating adult production. For instance, though development rates at 30 and 32°C were high, the actual numbers of adults produced at these temperatures were very low compared to lower temperatures. Thus the adult production rate should be expressed in terms of not only time to adult but also numbers surviving to adulthood.

The map generated using the temperature ranges for larval development, and assuming rainfall is not limiting, compared favourably with maps published using more sophisticated techniques. My zone map of suitable larval breeding sites was remarkably similar to the areas in the MARA/ARMA map depicting stable malaria. The southern limits coincided with areas of greater than 50% stability including north-eastern Namibia, northern and eastern Zimbabwe, Lesotho and northern South Africa including the east coast. Also, in the distribution maps produced by Lindsay and others (Lindsay *et al.*, 1998), all regions with a probability of *An. gambiae s.s* greater than 10% fell within the areas depicted by the larval map. This is evidence that climate is a first order determinant of the distribution and abundance of species. Further climate and vector parameters such as survival and reproduction may help construct improved analytical-based models of malaria transmission in Africa.

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Chapter 4

The effect of temperature on the production of *Anopheles gambiae sensu stricto*

Summary

Vector abundance is an important factor governing disease risk and is generally employed in modelling disease transmission. The rate at which the immature stages survive to adults dictates the size of the adult population and hence the intensity of disease transmission. Here, I examine how temperature influences the survival of larval stages and subsequent adult production of the world's most efficient malaria vector *Anopheles gambiae s.s.* Mosquitoes were reared at constant temperature between 10-40°C at 2° intervals and observed until the death of the last individual. Larvae developed into adults at temperatures between 16-34°C. Larval longevity was shortest (<7 days) at 10°, 12°, 38°, and 40°C and longest (>30days) at 14-20°C with larval deaths being the sole terminal event at all the cited temperatures except 18 and 20 °C. Within the temperature range at which adults were produced, larval mortality was highest at the upper range 30-32 °C, with death, rather than adult emergence, representing over 70% of the terminal events. The optimal survival temperatures were lower than the temperatures at which development was quickest, suggesting a critical relationship between temperature and the life cycle of the insect. These data provide fundamental information about *An. gambiae s.s.* adult productivity at different temperatures, which may facilitate the construction of process-based models of malaria risk in Africa and the development of early warning systems for epidemics.

Introduction

The rate at which new individuals are added to, or existing individuals removed from a population determines the probability of that population staying in existence (Krebs, 1972). The risk of infection with a mosquito-borne disease depends on the number of vectors per host (Garrett-Jones, 1964), and the size of the population of vectors transmitting the disease affects the stability of that disease. Temperature is critical here, as it has a major effect on the rate at which the immature stages of insects develop into adults (Lassiter *et al.*, 1995)

The influence of temperature on the aquatic stages of mosquitoes has been studied for a number of species, including *Aedes quadrimaculatus* (Huffaker, 1944), *Ae. aegypti* (Bar-Zeev, 1957; Tun-Lin *et al.*, 2000), *Taeniorhynchites brevipaplis* (Trpis, 1972), *Anopheles albimanus*, *An. quadrimaculatus*, *Culex pipiens*, *Cx. restuans*, *Cx. salinarus*, *Ae. Sollicitans*, *Ae. triseriatus* (Shelton, 1973) and *Wyeomyia smithii* (Bradshaw, 1980). These stages show enormous sensitivity to high temperature during development. Muirhead-Thomson (1940) subjected the early stages of *An. minimus* to slowly rising temperature after being held for 5mins at the highest temperature and observed that the thermal death points for the eggs, 1st instar, 4th instar and pupae were 43-44°, 42°, 41° and 41°C respectively. Similar trends with higher thermal death points have been reported for *An. subpictus* (Lal, 1953); *An. quadrimaculatus*, *An. freeborni* and *An. aztecus* (Barr, 1952) and an irregular trend for *Ae. aegypti* (Bar-Zeev, 1957). In pools of water in hot tropical climates maximum temperatures are usually maintained for only short periods each day so it is important for larvae to be able to survive such periods of exposure to sub-lethal temperatures (Muirhead-Thomson, 1940). High temperatures can usually be avoided by mosquito larvae in large or deep pools through vertical or horizontal migration (Haufe & Burgess, 1956). At low temperatures some species show more sensitivity than others. While larvae of *An. quadrimaculatus* are killed by exposure to 10°C (Huffaker, 1944), those of *Ae. aegypti* are killed by prolonged exposure to 8°C (Bar-Zeev, 1957) and of *An. culicifacies* by exposure below 5°C (Pal, 1945).

However, little is known about the quantitative impact of temperature on the survival of a wide range of mosquitoes (Clements, 1963; Mahmood, 1997), including the principal vector of malaria in Africa: *An. gambiae*. Also, most survival analysis of mosquitoes has been based on the adults (Clements & Paterson, 1981), mainly because adult survival affects the potential of mosquitoes to be vectors of disease organisms. Females that become infected when taking a blood meal must survive through the incubation period of the parasite before they can transmit the disease. However, the survival of the immature stages determines in the first place how many of the adults are available at a specific location, often an important measure of human biting rates.

The precise mechanisms used by insects to survive at high temperatures are still being investigated. One important factor is the production of heat shock proteins (hsps) which

have been associated with increased thermotolerance (Alahiotis & Stephanou, 1982; Stephanou *et al.*, 1983; Berger, 1984; Benedict *et al.*, 1991; Feder *et al.*, 1997). In selection studies involving the fruit fly, *Drosophila melanogaster*, cold-selected strains were more sensitive to heat killing and produced lower levels of hsps than heat-tolerant strains (Alahiotis & Stephanou, 1982). The onset of thermotolerance in *D. melanogaster* embryos occurred at gastrulation, the same stage at which they are able to synthesise hsps (Bergh & Arking, 1984). Also when *D. melanogaster* cells were treated with ecdysone, which is known to induce synthesis of small hsps, thermotolerance was increased (Berger, 1984). Medflies, *Ceratitis capitata,* that have been reared at a higher temperature were found to have higher levels of hsps and thermotolerance than larvae not pre-shocked (Stephanou *et al.*, 1983).

Insects may go into a state of arrested development or quiescence during adverse climatic conditions (Kovats *et al.*, 1999) to ensure their survival and reproduction at the end of such period. Inability to do this leads to death either directly from the adverse climatic factor or indirectly through its effect on other significant factors necessary for the existence of the insect.

Mathematical methods used widely in the explanation of survival and mortality patterns in wild mosquito populations have in the past been based on the exponential model, which assumes that mortality rates are independent of age (Macdonald, 1952). It is believed that in nature few organisms die of old age, most being killed by predators, disease or other environmental hazards before they become old (Krebs, 1972). Nonetheless, the probability of death has been shown to increase with age and a variety of patterns of survival can be observed (Clements & Paterson, 1981). Under these conditions, the mortality pattern of mosquitoes is best described by the Gompertz mortality function, which does not make any assumptions about independence of mortality and age of insect. The most convenient method for describing the mortality of a population is by using life tables (Southwood, 1978). Life tables provide an age specific summary of the mortality rates operating on a population and can either be static, when a cross section of the population at a specific time is investigated, or based on a cohort, when a given number of individuals in a population are followed throughout life. The features of the table include observations of number of individuals alive during a specific age interval from which the proportion surviving, the number dying, the rate of mortality and expectation of further life in each age interval can be

estimated. From these, survivorship curves can be generated. There are three general types of survivorship curves (Pearl, 1928). Type I curves represent the situation where mortality acts most heavily on the old individuals, Type II curves assume constant rate of mortality independent of age, and Type III curves indicate high mortality early in life followed by a period of much lower and relatively constant mortality.

Shelton commented that in general, the duration of development of the immature stages were longer at temperatures that produced more adults. (Shelton, 1973). On the other hand, temperatures that allowed the fastest rate of development resulted in decreased survival and smaller adults. In nature, a prolonged aquatic life may result in increased chances of stranding due to drying up of breeding sites or a reduction in numbers from predation or disease pathogens, though evidence for this is rare. This study was designed to investigate how temperature influenced larval life and adult production under laboratory conditions where food is unlimited and predators are absent. It seeks to show the maximum potential of vector abundance that will improve current estimations of the effect of changes in temperature on the vectorial capacity of *An. gambiae s.s.*

Methods

The mosquitoes used in this study were obtained from a colony of the 16CSS strain of *An. gambiae s.s* (see chapter 2). The colony was maintained at 26°C (±1 °C), 80% relative humidity and a 12:12hr light and dark regime on 10% glucose solution and defibrinated horse blood (OXOID, England).

Eggs produced by colony females were hatched at 24-26°C and two-day old first instar larvae placed at 16 constant temperatures ranging from 10-40°C (±1 °C) under a 12:12hr light and dark regime in programmable growth chambers (LMS cooled incubators, S. H. Scientific). Water temperature was monitored using data loggers (Tiny Talk II, Gemini, with accuracy ±0.5 °C). 30 first instar larvae were dispensed into larval bowls, plastic containers 15 x 10 x 8cm, containing 1L of aged tap water and 100ml of rearing medium before placing at the appropriate temperatures. The rearing medium, which served as the source of initial food for the first instar larvae, consisted of a bacterial soup produced from aged tap water and whole grass plants with soil attached to the roots (see chapter 2). About 10mg of powdered Tropical Fish Food (Tetramin[R], Germany) was dispensed on the surface of the medium in each larval bowl on a daily basis 24hours after the experimental bowls has been placed in the growth chambers and

continued till the death of the last individual immature stage. Any dead larvae or pupae were removed before the addition of feed each day and the rearing medium replaced every two days to prevent the formation of scum and accumulation of metabolites that may be toxic to the insects. Larvae were counted daily and categorised according to instar stages (WHO, 1975). For each temperature interval there were five replicates (n = 150 larvae) with an overall total of 2,400 larvae used in the entire study.

Survival analysis

Survival analysis is a technique for analysing "time-to-event" or "failure-time" data, which may or may not be related to survival and death in the usual sense. The value of a survival time variable must be larger than zero, i.e. a zero or a negative value is not allowed. There are three major types of survival analysis techniques that are routine epidemiological tools, differing in the assumptions that are made (see Collett, 1994). They include parametric t-tests, semi-parametric Cox regressions (Cox, 1972) and non-parametric Kaplan-Meier Method (Kaplan & Meier, 1958). The Cox regression models assume that for two observations with different values for the independent variables, the ratio of their hazard functions does not depend on time and that a log-linear relationship exists between the independent variables and the underlying hazard function. The Kaplan-Meier method does not make any assumptions about the survival distribution and was used in this study. In calculating a Kaplan-Meier survival curve, probability of death is obtained from the proportion of at risk individuals that die. The cumulative survival probability is initially 1 and here it is presented as proportion of larvae alive over time. Since for some individuals the terminal event was development into adults and not death, the cases were considered to be censored making actuarial life table methods unsuitable. The Kaplan-Meier procedure generates time-to-event models in the presence of such censored cases and is based on estimating conditional probabilities at each time point when an event occurs and uses the product limit of those probabilities to estimate the survival rate at each point in time. Censoring is assumed to happen at the end of the day such that the number at risk the next day is reduced by the number lost up to the end of the previous day. To test the null hypothesis that two survival curves (e.g. between temperature regimes) are identical the log rank test was used. The test is based on a comparison of the observed and expected numbers of death, with the expected number calculated under the assumption of no difference in survival between groups. The test statistics is compared with the critical values of a Chi-square

distribution. The larger the discrepancies between the observed and expected number of deaths in the groups, the more likely the statistics will be larger than the critical value of a Chi-square distribution with 1 degree of freedom (in the case of two groups); and therefore more likely to reject the null hypothesis of no difference. The two main assumptions used in this test are that the survival times are ordinal or continuous and the risk of an event in one group relative to the other does not change with time. The SPSS statistical package (Version 10.0 for Windows, SPSS Inc., Chicago) was used in the survival analysis.

The mean daily expectation of life (E_x) was calculated for each temperature using Microsoft Excel 2000 (Microsoft Corporation) from the expression

$$E_x = T_x / n_x$$

where T_x is the cumulative sum of L_x from the day the last individual died, and n_x is the number alive at the beginning of that interval. L_x is the average number of individuals alive in each age interval. For instance, L_x for interval x is the sum of the number alive at beginning of that interval and the following interval divided by 2. That is,

$$L_x = (n_x + n_{x+1}) / 2$$

(Krebs, 1972). Expectation of life is defined here as the number of days an individual is expected to survive at each age.

A 3D surface describing daily survivorship at different temperatures was constructed using TableCurve[®]3D automated surface-fit equations. The data was processed by all the pre-defined equation sets and the output digitally enhanced by the non-parametric interpolates to uniform grid, which independently sets the x and y points in the grid.

Results

The length of each larval stage was recorded as number of days before death or some other terminal event such as development into adult or loss during observation. The mean survival at each temperature is given in Table 4.1.

Table 4.1 Summary of life table attributes of larvae of *An. gambiae s.s.* at different constant temperatures.

Temperature (°C)	Mean Survival time in Days (95% confidence interval)	Range of larval mortality (Days)	Proportion of terminal events occurring as larval mortality (%)	*Equality of survival distributions
10	2.7 (2.6-2.8)	2-5	100.0	a
12	3.7 (3.6-3.9)	1-6	100.0	-
14	20.5 (19.3-21.8)	5-42	100.0	-
16	25.5 (24.4-26.5)	9-39	100.0	b
18	24.9 (23.8-26.2)	10-38	58.0	b
20	24.9 (23.6-26.4)	3-31	24.7	b
22	18.1 (17.5-18.6)	5-20	24.0	-
24	16.4 (15.9-16.8)	6-18	20.7	-
26	13.5 (13.2-13.9)	5-15	27.3	-
28	11.0 (10.6-11.4)	3-14	33.3	c
30	11.2 (10.8-11.5)	4-16	72.7	c
32	10.2 (9.9-10.50	5-13	70.0	-
34	8.9 (8.5-9.3)	4-14	100.0	-
36	6.9 (6.8-7.2)	4-10	100.0	-
38	4.8 (4.6-4.9)	3-7	100.0	-
40	2.8 (2.6-2.9)	2-4	100.0	a

*no significant differences in survivorship between temperatures with same letter, Log Rank Statistic U and P values for a, b and c: 0.39 and 0.5323; 1.41 and 0.4932; and 0.72 and 0.3954 respectively. The symbol (-) means survivorship was significantly different from all others

There were no within group differences in survival distributions for all five replicates at each temperature. Thus the replicates were combined and treated as a single population. Generally, larval life decreased with increased temperature. Survivorships for 3 sets of temperatures were similar (Table 4.1). When all data were considered the differences between groups were significantly different $U= 4457.26$, $P<0.0001$.

A 3D presentation of age-specific survivorship is given in Fig. 4.1. Survival at the early age seems to be a constant trend at all temperatures. Depending on the temperature, mortality sets in earlier or later forcing the surface to take the downward slope of its sigmoid shape. The tails of the curves are short for the extreme temperatures, but long or moderate for the lower moderate and upper moderate temperatures. Generally, survival is extended between $14°$ and $20°C$, but declines appreciably at lower and higher temperatures.
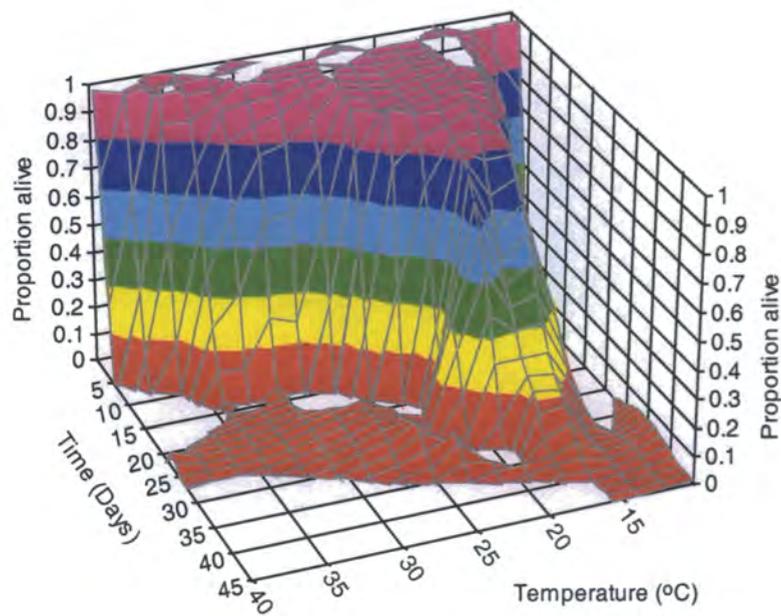
Fig. 4.1 Age specific 3D survivorship curves for the aquatic stages of *An. gambiae s.s.* at various constant temperatures.

Trends in age -specific life expectancy clearly demonstrate that mortality was influenced by age (Fig. 4.2). The curves for some of the temperatures were removed for clarity. The older the individual, the fewer the number of days it is expected to survive. In general, expectation of life declined in a linear manner with age, although at temperatures between 14-20°C, survival of older individuals was greatly extended. The maximum life expectancy throughout the temperature range was about 25 days at 16°C. The shortest length of expected life was about 2 days at 10°C and 40°C.
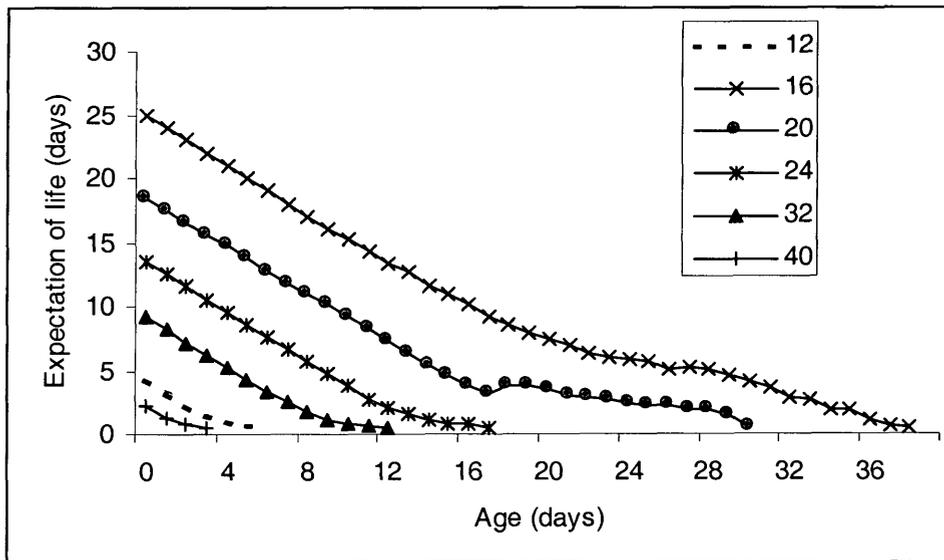
Note: no adults were produced at 12, 16 and 40°C despite the lengths of immature lives.

Fig. 4.2 Life expectancy of the aquatic stages of *An. gambiae s.s.* at different representative temperatures (selected for clarity).

Discussion

Water temperature had a marked effect on the survival of the aquatic stages of *An. gambiae s.s.* At the extreme cold (10-12°C) and hot (38-40°C) mean temperatures all larvae died within a few days. The low temperature range is often not experienced at *An. gambiae* breeding sites while the high temperatures are frequent occurrences during the dry season in most tropical regions (Muirhead-Thomson, 1951; Young, 1975). However, in nature, these high temperatures occur only for a few hours and larvae may survive these short periods. At temperatures of 14-20°C larval survival was prolonged, with individuals living for as long as 40 days. Previous work has shown that at 14° and 16°C these insects survive as second or third instar stages (see Chapter 3) and that adult development begins at 18°C. Whether larvae at 14-16°C failed to develop into adults due to physiological difficulties or whether it is an adaptation to ensure that adults develop only when conditions are suitable for flight and host location needs to be investigated. Overall larval mortality was highest at temperatures <18° and >32°C. Reports from work on the survival of *Cx tarsalis* associated temperatures < 12°C and ≥ 32°C with high larval mortality (Bailey & Geike, 1968; Reisen *et al.*, 1984). This supports the view that the lower threshold for survival of insects normally has a broader range than the upper threshold for most insects (Buxton, 1933; Heinrich, 1981).

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Within the temperature range at which adults were produced (18-32°C), larval mortality was highest at the upper range 30-32 °C, with over 70% of the terminal events being deaths. At these higher temperatures few larvae survived the period needed for adult development and although development times were shorter (see Chapter 3), very few adults were produced. As the body temperature of an insect increases, there is an increase in both metabolism and respiration up to a critical thermal limit. It has been reported that death occurs soon after respiration rates begin to drop even if the insect is returned to normal temperatures, indicative of systematic cell deaths at the high temperatures. (Neven, 2000). Such elevated temperatures may also affect the nervous and endocrine systems, which are responsible for directing insect metamorphosis (Neven, 2000) and could result in developmental defects causing death (Neven & Rehfield, 1995).

It has been reported that larvae that survive at high temperatures produce smaller and less successful adults (Shelton, 1973). Thus it is possible that certain physiological changes at such temperatures prevent the immature stages from becoming adults. The change from 4th instar to pupa is a complex process (Lassiter et al., 1995) and the larvae must attain a certain critical mass during development before they can pupate (Clements, 1992). At high temperatures the amount of mass they can accumulate is reduced (Chambers & Klowden, 1990). Thus development from the 4th instar stage at these temperatures is impeded. It was observed at the high temperatures that most 4th instar larvae died during pupation or pupae failed to emerge into adults. Another possibility is that when development takes place at a rapid rate, the organism may be unable to sustain the accompanying nutrient intake, metabolism or accumulation prior to pupation (Korochkina et al., 1997) and as a result fails to develop.

The cessation of development at 14-16°C with continuation of life for a long period may have important public health consequences in highland areas prone to epidemics, that is, if such individuals have the potential of developing fully with improved environmental conditions. B▶ dker (2000) recorded mean temperatures of this order at high altitude villages (>1400m) during the cold months in the Usambara Mountains of Tanzania. At these sites he attributed the low entomological inoculation rates to the low anopheline densities during the cold months. In such areas, cold conditions could maintain larval populations of *An. gambiae s.s.* and any rise in temperature could see a rapid increase in mosquito abundance. It should be mentioned however that data obtained from

meteorological stations might not directly represent the conditions experienced by the aquatic stages of the insect at the breeding sites. Proper surveillance of water temperatures must be carried out and a clear relationship established so that meteorological data could be correctly utilised in predicting favourable conditions for larval development and adult production at these highland regions.

Information on the relationship between temperature and larval survival and adult production will be useful in developing processed-based models of malaria transmission.

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Chapter 5

Comparing the rate of development and survival of immature stages of *Anopheles gambiae* under natural and laboratory conditions.

Summary

Studies have been carried out previously to determine how temperature influences the rate of development and survival of immature stages of *Anopheles gambiae sensu stricto* under controlled laboratory conditions. The current investigation was designed to examine whether results obtained in the laboratory under constant and daily fluctuating temperatures parallel those that occur naturally in the field. Studies were carried out in the laboratory in the UK and in the field in The Gambia during the rainy season when breeding of *An. gambiae* is common. In the laboratory, fluctuating the water temperature by 1-6 ° around a mean of 26°C did not affect mean development time or larval survival. Studies in the field showed that the mean water temperature of breeding sites in ricefields (27.4 °C; 95% CIs = 25.3-34.5 °C) were similar to that of puddles (27.1 °C; 20.7-36.9 °C). Whilst the rates of larval development were slightly faster in the puddles (11.0 days, 95% CIs = 10.8-11.2) than ricefields (11.5 days, 95% CIs =11.2-11.7), larval mortality was considerably less in the puddles (46.7% mortality) than in the ricefields (75% mortality). Development rate models generated from laboratory data were compared with data collected from the two types of breeding sites. The best model for the puddle sites was based on half-hourly water temperatures and rate models for each stage of development, whilst the best at the ricefield was based on the half-hourly temperatures and the overall egg to adult development rate model. The differences between the observed and predicted development times for the best models were less than half a day. Thus development rate models generated in the laboratory can, to a certain extent be applied to the natural environment of the insect.

Introduction

There has been a considerable effort to improve our understanding of how climate change may affect the transmission of malaria (Lindsay & Birley, 1996; Craig *et al.*, 1999; Martens *et al.*, 1999; Rogers & Randolph, 2000). Evidence of a possible warming of the earth with the potential for changing patterns of malaria, especially at

higher altitudes and latitudes, has generally prompted this interest. Several aspects of the plasmodium parasite, the mosquito vector and the human host, which are important in disease transmission, are directly affected by environmental factors such as temperature and rainfall. It is the relationship between changes in climate factors and transmission attributes that are used to make logical predictions as to how climate change may affect transmission. One important transmission attribute is the number of mosquitoes per human host, which is determined by the rate at which new individuals are added to the vector population. Temperature has a significant effect on the rate at which the immature stages of mosquitoes develop (Brust, 1967; Trpis & Shemanchuk, 1970; Shelton, 1973). I have described mathematically how temperature governs the rate of development of the immature stages of *Anopheles gambiae s.s.* under controlled laboratory conditions (see Chapter 3) and found that the overall rate of development increased with increasing temperature within a lower threshold of 16°C and an upper lethal temperature of 34°C while the thresholds for the individual stages differ from stage to stage and range from 14° to 40°C.

In nature, most insects experience regular fluctuations in temperature, which may produce different effects on development from that of exposure to a constant temperature. Hoffman (1986) reported that temperatures fluctuating on a day-night cycle stimulated egg production of crickets by up to 3 fold compared to constant temperatures. By analysing the activity of the cricket neuroendocrine system, he suggested that the effect of fluctuating temperature acts via the endocrine system rather than a direct effect of temperature on the ovary. In studies of the fruit fly, *Dacus tryoni*, it was found that development took place at temperatures below the calculated threshold of 13.5°C if such low temperatures alternated diurnally with higher temperatures (Meats & Khoo, 1976). The aquatic stages of mosquitoes are exposed to daily temperature cycles consisting of a warmer and a colder phase. The water temperature at the breeding sites are related to the ambient temperature, the surface area and depth of the breeding site, the biotic component and other climatic factors such as rainfall and insolation. The rate of development may be faster or slower at a given thermoperiod than at an intermediate constant temperature depending on the duration of the thermophase and the cold phase (Clements, 1992). While some studies report an increase in development rate of insects under fluctuating temperatures compared to the corresponding constant mean temperatures others report a decrease or no effect (Bailey & Geike, 1968; Milby & Meyer, 1986; Al-Saffar *et al.*, 1995; Joshi, 1996). Cossins and Bowler (1987)

suggested that the response of aquatic organisms to fluctuating temperature regimes is closer to that predicted from the median temperature, and is presumably a reflection of their more thermostable environment where fluctuating temperatures are not experienced.

Using rates of development under constant-temperature regimes to predict rates of development under fluctuating-temperature regimes assumes that development rates are the same at all temperatures in the fluctuating regime as those in the constant-temperature regimes. Predictions based on this assumption are difficult to interpret especially when the fluctuations are of high amplitude or include high or low extremes. Such difficulties are further compounded by the non-linearity inherent in the temperature-rate relationship of insect development and the phenomenon used to describes these difficulties in interpretation is known as the rate summation or the Kaufmann effect. (See Hagstrum and Milliken, 1991; for a review of these methods and the consequences of the Kaufmann effect). Attempts have been made in the past to model insect development times at fluctuating temperatures using data derived from constant temperature studies with various degree of success (Hagstrum & Milliken, 1991; Worner, 1992; Li *et al.*, 1993; Al-Saffar *et al.*, 1995). Hagstrum and Milliken (1991), for instance, produced poor predictive models of development times of the red flour beetle, *Tribolium castaneum*, and 16 other species at fluctuating temperatures over a broad range of mean temperatures or amplitudes of fluctuating temperatures. They reported that for the same mean temperature, development times at constant temperatures tended to be shorter above 25-30°C range and longer below this range than at fluctuating temperatures.

There is thus great complexity in relating data obtained from fluctuating temperature regimes in the field to constant temperatures in the laboratory. If the lowest temperatures of two sets of fluctuating regimes with the same mean are different, the overall rate of development may be different. A wider range around a mean optimum temperature for development of an organism may produce a significantly different overall development rate compared to a narrower range. In the case of the wider range, there may only be a small increase in rate during the thermophase but a large reduction at the cold phase leading to a reduced net effect. In the case of the narrower range, the increase in rate at the thermophase may just cancel out the decrease at the cold phase. What happens at mean temperatures that are not optimal will be difficult to generalise.

A better estimate of development at fluctuating temperatures will be to sum the fraction of development that would have been completed in the time spent at each individual temperature based on the known rates at such constant temperatures (Cossins & Bowler, 1987).

An. gambiae mosquitoes naturally occur in a variety of water habitats of which the shallow sun-lit pools are most favoured. Such water bodies originate from a range of sources including borrow pits, drains, car tracks, hoof prints near water holes, the overflow of rivers, pools left behind by receding rivers, and rainwater collecting in natural depressions (Gillies & de Meillon, 1968). Little is known about the effect of micro-climatic conditions on *Anopheles* larval growth and survivorship under natural conditions (Collins *et al.*, 1985; Wallace & Merritt, 1999).

In The Gambia, the *An. gambiae* complex is represented by *Anopheles gambiae s.s.*, *An. melas* and *An. arabiensis* (Bryan, 1983; Lindsay *et al.*, 1993). *An. melas* is a saltwater breeder and dominates in highly saline areas of the flooded alluvial plains bordering the river whilst *An. gambiae s.s.* and *An. arabiensis* are more common in the rain fed rice fields along the edge of the alluvial plains (Bøgh *et al.*, 2001). Unlike most other malarious regions of Africa, rain-fed puddles are not the most important source of mosquitoes in The Gambia. Any small collection of water in the alluvial soil quickly seeps away as water retention is poor. However, in some areas, for example, in borrow pits, large puddles might linger enough for the completion of the insect's life cycle. This study will compare development and survival rates obtained from controlled experiments in the laboratory with rates at the typical *An. gambiae* breeding sites in Africa; rain fed rice fields and artificial puddles. In addition studies were carried out in the laboratory to examine how diurnal changes in temperature affect larval development and survival.

Methods

Study site

The study was carried out on the edge of Farafenni town (13° 34' N and 15° 36' W), situated north of the River Gambia. The Gambia is located in the Sahel and has the characteristic two-season climate: dry and wet. The rainy season typically lasts from June to November (Hutchinson, 1985) with an annual precipitation of 600-800mm. Maximum temperature remains high during the dry season; mean monthly maximum

temperature 38.8-40.2°C, while the relative humidity falls to its lowest; mean monthly minimum humidity 28.4-46.5% (Lindsay *et al.*, 1989). Due to tidal differences, saltwater intrusions occur at the lower reaches of The River Gambia. In the dry season the salt front can travel as far as 200 km up river, creating large areas of saltmarsh and mangrove forests immediately adjacent to the river. However, in the rainy season the situation is reversed by the outflow of freshwater that makes the river and adjacent flooded areas markedly less saline (Sylla *et al.*, 1995).

Puddle site

The puddle sites were situated within the Medical Research Council (MRC) compound at Farafenni. To mimic rain-fed puddles, earthenware round-bottomed pots, 30cm wide and 15cm deep, containing some 50ml of soil, 3 whole grass plants, both taken from the vicinity, and rainwater, were placed in depressions in the soil (Plate 7).

Plate 7. Puddle site

A fence was built around the entire breeding area to prevent domestic animals from disturbing the experiments. A plastic sheet was used to cover the entire area when it rained so that the pots did not overflow with water and risk losing the larvae.

Ricefield site

The rice field sites were situated 500m outside the MRC compound in Farafenni. The unshaded fields consisted of an overall surface area of over 0.5 km² with small-

interconnected patches. The water depth at the edge was about 90cm. Rice plants; 30cm above the water surface, and other weeds of similar heights were present. Larval entrapments were placed along the edges of these flooded fields and the insects reared within these enclosures (Plate 8). The entrapments were made from fine polyester mosquito netting of mesh size $0.2mm^2$ sewn into a cylinder shaped sack of 30cm diameter and 60cm height. Flexible wire mesh was built around each bag to prevent it from collapsing. The open end of the bag had an elastic band fastened round it, which was pulled out and wrapped over the edge of the wire mesh to enable the bag to fasten securely to the mesh. The bag was tied to the wire frame with string. Iron rods attached vertically to the base of the entrapment were used to anchor the bag into the mud. The entrapment was placed in the water such that half of it projected above the water to avoid overflow and thus loss of larvae. Water freely passed through the netting but larvae were trapped inside.

Plate 8. Ricefield

Study design

Origin of mosquitoes

Immature anopheline stages used in these experiments were obtained from F1 generations of wild-caught, blood-fed, females. The local village of Bambally was visited at 6am in the morning and blood-fed anophelines caught from under bed nets using an aspirator and torchlight. The insects were transported to the insectaries at Farafenni where they were kept at 27°C and 85% relative humidity and allowed to

oviposit. The eggs were hatched and the first instar larvae fed on bacteria from a medium containing 3 whole grass plants with soil attached to the roots and 4 L of water in a large metallic bowl 50cm diameter and 30cm depth. Older larvae were fed on fish food (Tetramin[R], Germany) ground in a powder. Pupae were hatched in new cages kept separately from the potentially infected wild population. The adults produced were fed on my arm and the eggs and larvae produced were used in the experiments.

Larval experiments

Six replicates were performed at each site. For each replicate, 30 1-2 days old first instars were dispensed in either the entrapment or the clay pot. Larvae were counted each day and categorised according to instar stages (WHO, 1975). The pots and entrapments were covered with netting at dusk, to prevent the oviposition of wild females. When pupae started developing, exit traps were placed above each set up to collect emerged adults. For some experiments, pupae were removed and allowed to hatch in the insectaries. Emerged adults were collected and stored in a tight lid tube containing anhydrous copper sulphate for species identification. Both field sites were surveyed between 2nd and 25th October 2000.

Species identification

A diagnostic ribosomal Deoxyribonucleic acid (rDNA)-polymerase chain reaction (PCR) assay was utilised to identify all the species of the gambiae complex that were represented among the adults obtained (Scott *et al.*, 1993). The method is based on species-specific nucleotide sequences in the ribosomal DNA intergenic spacers. DNA was extracted from each adult mosquito using Instagene matrix, and the extracted DNA mixed with a cocktail of pcr reagents. Species-specific primers for *An. gambiae s.s*, *An. melas*, *An. arabiensis* and *An. quadriannulatus* and a universal primer that anneals to the same position of the rDNA of all four species were used. The resulting pcr product was run on 2.5% agarose gel containing ethidium bromide and the amplified fragments visualised by short-wave ultraviolet light.

Temperature data

Both ambient and water temperature were recorded continuously at 30min intervals at both breeding sites using tiny talk data loggers (Tiny Talk II, Gemini data loggers, UK). Loggers were placed at the sites in the water at a depth of 5cm or hung in the air 30cm above the water and half-hourly records of temperature obtained for the period of the

study. There was a spell of rainfall during the study from 13th to 18th October but the loggers used were unaffected. Data from the temperature loggers was downloaded using the OTLM software and processed in Microsoft Excel, 2000.

Laboratory studies involving fluctuating temperatures

The effect of alternating temperatures on larval development was observed in the laboratory. Using programmable growth chambers (LMS cooled incubators, S. H. Scientific), three sets of alternating temperatures 26 ±1, 3 and 6°C were investigated. The temperature and light regime consisted of 12 hours of the upper temperature range coinciding with the 12 hours of light and 12 hours of the lower range coinciding with 12 hours of dark. The detailed procedure in larval development studies in the laboratory has already been described (see Chapter 3), the only difference being that the rearing medium in this study was made of 5 drops of Liquifry No2 for live bearers (Interpet Ltd, Surrey, England) per 1L aged tap water per day. Six replicates were performed for each alternating temperature regime and each consisted of the observation of the development of 30 *An. gambiae s.s.* from egg to adult. Larvae were reared in experimental bowls and fed daily on 10mg of Tropical Fish Food (Tetramin[R], Germany). These immature stages were observed daily and categorised according to instar stages (WHO, 1975). Any dead individuals were removed before the addition of feed and the rearing medium was replaced every two days.

Statistical analysis

The SPSS software (Version 10 for Windows, SPSS Inc., Chicago) was used for all descriptive statistics and tests. T-tests were used to compare means of water temperature at the puddle site and the ricefield site, and to determine whether the water temperatures at the two sites had the same temporal distribution. The Paired-Samples T Test procedure compares the means of water temperature at both sites for a single half-hour, computes the differences between values of the two sites for each half-hour and tests whether the average differs from zero. Analysis of variance for emergence times by the sites (i.e. the two field sites and the laboratory 26°C constant and 26± 6°C fluctuating temperatures) and the six replicates was performed using the General Linear Model (GLM) Univariate procedure. The data was first weighted to accommodate the individuals that failed to develop and for which data was unavailable. The Type III sums of squares was employed to test the significance of the within site and between

sites variation based on an overall F statistic. The one-way ANOVA procedure was used to test for variation in larval mortality among the sites and the Bonferroni post hoc test was used to evaluate differences among means of specific sites. The Bonferroni test, which is based on the Student's t statistic, adjusts the observed significance level for the fact that multiple comparisons are made. The Levene statistic and the spread-versus-level and residual plots were used for checking assumptions about the homogeneity of variances for both adult emergence times and number of larvae surviving to adults at all sites.

Model testing

To predict development times in the field, three models based on constant temperature studies in the laboratory (Chapter 4) were tested. All calculations were done in Microsoft Excel. Firstly, the mean temperatures at the puddle and ricefield sites were directly substituted in the overall development rate model yielding predicted development times at each site. The model was based on a 3rd degree relationship between development rate from egg to adult (R) and temperature (t).

$$\text{Models 1 \& 2; } R = a + bt + ce^{t} + de^{-t}$$

where R is rate of development from egg to adult, t is temperature and the value of the coefficients are: a = -0.05078, b = 0.005202, c = -2.1625E-16, d = -282723.77 (Goodness of fit, r^2 = 0.992 and F = 245.6)

Secondly, the individual half-hourly temperatures recorded in the field for each study period were separately incorporated into the above overall rate model. This provided half-hourly development rates that were summed up for each site to produce overall development times.

Finally, models for each stage of development were used instead of the overall rate model. The half-hourly rates were summed up and after the completion of each stage, the next stage specific model was introduced at the last temperature value. This continued until the last stage of development, that is the hatching of pupa to adult. The development time at this stage was then the sum of the development times for all the individual stages. Each stage development time i.e. egg to 1st instar larvae, 1st instar to 2nd instar, 2nd instar to 3rd instar, 3rd instar to 4th instar, 4th instar to pupae, and pupae to adult followed the model;

$$\text{Model 3; } D = a + b/(1 + (t/c)^d)$$

where D = development time, t = temperature, and a, b, c and d are coefficients which differ for each stage (see Chapter 3; Table 3.2).

Results

The mean water temperatures at the puddle and ricefield sites were respectively 27.1 and 27.4 °C lying between laboratory investigated mean temperatures of 26 and 28 °C. A summary of the ambient and water temperature data recorded for the entire period of each site is given in Table 5.1. The extent of water temperature fluctuation was greater at the puddle site than at the ricefield site.

Table 5.1 Summary of temperature data recorded at the experimental breeding sites of *An. gambiae* in The Gambia during the entire study period.

| Site | Temperature °C | | | |
	Minimum.	Maximum.	Mean	Standard error
Water puddle	20.7	36.9	27.1	0.151
Water ricefield	25.3	34.5	27.4	0.083
Air puddle	19.0	39.0	26.1	0.200
Air ricefield	20.7	44.2	27.4	0.183

Air was warmer than water from late morning to early evening but colder at night and early morning. The air temperature will not be discussed further as it is possible that the record also included radiant heat since the metal probe was in direct sunlight. There was no statistically significant difference between the overall mean water temperature at the puddle site and the ricefield site (t = -1.928, df =1311, p=0.054). Also, 664 pairs of half-hourly water temperature data taken simultaneously at the two sites during the course of the study were compared and the differences were not statistically significant (Paired T-test; t = -1.528, p=0.127). However, mean hourly temperature shows that the puddles became warmer and colder quicker than the ricefield. Ricefield water temperature was much more stable than the puddle temperature.

The brief rainfall had a significant effect on temperature at both sites (Fig. 5.1). Overall mean temperatures before and after the rains were significantly different at both sites (puddle; t =9.86, df = 647 and ricefield; t = 22.5 df = 662)
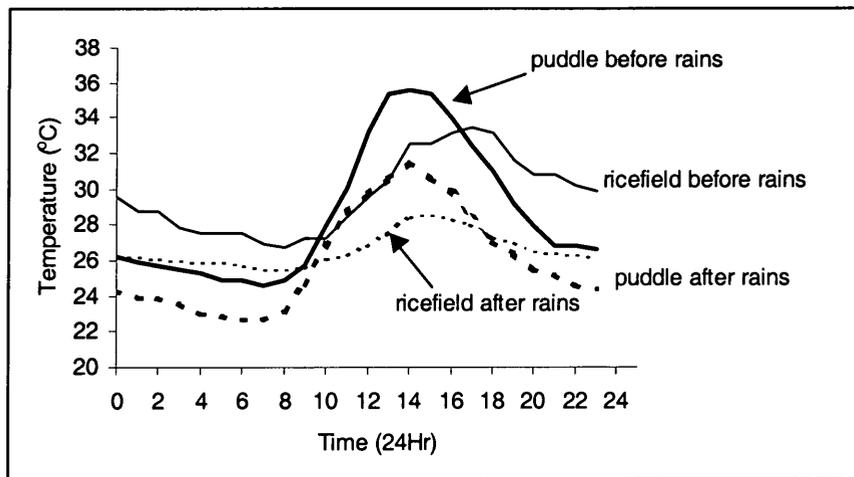
Fig. 5.1 Effect of six days of rainfall on water temperature at the puddle and ricefield survey sites in The Gambia.

The development time and proportions of larvae developing to adults in the field is presented alongside laboratory data for 2 constant and three fluctuating temperatures in Table 2. The mean temperatures and ranges for all the studies are also provided. Development rates were faster at the puddles sites than the ricefield (GLIM; F= 10.276, p=0.002, after allowing for variation between replicates). The differences in development times between the 3 fluctuating temperatures ± 1, 3 or 6°C were not significant (GLIM; between groups F=1.692, p= 0.185 and within groups or replicates F=0.758, p=0.581; see table 2 for means and 95% confidence intervals)

Survival at the ricefield was very low compared to the puddle sites (One-way ANOVA F = 13.939, p = 0.004). Also, the mean numbers of adults emerging at the three fluctuating temperatures were different (ANOVA; F=13.195, p<0.001). Post hoc Bonferroni analysis identified mean survival to be higher at 26 ± 1°C than either 26 ± 3°C or 26 ± 6°C while no difference was observed between 26 ±3°C and 6°C. More larvae survived in the laboratory than in the field. The numbers of adults that emerged in either field sites were consistently lower than those in the entire laboratory studies, with mean survival differences ranging from about 12.7% to 65.5% (Table 5.2).

The development times predicted for the natural environment of the insect using the mean temperature (model 1), the individual fluctuating temperatures in the field (model 2) and finally each half-hourly temperature and the stage by stage development rates (model 3) are presented in table 2. The mean temperature model produced the best

overall result but taking each site separately, one of either half-hourly model produced the closest development time compared to observed values. On the whole the models produced results that were approximately within 1 day of the observed values.

Table 5.2 *Anopheles gambiae s.s.* mean adult emergence times (days) and proportion of immature surviving to adult in the laboratory and at field sites in The Gambia with predicted values for field studies based on laboratory models.

Larval conditions	Mean Temperature & range (°C)	Mean Development time (95%CI)	% survival	Predicted development times (difference with observed values)		
				Model 1	Model 2	Model 3
Constant 28 °C	28 (26-29)	9.8 (9.4-10.0)	66.0			
Constant 28 °C	26 (25-27)	11.5 (11.2-11.9)	72.6			
Fluc-1 26 ± 1°C	25.8 (25.1-26.8)	10.3 (10.1-10.5)	90.5			
Fluc-2 26 ± 3°C	26.2 (23.1-28.7)	10.5 (10.3-10.7)	80.0			
Fluc-3 26 ± 6°C	25.7 (19.6-32.2)	10.5 (10.3-10.7)	71.0			
Puddle sites	27.1 (20.7-36.9)	11.0 (10.8-11.1)	53.3	10.8 (+0.2)	12.2 (+1.2)	11.2 (-0.1)
Ricefield sites	27.4 (25.3-34.5)	11.5 (11.2-11.7)	25.0	11.1 (+0.4)	11.2 (+0.3)	10.4 (+1.1)

Models were tested only for the field studies.

From a sample of 143 adult mosquitoes identified by pcr analysis, 93% were *An. gambiae s.s.*, 4.6% *An. melas* and 2.3% *An. arabiensis* and these proportions did not differ when the samples were split into puddle and ricefield.

Discussion

The mean water temperatures were similar at the puddle site and in the ricefield, but the daily range in temperature was 7°C greater in the puddles than the ricefield. Water temperature was thus more stable at the ricefield than the puddle. This may be due to the fact that the water body at the ricefield was larger and contained more biotic components including rice plants, weeds and other aquatic fauna. This observation is in agreement with studies by Haddow (Haddow, 1943) who reported that vegetation present in tropical pools had an insulating effect on temperature. The temperature range he observed in a grassy pool was less than half that observed in an open pool. On the contrary, Wallace and Merritt (Wallace & Merritt, 1999) reported more variation in surface water temperatures in vegetated habitats compared to open water habitats in a temperate pond. The brief rainfall during the course of the study reduced mean water temperature at both field sites and the amplitude of the diurnal fluctuations at the ricefield. Mean hourly temperatures fell by as much as 6°C after the rains while the range of water temperature at the ricefield reduced from 5.7-8.1 °C. Qualitatively similar data has been reported for ponds in Kenya (Young, 1975) where the arrival of the rainy seasons reduced the average daily water temperature by 2-3°C and the amplitude of diurnal fluctuations from 3-4°C to 1-1.5°C.

Larvae developed faster at the puddle site than the ricefield even though mean temperatures were not different. A plausible explanation for the faster rate at the puddle site could be the higher temperatures observed at these sites. There were longer periods spent at relatively higher temperatures at the puddle site than the ricefield although longer periods were also spent at lower temperatures. However, the models developed for estimating development suggest that development would not occur more quickly in the puddle than in the ricefield sites. Wallace and Merritt (1999) found larvae of *An. quadrimaculatus* developing faster in the open water habitat than the vegetated habitat although they reported higher fluctuations at the vegetated habitat.

In the laboratory, alternating temperatures around a mean of 26°C did not have a significant effect on mean development time but affected the proportion of larvae surviving to adults. It is possible that the long period spent at the higher ranges of 26 ± 3 °C and 26 ±6 °C were not suitable for pupal emergence. Death at pupation or pupa failing to hatch was observed from my previous study at constant temperatures of 30°C and above (see Chapter 4). The difference in mean development times between these

fluctuating temperature studies and the constant temperature of 26°C could be attributed to the change in larval feed. Liquifry was used instead of a bacterial suspension produced from water and grass. This highlights the importance of food quality in the development of mosquitoes.

Using a single mean for the entire study period to describe development models of a short growing organism such as *An. gambiae*, may miss out the effects of alternating temperatures. So even though this method yielded rates that were closest to the observed field values its use from a biological viewpoint is limited. When the individual fluctuating temperatures were considered, a more complex result was obtained. Rate became faster at the ricefield than at the puddle for the overall development rate versus temperature model. The overall development rate model had a lower limit and an upper limit for development. Any temperatures outside these limits are not considered as appropriate for development and are thus not included in the calculations. In these circumstances higher minimum temperatures, as was the case in the ricefield, are more important than higher maximums. Thus the ricefield temperatures gave faster development. The overall development versus temperature model considered temperatures at which full development from egg to adult took place. This means that certain low or high temperatures at which development of certain stages may take place were discarded. For instance although development into adult does not take place at 14°C, development from egg to second instar does. Thus the relationships between development rates of the individual stages and temperature were employed in the third model. This is the best model from a biological viewpoint as it incorporates not only the changing temperature but also the requirements of all the stages in the development of the insect. Here, predicted development times were similar to the observed values at the puddle site than they were at the ricefield. One complication of predicting development in the field is that factors other that temperature may be important. For example, development is affected by the quantity and quality of food, and both are apt to differ from the laboratory conditions used to establish the model. It is possible that due to the nature of the ricefield sites these factors were more important at the ricefield than the puddle site and was responsible for the differences in prediction quality between the two sites.

On the whole the first model made better predictions than those utilising rate summation of instar stages or individual fluctuating temperatures. This was expected since the first

model only used mean temperatures at the breeding sites and the model was based on mean temperatures in the laboratory. The models using fluctuating field temperatures can be improved if similar laboratory studies were conducted that incorporate fluctuating temperatures that more closely reflect field conditions. This means investigating several median temperatures with various amplitudes of fluctuations in the laboratory and using the outcome to develop the model.

More larvae survived to adults in the laboratory than in the natural environments of the insects. This is not an unusual phenomenon as the absence of natural enemies in the laboratory is bound to increase the survivorship of the immature stages and hence their emergence to adults. In natural settings the presence of natural enemies means that r-strategists, like mosquitoes, will have to produce vast numbers of progeny to dilute the effect of predation. The most common predators in the vicinity of the study area were larvae of Odonates and small water beetles. Fish were not seen but it has long been reported that they form a significant source for predation on mosquito larvae (Christophers, 1960). It has been reported that fungi and microsporida are known to cause high larval mortalities at *An. gambiae* breeding sites (Rodhain & Gayral, 1971; Service, 1973; Robert *et al.*, 1988). The slimy deposits found at the point of contact between the water surface and the netting of the larval entrapments at the ricefield are indicative of the growth of fungi, which may be responsible for the low survival rates at the ricefield compared to the puddle sites.

Interpreting the relationships between laboratory and field data is a very complex one that needs to be treated with caution. It is impossible to completely mimic field conditions in the laboratory. Also, temperatures measured by probes in the field may not be the same as those experienced by the larvae resting at the water surface. In Queensland, Australia, Tun-Lin and others (Tun-Lin *et al.*, 2000) studying development and survival of immature stages of *Aedes aegypti* in the laboratory and various natural breeding sites of the insect, found that the nature of the breeding site, especially with respect to availability of nutrients, was more important than temperature. For instance, they found development to be five days quicker at a site with large amounts of detritus than an open site with less nutrients even though the detritus site had a mean temperature 2 degrees lower. At some of the natural breeding sites, they found development and survival to be similar to the equivalent laboratory temperature while at others they were better than the laboratory temperature. Here it is possible that

development and survival was better at the puddle site than the ricefield because being a smaller body of water there could well be more nutrients present per unit of surface area.

In this study the puddle site was made as similar as possible to the laboratory with the exception of temperature conditions. The earthenware pots used it the study are generally used to keep water in homes and *An. gambiae* has been found breeding prolifically in such pots in Nigeria (Bruce-Chwatt, 1957). The striking resemblance of this data with my laboratory model indicates that other factors and not fluctuation of temperatures are responsible for differences between laboratory and field data.

This work has demonstrated that data collected in the laboratory can to a certain extent be extrapolated to the natural environment of the insect. Insect data obtained from a laboratory in Durham, UK predicted, with less than 3hrs precision, emergence times of mosquitoes bred in the field in Farafenni, The Gambia. The range of temperatures observed at the breeding sites in the field are the temperatures at which *An. gambiae s.s.* thrives, making extensions of my laboratory study to this field situation possible. Although development rates can be modelled fairly well from laboratory data, larval survival cannot. It is very difficult to simulate biotic components in the laboratory but carefully controlled investigations such as the use of microcosms in an exterior environment may answer this every important question about the numbers of mosquitoes produced at breeding sites at different temperatures.

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Chapter 6

Mortality rates of adult *Anopheles gambiae sensu stricto* under controlled conditions of temperature and humidity.

Summary

To transmit malaria, the vector must outlive the extrinsic life cycle of the malaria parasite. The length of life of the vector therefore determines whether it has the potential to effect an infective bite. The climatic conditions of the environment affects the life cycle of the vector and it is important to know, especially for areas on the fringes of transmission, which conditions of temperature and humidity are suitable for disease transmission. The survival and mortality rates of adult *Anopheles gambiae sensu stricto* fed on glucose solution were monitored at combinations of temperatures from 0-45°C at 5° intervals and relative humidities of 40%, 60%, 80% and 100%, in environmental chambers. Survival was highest between 15-25°C and was lowest (less than 24hours) at 0°C and 45°C, while generally increasing at higher humidity. Females lived longer than males although the differences were mostly not significant at extreme temperatures and humidities. Maximum longevity was at 15°C and 100% humidity. Knowledge of the survival probability of the insect at the various microclimatic conditions, coupled with the effect of temperature on the sporogonic cycle of the malaria parasite can provide the basis for future process-based models of malaria transmission.

Introduction

Insects rely on environmental temperature for all metabolic life processes. Different insects are adapted to survive and reproduce at specific temperature ranges and thus have different thermal limits. Some insects can withstand sub zero temperatures mostly by super-cooling which prevents their body fluids from freezing and causing death. For instance, female *An. maculipennis* was reported to survive for months at -18°C (Maslow, 1930). Investigating cold tolerance in *Drosophila*, Karan and David (2000) concluded that the capacity of the insect to tolerate cold stress was modified in an adaptive way by the rearing temperature. They found that adults grown at the lower thermal limit were most tolerant to cold compared to those reared at the higher thermal

limit, possibly because of the greater accumulation of lipid reserves in cold conditions and the lower activity of these insects (David *et al.*, 1983).

High temperatures may denature proteins, alter cell membranes and enzyme structures and properties, and the wax complex of the cuticle and cause a rapid loss of body fluids (Roti Roti, 1982; Hepburn, 1985; Bowler, 1987). When exposed to high temperatures, insects may therefore be killed by these processes or by desiccation due to evaporation. The precise mechanisms by which insects can tolerate high temperatures are uncertain. In situations where temperatures increase gradually, exposed insects will have a greater disposition to survival at extreme temperatures; a process termed acclimation, compared with instantaneous exposure. Adult *Aedes punctor* kept for 24hrs at $10^{o}C$ were observed crawling actively at $3^{o}C$, but others kept at 24°-$28^{o}C$ for the same length of time had a still-coma at $5.5^{o}C$ (Mellanby, 1940). A behavioural feature commonly employed by certain insects is relocation to suitable microhabitats such as burrows or small crevices when ambient temperature is raised (Cloudsley-Thompson, 2001). One common physiological response to heat stress is the synthesis of heat shock proteins (hsps). These proteins, now better known as stress-induced proteins, assist in protein folding at high temperatures and act as molecular chaperones by binding to and stabilising unfolded proteins (Feder *et al.*, 1997). For most organisms, hsps are synthesised when ambient temperatures exceed the normal temperature optimum for the survival of the organism. Certain insects, such as *Cataglyphis* ants in the Sahara, can accumulate high levels of hsps before they leave their cool burrows to forage in the external heat (Gehring & Wehner, 1995). Whiteflies, *Bemisia argentifolii*, have been shown to synthesise and accumulate protective polyhydric alcohols, such as sorbitol, in response to increased temperature (Wolfe *et al.*, 1998; Salvucci *et al.*, 2000). These polyols are known solvent modifiers that protect the native structure of proteins from thermal denaturation (Back *et al.*, 1979). In white flies sorbitol has been shown to act as a thermoprotectant *in vivo*, protecting whitefly proteins from thermal denaturation (Salvucci, 2000).

Small insects are particularly limited in their ability to control the temperature of their bodies to any great extent whether by behavioural or biochemical means. The physiological processes in mosquitoes for instance are slowed down at temperatures higher than $30^{o}C$ (Trpis, 1972) with obvious modification of their metabolism. The development and survival of mosquito vectors is therefore critically dependent on

ambient temperature. The relation between temperature and survival is generally direct at moderate temperatures: the higher the temperature the shorter the life.

The small size, relatively large surface area and the tracheal system of respiration, make insects particularly susceptible to desiccation. Low moisture content of the air can affect the physiology, longevity and oviposition of many insects. The importance of humidity in the longevity of mosquito species has long been recognised. Gill (Gill, 1921) reported that at 27°C a mean relative humidity of not less than 48% was essential to the existence of *Culex fatigans* for a period of 5 days. Lower humidity resulted in premature death. He also noticed that mosquitoes did not feed when the daily mean humidity was below 40% but fed readily when it was over 50%. High survival rates at high humidity has also been reported for *An. culicifacies* (Mayne, 1930; Pal, 1943; Siddons, 1944) and for *An. atroparvus* (Lesson,1939). The amount of moisture in the air can thus act as a limiting factor in the survival and distribution of species; Forest species being more susceptible to humidity changes than those living in dry areas. Like most other insects, mosquitoes are able to survive only for short periods at low humidities, probably because of inability to control water loss through evaporation (Wigglesworth, 1939). Laboratory experiments with anopheline mosquitoes indicate that, in general, the higher the humidity, the longer the longevity of adults. Note, however, that in some selection experiments, female *Cx. pipiens fatigans* (Thomson, 1938) and *An. quadrimaculatus* (Platt *et al.*, 1957) have been found to avoid very high humidities with detrimental effects reported for *An. culicifacies* at 100% humidity (Pal, 1943; Siddons, 1944). This suggests that high humidity conditions are not always favourable.

It is very difficult to separate the influence of temperature and of humidity on mosquito longevity. Presumably there is an optimum temperature and humidity for each species of mosquito, probably related to rate of water loss. At optimum humidity, the survival of *Aedes polynesiensis* was found to be inversely proportional to temperature (Ingram, 1954) and at moderate temperatures the survival of *Ae. aegypti* was roughly inversely proportional to the relative humidity (Lewis, 1933; Bar-Zeev, 1957). However, there does not seem to be a direct relationship between longevity and relative humidity. At moderate temperatures, the survival of *Ae. aegypti* females was increased by high relative humidities but at extreme temperatures 0.5° and 40 °C survival was independent of relative humidity (Bar-Zeev, 1957). Humidity may or may not affect the thermal death point of an insect. In *An. quadrimaculatus* all females exposed to 41 °C died

within 4 minutes at low relative humidity but some survived for 13 minutes at high relative humidity (Platt *et al.*, 1957). Humidity certainly affects survival at moderate temperatures. Longevity of *An. subpictus* at 25°-35 °C increased with rising relative humidity up to 70% but at 90% longevity was reduced (Metha, 1934).

The average environmental conditions prevailing at a site are not always the conditions at which mosquitoes live. It is not uncommon for vectors to avoid extreme temperatures by resting in more favourable microclimates. It is therefore important that these microclimates be established or at least considered in the overall picture of global climate change and insect vectors (Lindsay & Birley 1996). This study looked at how temperature and humidity interact to provide the best conditions for survival of *An. gambiae s.s.*

Methods

Source of adult mosquitoes

Test mosquitoes were obtained from the 16CSS strain of *An. gambiae s.s.* maintained at 26°C ±1 °C and 80% relative humidity (see Chapter 2 for details). Briefly, eggs were hatched at 25-26°C and the larvae reared on fish food. Pupae were collected daily and allowed to hatch at 26°C in colony cages. On the second day of eclosion, adults were removed from each colony cage and used in the tests.

Materials, environmental conditions and test design

The test cages were 15cm^3 and housed in rectangular glass chambers (39cm x 20cm x 22cm) , which provided the required humidity. Each test cage held one sugar fountain with 10% glucose solution that served as the only food source for the mosquitoes. Sugar was topped daily and the lint changed weekly.

For 100% relative humidity a bowl of distilled water was placed in the glass chamber while for lower humidities, various dilutions of strong potassium hydroxide solution were used to provide the correct humidity (see Chapter 2). Humidity within each glass chamber was monitored using humidity loggers, and the solutions were replaced whenever the humidity readings fell outside (± 10%). The required temperatures were provided by the environmental chambers (Cooled Incubators, LMS Ltd, Kent, UK). They are dual temperature and illumination programmable growth chambers with an

accuracy of ±0.5°C. Each chamber was adjusted to the appropriate constant temperature and a 12:12hr light and dark regime. Temperature was monitored using loggers.

Adult mosquito mortality was investigated for 40 combinations of temperatures 0-45°C at 5° intervals and humidities 40-100% at 20% intervals. Adult mosquitoes taken from the colony cages were transferred to test cages, which were placed inside the glass chambers providing the required humidity. The glass chambers were then shelved in the growth chambers at the appropriate temperatures. For each combination of temperature and humidity, four sets of replicate tests were performed. Each replicate consisted of a total of 50 adults; 20 males and 30 females. These were monitored daily up to the death of the last individual. In total 8,000 adult mosquitoes were investigated in this study.

The number of dead mosquitoes was recorded daily and each dead individual sexed and removed from the cage. Losses due to accidents such as the tipping over of the sugar fountain in the cage or insects escaping from the cages were also recorded. During the observation, cages were removed from the environmental chamber and the top opened for about 1 minute to replenish the air in the chamber. For 0° and 5°C individuals presumed dead were placed into separate cages outside the chambers at 26°C and observed at 1 hr intervals for 5 hrs. Any individuals still alive were removed from the records and the cage they came from and replaced by a new batch of insects. This happened twice in the entire experiment and at 5°C only.

Data analysis and statistics
Adult survivorship curves were generated by the Kaplan-Meier technique in SPSS (Version 10.0 for Windows, SPSS Inc., Chicago) statistical package. Log rank statistics based on the Kaplan-Meier procedures were used to compare mortality probabilities between the different temperature regimes within each level of humidity. Non-parametric Mann-Whitney U test was used to compare mean survival times between male and female mosquitoes at all the microclimates investigated. Interaction between temperature and humidity on survival was tested using a simple factorial model after log transformation of the data. The General Linear Model (GLM) Univariate procedure and the Type III sums of squares were employed.

The mean daily expectation of life (E_x) at each combination of temperature and humidity was derived from the expression

$$E_x = T_x / n_x$$

where T_x is the cumulative sum of L_x from the day the last individual died, and n_x is the number alive at the beginning of that interval. L_x is the average number of individuals alive in each age interval. (Krebs, 1972) All calculations were performed in Microsoft Excel 2000.

The automated Fill Sparse Grid surface-fit equations and the Renka 1 algorithm in TableCurve[®]3D was used to produce a uniform surface that described the combined effect of temperature and humidity on mean mosquito survival.

All combinations of temperature and relative humidity were converted to saturation deficit and regressions were performed using expectation of life as the dependent variable to elucidate the response of the mosquito to saturation deficit. Saturation deficit (SD) in millibars was obtained from saturation vapour pressure (SVP) and vapour pressure (VP) calculations (Unwin, 1980):

$$Log_{10} SVP = 9.24349 - (2305/t) - (500/t^2) - (100000/t^3)$$

$$VP = SVP * RH / 100;$$

$$SD = SVP - VP$$

where t = temperature in $°K$ $(°C+273)$ and RH = relative humidity (%)

Various categories of temperature and humidity were tested to see the conditions under which adult mosquitoes are more responsive to saturation deficit.

Results

Temperature and humidity had a significant effect on the mortality of adult *An. gambiae s.s.* High temperatures and low humidities resulted in short lives. Adults survived for less than 24 hrs at 0 and 45°C but lived for more than 1 day at 5 and 40 °C although at these temperatures they were unable to fly and were all remained on the floor of the cage only capable of leg movements. At temperatures of 25 °C and greater, mean survival was higher at 80% humidity than at 100% humidity. For all other temperatures a linear relationship existed between mean survival and humidity (Fig. 6.1). Log rank statistics indicated significant differences in mean survival time between all temperatures when compared at each humidity except for 0 and 45°C for all humidities.
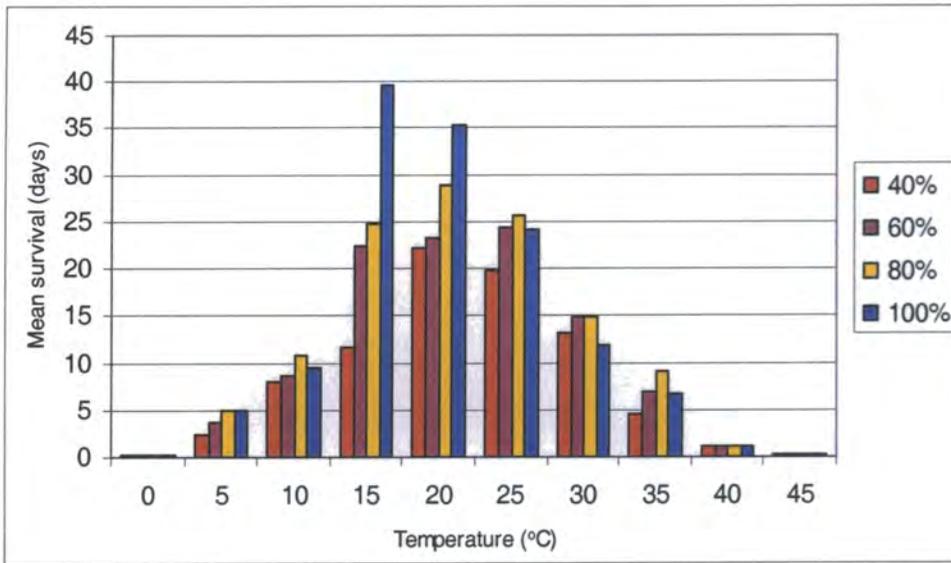
Fig. 6.1 Illustration of the effect of increasing humidity on the survival of adult *An. gambiae s.s.* at different constant temperatures.

A normal distribution of mean survival existed between the temperature ranges investigated at all % humidities. Survival was lower at both extremes and peaked towards the middle (Fig. 6.2).
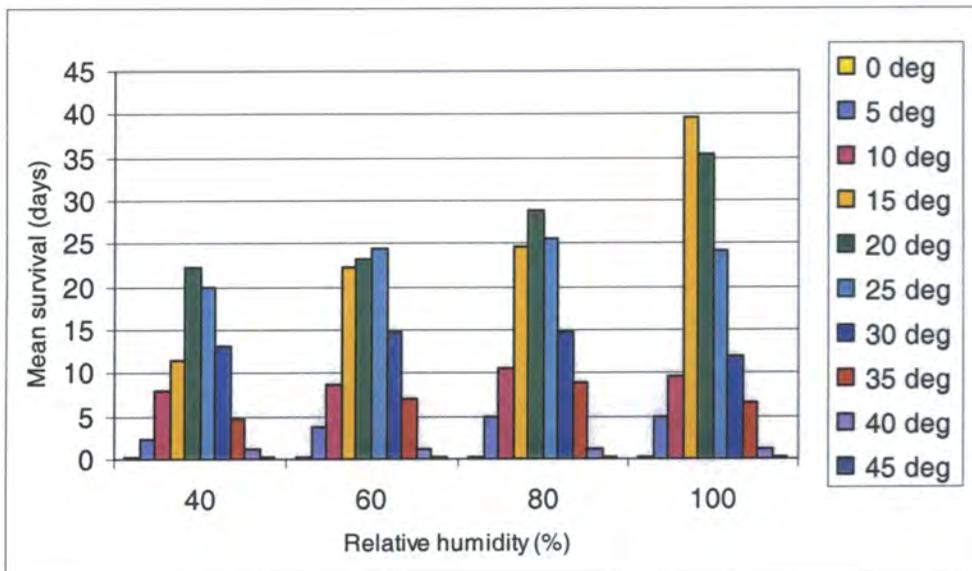
Fig. 6.2 Effect of increasing temperature on the survival of adult *An. gambiae s.s.* at various humidities.

The mean survival times according to sex is presented in Table 6.1 together with results of the Mann-Whiney U test on differences between sexes at each node of study. Females lived longer than males on most occasions. However at the temperature and humidity extremes, the difference in survival was not significant on most occasions.

Table 6.1 Longevity (mean survival time in days) of male and female *An. gambiae s.s.* at various combinations of temperature and relative humidity.

Temperature (°C)		40% RH Mean	±SE	60% RH Mean	±SE	80% RH Mean	±SE	100% RH Mean	±SE
5	Male	2.22	0.08	3.61	0.10	4.97	0.14	4.99	0.18
	Female	2.41	0.06	3.76	0.09	5.04	0.13	5.06	0.15
	P	0.063		0.192		0.760		0.757	
10	Male	8.06	0.29	8.56	0.44	10.35	0.38	9.17	0.35
	Female	7.82	0.24	8.80	0.32	10.87	0.32	9.78	0.32
	P	0.595		0.619		0.288		0.250	
15	Male	9.59	0.65	17.10	0.83	21.69	1.17	39.26	1.53
	Female	12.89	0.60	25.77	1.04	26.66	0.98	39.92	1.12
	P	<0.001		<0.001		<0.001		0.73	
20	Male	20.76	1.34	21.09	1.32	24.55	1.44	34.90	1.76
	Female	23.15	0.97	24.52	0.84	31.77	1.10	35.61	1.18
	P	0.133		0.037		<0.001		0.762	
25	Male	17.87	1.08	23.10	1.08	22.85	1.32	24.49	0.94
	Female	21.23	0.89	25.22	0.84	27.52	1.05	23.89	0.64
	P	0.015		0.16		0.003		0.516	
30	Male	13.61	0.69	13.62	0.72	13.57	0.64	10.74	0.46
	Female	12.83	0.45	15.68	0.54	15.61	0.55	12.57	0.42
	P	0.492		0.016		0.022		0.004	
35	Male	4.41	0.15	7.00	0.25	9.15	0.26	6.32	0.28
	Female	4.70	0.11	6.89	0.19	8.88	0.25	6.73	0.21
	P	0.074		0.678		0.604		0.34	
40	Male	1.09	0.03	1.06	0.03	1.15	0.04	1.11	0.04
	Female	1.12	0.03	1.06	0.02	1.20	0.04	1.15	0.03
	P	0.511		0.903		0.368		0.395	

P-values derived from the Mann-Whitney U Statistics shows the significance levels for the differences in mean survival between sexes. SE= standard error of the mean.

Age specific life expectancy of adult *An. gambiae s.s.* at the various temperatures and each relative humidity is given in Fig. 6.3. Survival increased with an increase in relative humidity. Within each level of relative humidity, mortality was influenced by age. Overall, expectation of life declined in a linear manner and older individuals had

less number of days available to them for survival. The maximum life expectancy throughout the entire investigation was 39 days at 15°C and 100% relative humidity and the least was less than 24hrs at 0°C and 45°C at all humidities. Individuals lived longer with increase in relative humidity, whilst with temperature there is an optimum above which survival decreases. The age-specific survivorship curves at the various temperatures for each relative humidity are presented in Fig. 6.4. Survival was lower at the extreme temperatures compared to the moderate temperatures. At all humidities greater than 40%, survival was extended for the temperature range of 15 -25°C.
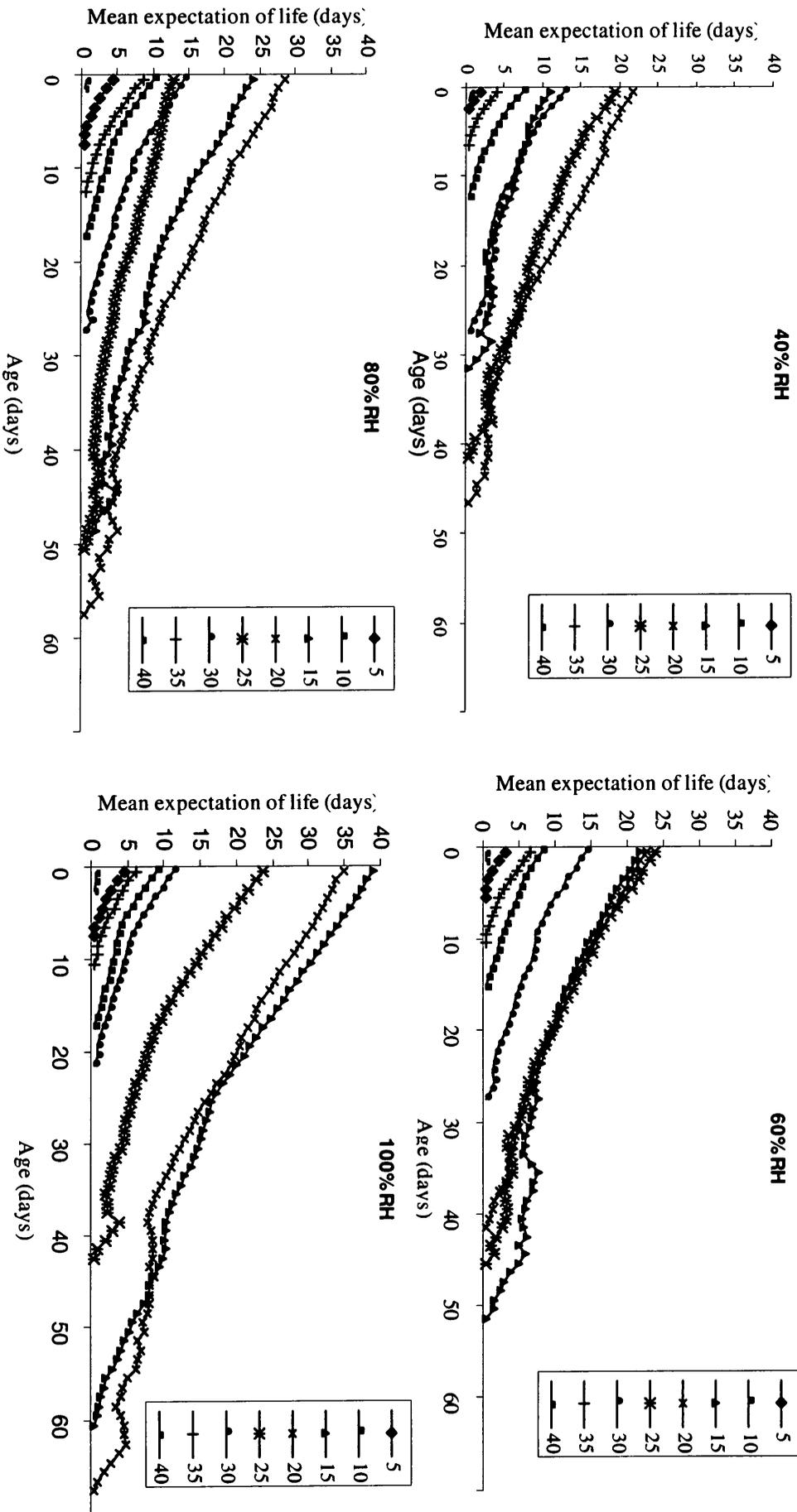
Fig. 6.3 Expectation of life of adult *An. gambiae s.s.* at different temperatures for the 4 relative humidities investigated

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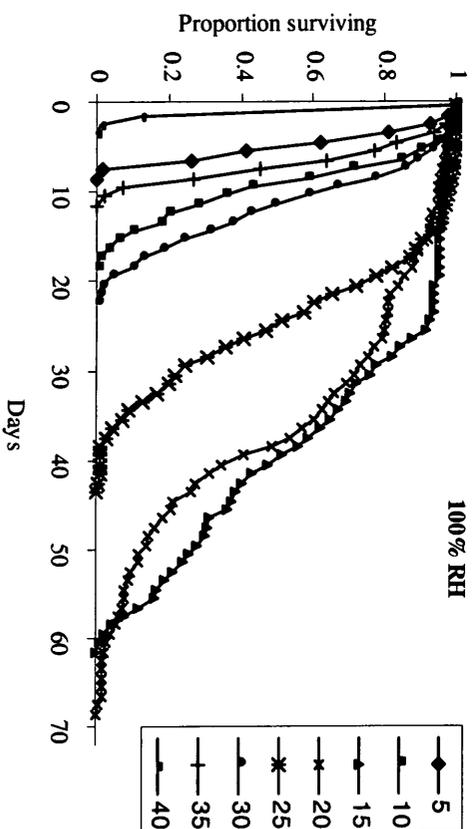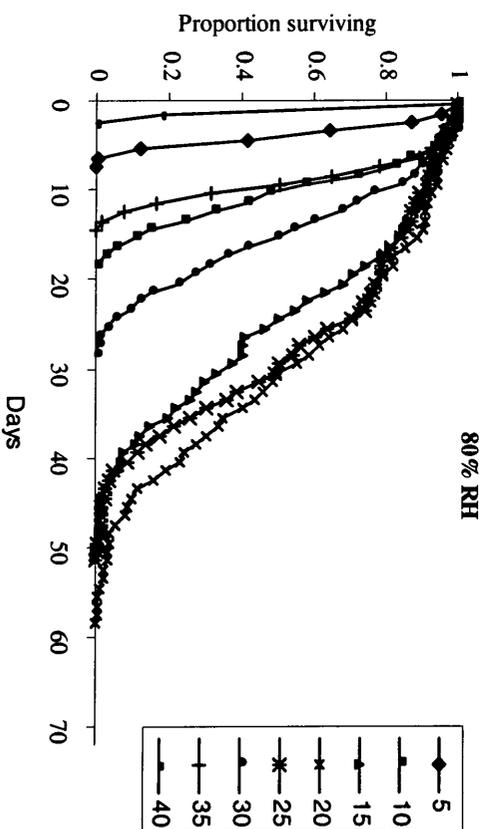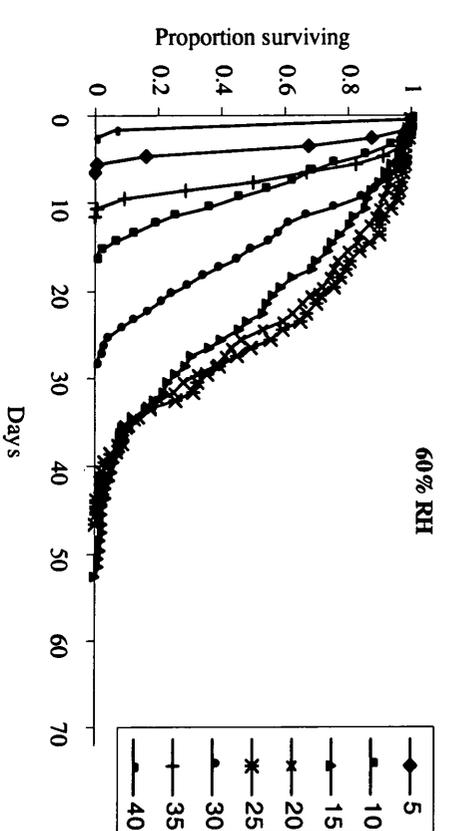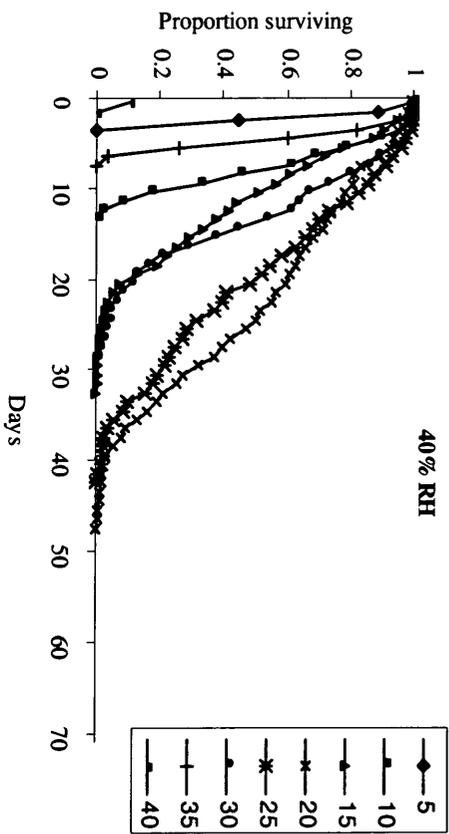
Fig. 6.4 Survivorship curves for adult *An. gambiae s.s.* at different temperatures for the 4 relative humidities investigated.

From the the GLM univariate analysis of variance for survival times by temperature, humidity and the interaction between the two it was found that both temperature (F = 3329, d.f. = 7, P < 0.001) and humidity (F = 233, d.f .= 3, P < 0.01) on their own had significant effects on survival and the interaction between the two factors (F = 34, d.f. = 21, P < 0.001) also resulted in significant effects. The intensity as shown by these statistics is in the order temperature > humidity > temperature*humidity.

The combined effect of temperature and humidity on mean adult survival is clearly described by the 3D surface presented in Fig. 6.5. Survival was maximal between 15 and 25°C and 80-100% humidity.
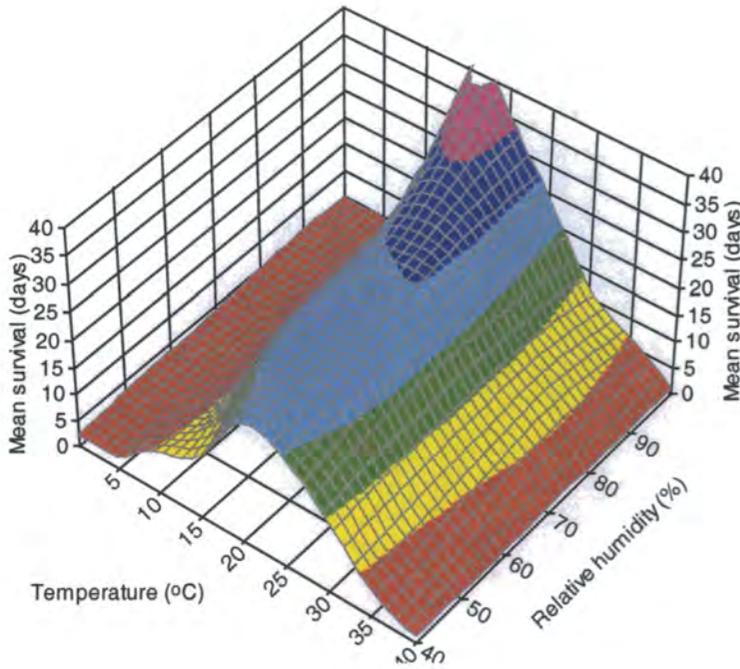
Fig. 6.5 Illustration of the combined effect of relative humidity and temperature on mean adult survival time for *An. gambiae s.s.* under controlled laboratory conditions.

There was no significant difference in the expectation of life in relation to saturated deficit, when data from all temperatures and humidities were considered ($R^2 = 0.09$, F = 3.265, P = 0.081). However, when cold temperatures (<15°C) were excluded expectation of life was negatively correlated with saturation deficit ($R^2 = 0.289$, F = 8.926, P = 0.007). In addition, the removal of data recorded at 100% relative humidity

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further improved the model fit ($R^2 = 0.495$, $F = 15.661$, $P = 0.001$). See Fig. 6.6 for trends in relationship between expectation of life and saturation deficit.
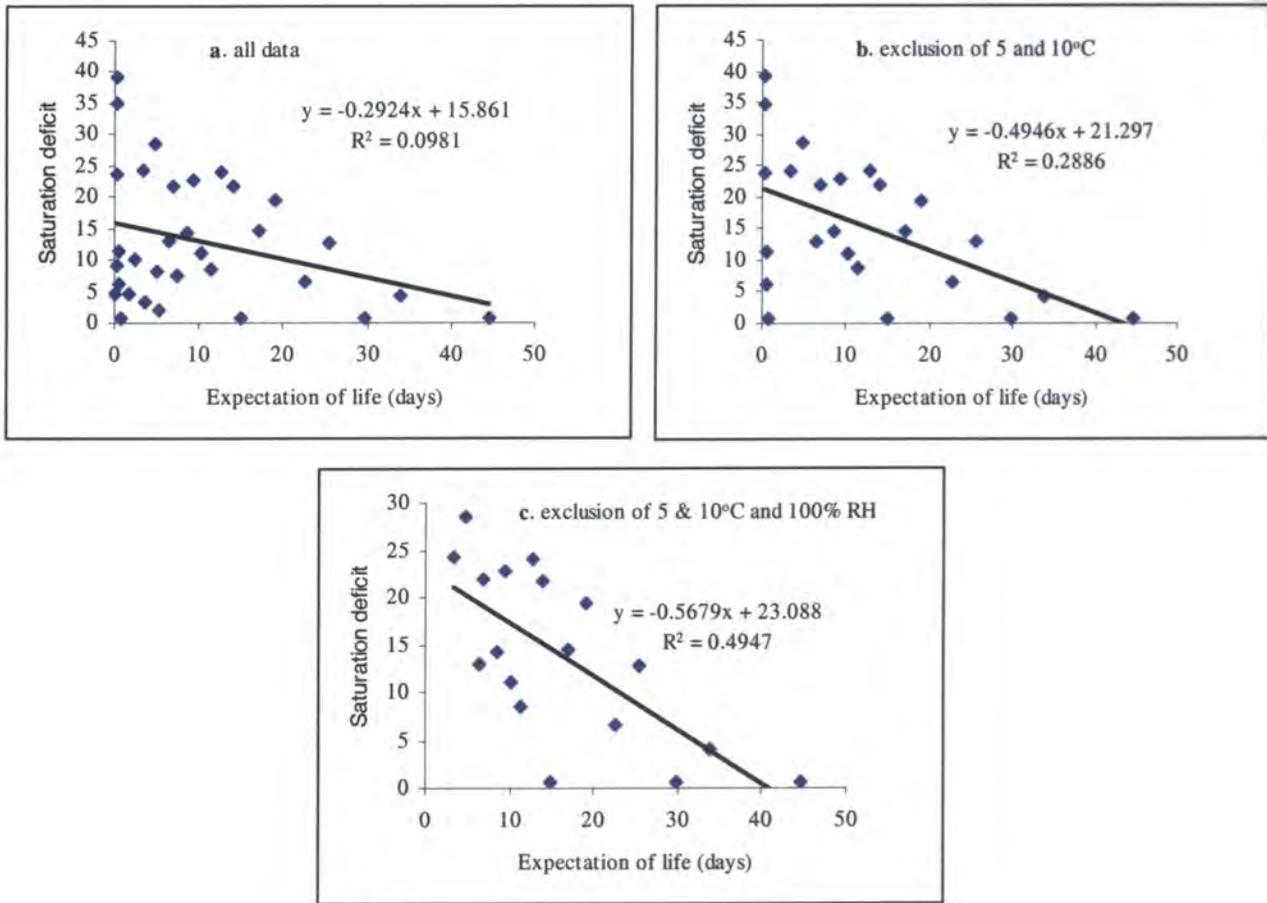
Fig. 6.6 Trends in the response of *An. gambiae s.s.* expectation of life to saturation deficit at various combinations of temperature and relative humidity. (Both the equation of the trend and the strength of the relation are given).

Discussion

Insects survive in a wide range of temperatures but most do not survive above 40-45°C (Buxton, 1933; Heinrich, 1981). The precise reason why death occurs at these high temperatures is still debated. Two important mechanisms suggested include the effects of heat on macromolecules (Roti Roti, 1982) and the damage of the cell membrane that ensues as a result of high temperatures (Bowler, 1987). As temperature increases, the pH and ion concentrations within the insects are altered, and the functioning of macromolecules such as proteins, DNA, lipids and carbohydrates and cellular structures such as cell and nuclear membranes are affected.

The insect's cuticle is also sensitive to temperature changes. High temperatures can alter the wax complex to become more fluid and may lead to desiccation (Hepburn, 1985). The effects of high temperature on insect mortality in low humidity environments may be compounded with desiccation stress (Beament, 1959). Low humidity combined with high temperature shortened the duration of life of *An. gambiae s.s.* adults. At such adverse conditions the insect may be unable to cope with the rapid rate of evaporation and consequent loss of fluids from its body. Metha (1934) working on *An. subpictus* in India reported that if loss of water was compensated by keeping the mosquitoes at higher humidities a gradual increase in longevity resulted. On the other hand, a high temperature treatment in a highly saturated environment may lead to drowning, primarily due to the loss of cuticular protection of the spiracles leading to the tracheoles (Neven, 2000). This may explain the reductions in mean survival that occurred at 100% humidity from 25-35 °C. There is also the chance of fungal growth which may be detrimental to the survival of the insects.

It has been reported previously that female mosquitoes live longer than males (Clements, 1963; Lansdowne & Hacker, 1975). A similar observation on difference in mean survival of females and males was observed here. It is important to note that since there was no blood feeding in these experiments, females could be predisposed to live longer probably by differences in nutrient accumulation between sexes during immature development, than by merely the rich protein they obtain from blood feeding.

In the field, one would expect that the life expectancy of a given mosquito population would vary with densities of parasites and predators. There is little evidence, however, that such is the case with adult mosquitoes. It seems probable that climatic factors play a far more important role in the survival of mosquitoes than do parasites or predators (Bates, 1949).

The length of life of a mosquito is important because it determines whether the individual has the potential to transmit malaria. For a species to be an efficient vector, it has to outlive the incubation period of the parasite. Thus the duration of the life of an adult mosquito is important not just for the reproduction and survival of

individuals, but also for the epidemiology and transmission of malaria. Temperature and humidity has significant effects on the activity as well as the length of life of the mosquito. *An. gambiae* thrive in humid and warm environments where in nature they could survive long enough to ensure reproduction and transmission of the disease. The survival of adults must also be considered with respect to the incubation period of the parasite at the prescribed temperature since survival of the vector alone in the short term would not be significant if the parasite is unable to survive. Temperature plays a paramount role in the speed of development of *Plasmodium* parasites (Macdonald, 1957; Detinova, 1962). The lower the temperature the slower the growth of the organisms. Each *Plasmodium* species has its minimum threshold for development. Gill reported that for transmission of *Plasmodium vivax* by *An. maculipennis*, a mean temperature of 22°C and a mean relative humidity of 50.8% was highly favourable. He stopped short of suggesting the optimum conditions of temperature and humidity for the transmission of disease but approximated the minimum requirements for disease transmission to a monthly mean temperature of 16°C and humidity of 63%. He concluded that when either of the factors fall below their critical minimum values a period of interrupted transmission occurs (Gill, 1921). The minimum temperature requirements for the complete development of the two main malaria parasites transmitted by *An. gambiae s.s., P. falciparum* and *P. vivax* has been estimated as 16° and 14.5°C respectively (Molineaux, 1990). At both temperature thresholds, the expectation of life of adult *An. gambiae s.s.* from time of emergence was less than 25 days at 40- 80% humidity and just under 40 days at 100% humidity. The duration of the sporogonic cycle at these temperatures may be over 40 days (Macdonald, 1957) meaning that at such temperatures the vector may not live long enough to be infective.

The vector should be able to locate and feed on a host if the specific microclimate is to be regarded as appropriate for the transmission of disease. At low temperatures (5-10°C), activity of the insects was restricted to limb movements. It is necessary to find out whether they do feed and their feeding frequencies at the different microclimates at which expectation of life was greater than a week. This should however not be overemphasised for endophilic feeders, as in nature what would be crucial is the ability of these insects to fly from their cold hiding places into warm homes where conditions will be suitable for feeding.

The result from this work has thrown light on the survival potential of the insect at various temperatures and humidities. Further analysis of this data with input from data on *An. gambiae s.s.* growth rate or speed of development of individuals and reproductive output at different microclimatic conditions could produce valuable information on the net population changes of the insect or the innate capacity of increase of the population under these conditions. This would immensely contribute to current process based models of the vectorial capacity of this insect and may help produce more accurate models for assessing the relationship between malaria and global climate change. Knowledge of the minimum conditions for disease transmission and the survival of adult insects have practical importance in determining the time of year and for how long transmission can occur at different places. This is significant in appropriating resources and executing disease control programmes.

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Chapter 7

Mapping distributions of chromosomal forms of *Anopheles gambiae, sensu stricto,* in West Africa using climate data[*]

Summary

The mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae), the principal vector of malaria in West Africa, comprises several chromosomal forms (e.g. Bissau, Forest, Mopti, Savanna) associated with climatic zones. That climate is an important determinant of insect distribution is shown here by using climate data to map the geographical distribution of these chromosomal forms in West Africa. The climate at 144 sites surveyed for mosquitoes in West Africa between 1971-92 was determined using computerised climate surfaces. Forest and Bissau forms occurred at relatively wet sites (median annual precipitation 1325mm and 1438mm, respectively, Inter-Quartile Ranges 1144-1858mm and 1052-1825mm), whilst the Mopti form was found at dry sites (annual 938mm, IQR 713-1047mm) and the Savanna form at sites intermediate between the wet and dry forms (annual 1067mm, IQR 916-1279). Logistic regression analyses of the climate variables were carried out on a stratified random sample of half the sites. The resulting models correctly classified over 80% of the sites for presence or absence of each chromosomal form. When these models were tested against excluded sites they were also correct at over 80% of sites. The combined data produced models that were correct at over 86% of sites. Mean annual precipitation, evapotranspiration, minimum temperature and maximum temperature were the most important climate variables correlated with the distribution of these forms. Logistic models were used to map the distribution of each chromosomal form within the reported range for *An. gambiae s.s.* in West Africa employing a geographical information system. The surfaces produced indicate that each chromosomal form favours particular climate envelopes in well-defined ecoclimatic zones, although these forms are sympatric at the edges of their ranges. This study demonstrates that climate can be used to map the distribution of chromosomal forms of insects across large areas.

Introduction

Mosquitoes of the *Anopheles gambiae* complex are amongst the most important vectors of malaria in tropical Africa (White, 1974; Coluzzi, 1984). The complex consists of at least six sibling species exhibiting varying degrees of ecological and behavioural differences and, consequently, important contrasts in their vectorial efficiency. They include *An. gambiae* Giles *sensu stricto*, *An. arabiensis* Patton, *An. quadriannulatus* Theobald; comprising two allopatric taxa one in Ethiopia and the other in southern Africa (Hunt *et al.*, 1998), *An. melas* Theobald, *An. merus* Donitz and *An. bwambae* White (Gillies & de Meillon, 1968).

Sympatry of two or more of the sibling species is a common phenomenon among members of the complex (White, 1974, 1985; Lindsay *et al.*, 1998; Coetzee *et al.*, 2000). In Senegambia, for instance, *An. arabiensis*, *An. gambiae s.s.* and *An. melas* occur together at many sites (Bryan *et al.*, 1982). By definition, however, all sibling species in the complex are apomictic. Their chromosomes show fixed paracentric inversion differences between them, as well as intraspecific inversion polymorphism (Coluzzi, 1984). *An. arabiensis* and *An. gambiae s.s.* have the most polymorphic chromosomal inversions, especially in West Africa (Coluzzi *et al.*, 1979; Petrarca *et al.*, 1983; Coluzzi *et al.*, 1985; Bryan *et al.*, 1987). This intraspecific inversion polymorphism is widespread among *An. gambiae s.s.* and *An. arabiensis*, the two most important African malaria vectors, and mainly involves the chromosome arm 2R. In *An. gambiae s.s.*, there is presence of non-random mating with partial or complete absence of interbreeding between carriers of certain inversion karyotypes (Bryan *et al.*, 1982; Touré *et al.*, 1994; Touré *et al.*, 1998). The inversions most frequently observed in this species include j, b, c, d and u, on chromosome 2R and a, on chromosome2L. The five 2R inversions associate in various combinations (Coluzzi *et al.*, 1979). The chromosomal forms identified to date have been broadly differentiated into Forest and Savanna forms. Incomplete intergradation between some non-forest populations, characterised by different inversion karyotype frequencies, has led to their recognition as named forms Bamako, Bissau, Mopti and Savanna. Where they are sympatric, these forms differentially fluctuate seasonally (Touré *et al.*, 1994; Touré *et al.*, 1998). Point maps of sites where these different forms occur together (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985) show that their inversion frequency variations correlate well with environmental clines, indicating adaptations to climatic and ecological conditions (Coluzzi *et al.*, 1979; Thomson *et al.*, 1997).

Recently it has been shown that *An. gambiae s.s.* exists as two distinct molecular forms, designated M and S (Favia *et al.*, 1997), that are not clearly concordant with the different chromosomal forms (Touré *et al.*, 1998; Della Torre *et al.*, 2001; Gentile *et al.*, 2001). Thus the chromosomal forms may not be reproductively isolated throughout West Africa (Della Torre *et al.*, 2001). Instead they are more likely to represent adaptations to particular habitats since most inversions show distinct seasonal or geographical changes in frequency or both (Della Torre *et al.*, 2001). It has previously been shown how climate, particularly differences in wetness, can be used to map the distribution of *An. arabiensis* and *An. gambiae s.s.* (Lindsay *et al.*, 1998). This work was designed to examine whether defining the climate envelope suitable for each chromosomal form based upon point samples could generate good predictions of the distributions of these forms over much larger areas.

Methods

Data sources

Information on the occurrence of chromosomal forms of *An. gambiae s.s.* at locations in West Africa was obtained from published literature. Literature was sought by initial keyword searches on BIDS and MEDLINE using '*Anopheles gambiae*' and 'chromosomal inversion' as search terms. Studies were included in the analysis if the article contained information on the prevalence of the chromosomal forms or frequency of the inversion polymorphisms, and the names of sites where the mosquito samples were collected or a map identified the geographical locations of sites. Where a study did not report the presence of chromosomal forms, presence or absence of a form was determined using the following classification scheme developed from inversion frequencies proposed by Coluzzi and colleagues (Coluzzi *et al.*, 1985). Forest (2R and 2L standard arrangements or < 10% chromosome 2 inversions), Savannah (> 20% 2Rb and 2La or 2Rcu/2Rbcu), Bissau (> 20% 2Rd and <10% 2Rb, 2La), Mopti (> 20%2Rbc or 2Rbcu) and Bamako (> 20% 2Rj and 2Rcu or 2Rbcu). For studies presenting only names of study sites, geographical locations were obtained from the National Imagery and Mapping Agency database, accessed via the GEOnet Names Server.[#]

Climate data

Latitude and longitude co-ordinates were used to obtain climate data for each selected site. A computerised environmental database, the spatial characterisation tool, SCT

[#] Web address for National Imagery and Mapping Agency database: http://www.nima.mil/gns

(Corbett & O'Brien, 1997), operating within a geographical information system (GIS, Arc/Info, GIS Version 7.2; ESRI, Redlands, CA), was used to determine the values of climate variables at each site. Estimates of mean annual monthly values for precipitation (P); potential evapotranspiration (PE); mean minimum temperature (T_{min}) and mean maximum temperature (T_{max}) were obtained for each locality

Data analysis

Data for Bissau, Forest, Mopti and Savanna forms were analysed, but Bamako was excluded because of insufficient data (n = 6). Statistical analysis was performed using SPSS software (Version 10 for Windows, SPSS Inc., Chicago). Climate variables were not normally distributed and were described by the median and inter quartile ranges. Comparisons between forms were analysed using non-parametric statistics. The complete data set was divided into two using stratified random sampling to ensure that a similar proportion of positive and negative sites for each form was included in both subsets. Half the data (72 sites) was used for constructing the pilot model, whilst the remainder (72 sites) was used for validating the model. Because of the expected sympatry between chromosomal forms simple polychotomous logistic regression was inappropriate. Binary logistic, using stepwise backward selection, for each chromosomal form was therefore employed in order to determine the combination of climate variables that characterised the presence or absence of each form. The assumption made was that a form was present at a site where the calculated probability of finding a form (see below) was ≥0.5 and absent at any site where it was <0.5 . Standard diagnostics were used to determine the quality of the models. Logistic regression equations, based on the original exploratory data set, were used to map the probability of each chromosomal form within the climatically determined species range for *An. gambiae s.s* (Lindsay *et al.*, 1998) using the GIS.

Results

Climate characteristics

A total of 171 sites where surveys had been carried out were identified from the literature. Of these, the latitude and longitude were found for 144 sites, all of which occurred in the West African region (Fig. 7.1)
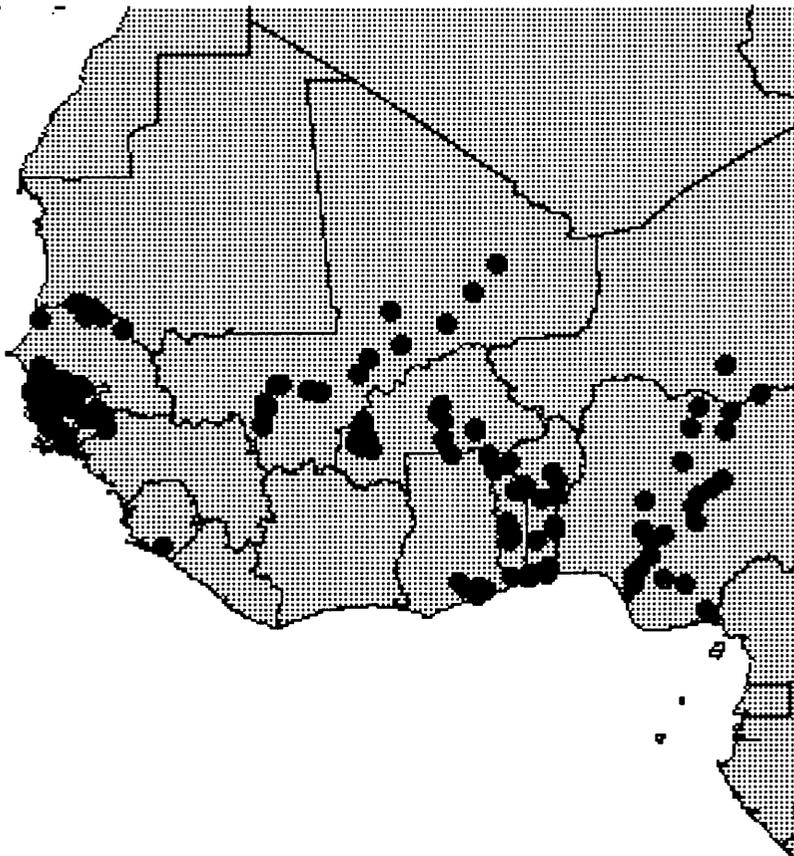
Fig.7.1 Distribution of survey sites included in the analysis

A summary of the countries, the actual number of sites for each country and the dates the surveys were carried out are provided in Table 7.1.

Table 7.1 Summary of data sources used for the analysis of sites with different chromosomal forms of *An. gambiae s.s.*

Country	No. of sites	Survey period	Reference
Benin	15	1984-90	(Akogbeto & Di Deco, 1995)
Burkina Faso	21	1984	(Petrarca *et al.*, 1986;
		1981-84	Robert *et al.*, 1989)
Ghana	8	unavailable	(Appawu *et al.*, 1994)
Guinea-Bissau	21	1981-82	(Petrarca *et al.*, 1983;
		1992	Fonseca *et al.*, 1996)
Mali	16	1981-88	(Touré *et al.*, 1994)
Nigeria	22	1971-77	(Coluzzi *et al.*, 1979)
Niger	1	1971-77	(Coluzzi *et al.*, 1979)
Senegal	18	1980-81 &	(Bryan *et al.*, 1982;
		1982	Petrarca *et al.*, 1987)
Sierra Leone	1	1990-91	(Bockarie *et al.*, 1995)
The Gambia	12	1979-81	(Bryan *et al.*, 1982)
Togo	9	1984-90	(Akogbeto & Di Deco, 1995)

The results of the univariate analysis are shown in Table 7.2. Forest and Bissau chromosomal forms were more prevalent in wetter regions, the Mopti form favoured dry and hot conditions and the Savanna form occurred between the two extremes.

Table 7.2 Comparison of climate variables between sites, with and without each of the four chromosomal forms of *An. gambiae s.s.*

		Precipitation (mm)	Evapotranspiration (mm)	Minimum temperature (^{o}C)	Maximum temperature (^{o}C)
Forest	Present	1352	1440	22.0	31.3
		(1144-1858)	(1412-1520)	(21.5-23.0)	(30.7-31.5)
	Absent	1040	1853	20.6	34.2
		(832-1251)	(1719-2059)	(20.1-21.4)	(33.0-34.8)
	p	<0.001	<0.001	<0.001	<0.001
Mopti	Present	938	2041	20.7	34.5
		(713-1047)	(1970-2312)	(20.1-21.4)	(34.1-34.8)
	Absent	1224	1663	21.0	32.8
		(966-1482)	(1486-1804)	(20.4-21.9)	(31.4-34.1)
	p	<0.001	<0.001	n.s	<0.001
Savanna	Present	1067	1769	20.6	33.5
		(916.7-1279.7)	(1621-1938)	(19.9-21.3)	(32.7-34.6)
	Absent	1116	1726	21.6	33.0
		(797-1839)	(1438-2230)	(20.8-22.2)	(31.3-34.7)
	p	n.s.	n.s.	<0.001	n.s.
Bissau	Present	1438	1723	20.6	33.0
		(1052-1825)	(1638-1771)	(19.5-21.3)	(31.5-33.6)
	Absent	1024	1847	21.0	34.2
		(850-1209)	(1497-2050)	(20.4-22.0)	(32.1-34.8)
	p	<0.001	n.s.	0.001	0.015

All the climate variables presented as medians of the mean annual monthly values for the sites, with inter-quartile range in parentheses.

Models

Four separate models representing each of the chromosomal forms were developed. The general formula for a binary logistic regression equation is:

$$\text{Probability} = 1/(1 + e^{-z})$$

where $z = \beta_{constant} + (\beta_1 \times P) + (\beta_2 \times PE) + (\beta_3 \times T_{min}) + (\beta_4 \times T_{max})$.

Thus z for the Savanna form in the final model (see Table 3)

$= 75.223 + (-0.008 \times P) + (-0.024 \times PE) + (-3.583 \times T_{min}) + (1.647 \times T_{max})$.

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Logistic regression coefficients of the explanatory variables for absence or presence of a form suggested by each model are shown in Table 7.3.

Table 7.3 Regression coefficients of climate variables included in the final logistic regression models for the distribution of the chromosomal forms of *An. gambiae s.s.*

Variable	Forest model	Mopti model	Savanna model	Bissau model
P	0.003 (0.028)	-	-0.008 (<0.001)	0.005 (<0.001)
PE	-0.021 (<0.001)	0.018 (<0.001)	-0.024 (<0.001)	-
T_{min}	-	1.053 (0.004)	-3.583 (<0.001)	-0.988 (<0.001)
T_{max}	-	-1.270 (<0.001)	1.647 (<0.001)	0.558 (0.023)
Constant	28.245 (<0.001)	-13.488 (0.021)	75.223 (<0.001)	-4.407 (0.567)

In parentheses, the significance level of the coefficient being statistically different from zero, based on the Wald Statistic

The percentage of sites correctly classified as absent or present for the different chromosomal forms in the pilot models was 94.4% for Forest, 87.5% for Mopti, 95.8% for Savanna and 84.7% for Bissau (Table 7.4). These models were based on the analysis of 72 sites from the original 144 sites. When the models were tested against the sites excluded from the analysis it accurately predicted 87.5% of Forest, 90.3% of Mopti, 80.6% of Savanna and 80.6% of Bissau. When the data were combined the models correctly predicted 87.5% of Forest, 88.9% Mopti, 87.5% of Savanna and 86.8% of Bissau. The significance levels in the models are imprecise since both the chromosomal and environmental data are likely to be spatially autocorrelated, and therefore not independent. However, at present there is not a satisfactory method for incorporating spatial dependence within logistic regression analyses (Lennon *et al.*, 2000)

Table 7.4 Results obtained from testing the distribution models for four chromosomal forms of *An. gambiae s.s.*

Form		Sites included in pilot model			Test sites for pilot model			Final model		
		No. of sites observed	Sites correctly classified		No. of sites observed	Sites correctly classified		No. of sites observed	Sites correctly classified	
			No.	%		No.	%		No.	%
Forest	Present	14	12	85.7	17	14	82.3	31	23	74.2
	Absent	58	56	96.6	55	49	89.1	113	103	91.2
	Total	72	68	**94.4**	72	63	**87.5**	144	126	**87.5**
Mopti	Present	25	20	80.0	22	16	81.8	47	38	80.9
	Absent	47	43	91.5	50	47	94.0	97	90	92.8
	Total	72	63	**87.5**	72	63	**90.3**	144	128	**88.9**
Savanna	Present	47	46	97.9	43	36	75.9	90	82	91.1
	Absent	25	23	92.0	29	20	83.7	54	44	81.5
	Total	72	69	**95.8**	72	56	**80.6**	144	126	**87.5**
Bissau	Present	23	17	73.9	20	17	70.0	43	29	67.4
	Absent	49	44	89.8	52	42	84.6	101	96	95.0
	Total	72	61	**84.7**	72	59	**80.6**	144	125	**86.8**

Mapping

Using the climate boundaries provided by the original exploratory model, probability surfaces describing the distribution of four chromosomal forms of *An gambiae s.s.* in West Africa were generated (Fig. 7.2).
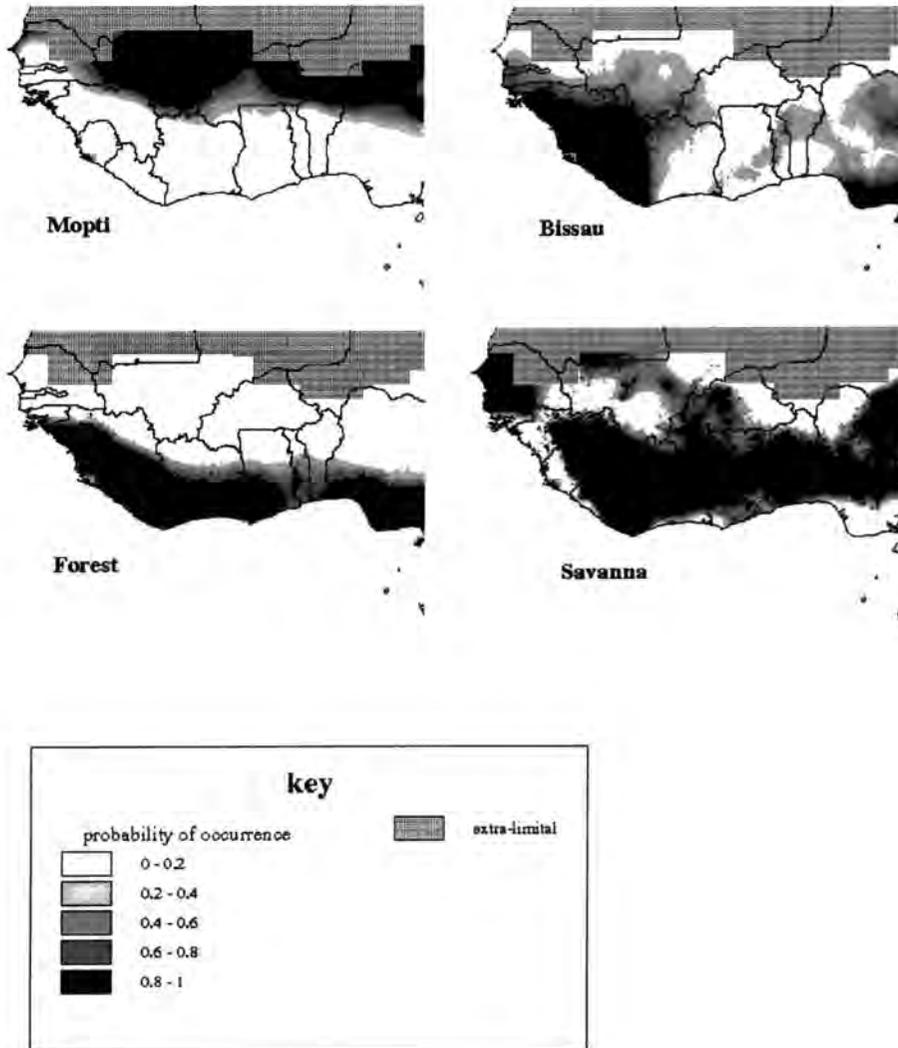
Fig. 7.2 Probability of occurrence of four chromosomal forms of *An. gambiae s.s.* mapped within the climate suitability zone for *An. gambiae s.s.* (Lindsay *et al.*, 1998)

The Mopti form is confined to the northern fringes of the Sahel, whilst the Bissau and Forest forms dominate to the west and south of the region respectively. The Savanna form, that has the widest distribution of all, dominates in the centre of the distribution of *An gambiae s.s.* .

Discussion

Climate is a first order determinant governing the distribution of insects and operates at a coarse spatial scale (Andrewartha & Birch, 1954; Sutherst *et al.*, 1995). It has previously been shown that climate data can be used to map the distribution and relative abundance of *An. gambiae s.s.* and *An. arabiensis* (Lindsay *et al.*, 1998), two of the most important vectors of malaria in the Afrotropical Region. This work is another demonstration of the utility of climate variables in mapping insect distribution.

It is impractical to produce precise distribution maps of the different chromosomal forms of *An. gambiae s.s.* across West Africa based solely on the analysis of field specimens. Reading *Anopheles* polytene chromosomes by microscopy (Coluzzi, 1968; Hunt, 1973) or molecular characterisation (Favia *et al.*, 1994) to identify the chromosomal forms are both skilled and labour intensive techniques. Here I augment studies of the distribution of chromosomal forms carried out over the past 30 years using climate data to define their differential geographical ranges. Climate variables used to generate the models were able to correctly predict the occurrence of the Bissau, Forest, Mopti and Savanna forms at more than 86% of sites. Thus the suggestion from many inversion polymorphism studies that the distribution of these genetic variants is climate related has been confirmed and exploited by this study. The distribution maps of chromosomal forms of *An. gambiae s.s* in West Africa produced confirmed that the Savanna form dominates across the Savanna belts, hence its name (Coluzzi *et al.*, 1985). To the north, Mopti has adapted to the hot and dry climate typical of the northern Sahel, associated with the seasonally flooded habitats flanking the Niger river system and artificially irrigated areas (Touré *et al.*, 1994; Touré *et al.*, 1998). The Bissau form is associated with western wet forests, whilst the Forest form is predicted more frequently in the wet southern and eastern forests of West Africa. Evidently each form has adapted to living in particular ecological

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regions, as summarised by the climate envelopes, and these distributions correspond with well-defined ecoregions for West Africa (Bailey, 1989). All chromosomal forms occur within the Humid Tropical Domain. Thus Mopti form occurs in the north of the Savanna Division, Savanna occupies the remainder of the Savanna Division, whilst Bissau and Forest forms are found in the Rainforest Division. Since within an area weather conditions change during the year, the relative abundance of the different chromosomal forms will vary accordingly as conditions change to suit one form over another. It is also likely that at the same place and time different forms are present that seek out their preferred climate envelopes in specific microclimates, thus each chromosomal form's climate preference may operate at a range of temporal and spatial scales from the micro to the macro level.

Although at least some of these chromosomal forms of *An. gambiae s.s.* are considered to represent divergent "incipient species" with very limited gene flow between them, notably Mopti versus Savanna (Chandre *et al.*, 1999; Della Torre *et al.*, 2001), however, current molecular markers M and S are not entirely concordant with their chromosomal differences, indicating variable degrees of introgression across their ranges (Black & Lanzaro, 2001). Thus the inversion karyotype differences apparently represent differential adaptations to particular ecological settings, rather than provisions of distinct mate-recognition mechanisms (Della Torre *et al.*, 2001; Gentile *et al.*, 2001).

Distribution maps of vectors are often important for understanding the epidemiology of the diseases they transmit. However, we know little about how the different chromosomal variants of *An. gambiae s.s.* may affect the epidemiology of malaria. There are indications that the 2La inversion in *An. gambiae s.s.* is associated with biting and resting indoors, perhaps in part reflecting their adaptations to drier environments (Coluzzi *et al.*, 1977; Coluzzi *et al.*, 1979; Coluzzi, 1982). In this situation strongly endophilic forms are likely to be more efficient vectors than exophilic ones since, if all else is equal, endophilic forms are more likely to feed on people, pick up malaria parasites and infect others. A study of *An. arabiensis* in southwestern Ethiopia found that DDT resistance levels were not uniform among the alternative chromosomal inversion arrangements, being associated with the 2Rb inversion (Nigatu *et al.*, 1995). Thus indoor spraying with DDT would not be as effective against individuals with this arrangement. However, the greatest significance

of the different chromosomal forms of *An. gambiae s.s* is that, together, they represent a stable vector system whereby different chromosomal forms can adapt rapidly to changes in weather and climate. Thus the overall intensity of malaria transmission and infection may change little within the region, even though the relative abundance of the different forms found there may vary appreciably in time and space. The distribution maps presented here provide a means for testing that assumption.

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Chapter 8

Conclusions

Temperature and humidity had significant effects on the development and survival of *Anopheles gambiae sensu stricto*. Between 18 - 28°C, the rate of development of the vector increased linearly with temperature. The lowest and highest developmental thresholds were 16° and 34°C respectively. Survival was highest at 20-26°C, but the fastest rate of development to adults took place at slightly higher temperatures, from 28-32°C. At these higher temperatures, most of the deaths occurred at the 4th instar, or pupal stages. It has also been observed that at high temperatures small and less fecund adults are produced (Lyimo, 1993). Thus when estimating mosquito production rates, the duration of the larval stages must be considered alongside the actual numbers developing into adults. The aquatic stages survived for extended periods, over 5 weeks, at 14° and 16°C without developing into adults. It would be appropriate to find out whether these larvae can develop into adults if temperatures are raised after such extended periods as this may have significance in highland regions in Africa that are prone to epidemics.

The relationship between laboratory-derived data and that obtained from the natural environment of the insect cannot be adequately defined. This is because the external environment contains many uncontrollable and confounding variables. Also, some of these variables, especially the biotic components, are hard to replicate in the laboratory. However, because we can control some of the factors in the laboratory, the data obtained can be cautiously applied in the field. There was a close relationship between development rates of *An. gambiae s.s.* in the field when tested with models obtained in the laboratory. Laboratory models however failed to predict the survival rates observed in the field. This can be attributed to the absence of the biotic component in my laboratory models. Predators such as dragon fly larvae, larvae of aquatic Coleoptera and aquatic species of Hemiptera as well as parasites and pathogens including fungi and microsporidia often occur at mosquito breeding sites and are among the most important causes of larval mortality at these natural sites (see Service, 1993). The effect of these organisms was not accounted for in my survival models.

Fluctuating temperatures around a mean optimum temperature of 26°C did not have a significant effect on mean development time but affected the proportion of larvae surviving to adults. The number of adults that emerged when the temperature was alternated by either ± 3°or ± 6°C were significantly lower than cycling the temperature by only 1°C around the mean. Since constant temperatures of >28 °C are not good for adult emergence, it is possible that the slightly extended hours spent at the high temperature ranges accounted for the lower adult numbers at 26 ± 3° and 26 ± 6°C. Cycling temperatures are the norm in natural breeding sites and their precise manner of occurrence could not adequately be replicated in the laboratory. This may also account for the differences in survival rates between larvae reared in the laboratory and field conditions. Temperatures observed at the breeding sites in the field were similar to those at which the vector flourished in the laboratory indicating that conclusions obtained from my laboratory study are applicable to the field situation.

High temperatures and low humidities were the most lethal conditions for adult *An. gambiae s.s.* Death at these conditions can mainly be attributed to cell death and the destruction of proteins and enzymes (Roti Roti, 1982; Bowler, 1987), as well as the alteration of the cuticle leading to desiccation (Hepburn, 1985). The best combination of temperature and humidity for the survival of the adults were 15-25°C, 80-100% Relative Humidity. High humidities were found to be lethal at high temperatures. Survival at low temperatures was complicated by the inactivity of the vector. Flight is important if the vector is to locate and feed on a potential host. Temperature and humidity significantly affected the length of life of adult female mosquitoes, and this has important consequences since it will affect reproductive potential as well as the chances of transmitting malaria. Since the incubation period of the malaria parasite in the vector is also affected by temperature (Macdonald, 1957; Detinova, 1962), this factor can be utilised in determining where or what time of year transmission may take place.

There is clearly a need to describe how changes in climatic factors may influence disease transmission. In regions that are likely to experience increased malaria as a result of climatic change, authorities would have the opportunity to take appropriate measures in advance, including stepping up awareness for reduction in the man induced factors responsible for climate change. Also knowledge of estimating current distributions over large areas will be of use to public health and disease control planners operating at international and regional levels.

Warmth and moisture affect the growth and survival of an organism (Uvarov, 1931; Andrewartha & Birch, 1954; Sutherst et al., 1995). Quantitative data on this influence has been utilised in mapping distribution of insect vectors or the disease they transmit (Sutherst, 1993; Martens, 1998; Lindsay et al., 1998; Craig et al., 1999). The use of climate and vector variables as opposed to intensive field surveys has won the attention of both control planners and those paying the bills. An. gambiae s.s. flourish within defined temperature and humidity conditions as shown by this study. Knowledge of these conditions can be employed to produce a climate envelope for their existence, which approximates to their distribution and thus distribution of the disease. The temperatures that support the development of An. gambiae s.s. obtained from the larva studies were used to map the distribution of this insect, the result of which was similar to the MARA/ARMA malaria distribution map, illustrating the relevance of laboratory derived data to actual field situations. There is a need to estimate the reproductive output of An. gambiae s.s. at different microclimatic conditions. Such data, if combined with information obtained here on growth and survival rates, would enable estimations of the innate capacity of increase of the insect population as well as the basic reproductive rate of the disease. Such estimations would enhance the construction of process-based malaria transmission models and help produce more accurate maps of the distribution of the insect in both time and space. The distribution of the chromosomal forms of An. gambiae s.s. across West Africa were defined by precipitation, evapotranspiration and temperature. The forms are adapted to well defined ecological clines (Bailey, 1989) depicted here by the predicted climate envelopes representing their distribution ranges. This work has demonstrated the use of vector parameters and climate variables in describing the distribution of insect vectors.

The temperature ranges for optimum growth and survival of An. gambiae s.s. found in this study are those that abound in most of sub-Saharan Africa. This highlights the inherent stability of malaria transmission in much of low-lying Africa. The situation in the highland regions of Africa is of concern as, although temperatures may currently not be suitable for the maintenance of An. gambiae s.s. populations, any shift in climate might pose serious consequences. Much of our current efforts are geared towards accounting for latitudinal shifts. What would happen at lower latitudes if temperatures increase further? The answer is probably not a lot, as any increased transmission is unlikely to substantially change the clinical picture. However, there is a need to expand on the response of the vector in such scenarios and predict what consequences this may

have on the distribution of the insect. Maybe temperature increase will reduce vector numbers and possibly transmission intensity. Laboratory data can be applied to field situations to a certain extent, but attributes such as vector survival would require robust field studies such as the use of aquatic microcosms to mimic biotic and abiotic factors in the natural environment of the insect.

Temperature and humidity significantly affects the growth and survival rates of *An. gambiae s.s.* The relationship between these climatic factors and the vector parameters provide a good foundation for mapping or predicting the distribution of this vector and of malaria.

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