Why does Anopheles Arabiensis predominate over An. Gambiae Sensu Stricto in hot and arid conditions?

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Why does *Anopheles arabiensis* predominate over *An. gambiae sensu stricto* in hot and arid conditions?

Matthew J Kirby

The mosquitoes *Anopheles arabiensis* and *An. gambiae s.s.* are two sibling species that are the most efficient vectors of malaria across Africa. Although they occur sympatrically over much of the continent, *An. arabiensis* predominates in hotter and drier conditions whilst *An. gambiae s.s.* is more abundant in wetter conditions. This study explored the physiological and behavioural factors responsible for the different spatial and temporal distributions of these species. In the laboratory adult *An. arabiensis* exhibit a three-fold longer survival than *An. gambiae s.s.* at high temperatures and low humidity. Moreover *An. arabiensis* is tolerant of high temperatures that are actively avoided by *An. gambiae s.s.* This behavioural response to high temperatures was also observed in adult populations of both species in the field in The Gambia. The interspecific difference observed in survival rates is not apparent in the 24 hour activity levels. Neither species could sustain periods of activity at temperatures above 30°C.

An. arabiensis loses water at a proportionately lower rate at 40°C and 30%RH, and contained a disproportionately greater reserve of accessible water, namely haemolymph, than *An. gambiae s.s.* Although heat-shocked groups of both species expressed higher levels of heat shock proteins when compared to control groups, this did not relate to improved knock-down resistance.

There were less obvious differences between the species in the larval stage. In the laboratory *An. arabiensis* larvae exhibited a greater survival to adulthood at 35°C than *An. gambiae s.s.*, when reared separately and together. However, in the field *An. arabiensis* did not dominate the hottest breeding sites in The Gambia in the dry season. Larvae of both species dived away from 40°C water surface temperatures, though for short periods only.

It is argued that a better tolerance of hot and drying resting conditions in *An. arabiensis* may result in longer-term improvements in survival rates compared to *An. gambiae s.s.*

WHY DOES *ANOPHELES ARABIENSIS* PREDOMINATE OVER *AN. GAMBIAE SENSU STRICTO* IN HOT AND ARID CONDITIONS?

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Table and Figure Legends

Declarations

None of the material contained in this thesis has been previously submitted for a degree at this or any other university. The findings of chapters III and IV have been published in modified form in the *Bulletin of Entomological Research* (2004) **94**, p 441-448

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Abbreviations

Standard S.I. units are used throughout the text. In addition the following abbreviations are used:

CI confidence interval
Hsp Heat shock protein
IQR Inter-quartile range
kdT_{50} Time (minutes) required to knockdown 50% of a cohort under specific conditions
LSHTM London School of Hygiene and Tropical Medicine
LT_{50} Time (minutes) required to kill 50% of a cohort under specific conditions
Mb Millibars
MARA Mapping Malaria Risk in Africa
MRC Medical Research Council
OR odds ratio
PCR Polymerase chain reaction
RH Relative humidity
SD Saturation deficit
SVP Saturation vapour pressure
T°C Temperature
VP Vapour pressure
WHO World Health Organization

Foreword

Malaria is a most devastating disease, threatening 40% of the world's population, causing more than 300 million acute illnesses and leading to over 1 million deaths per year (http://www.rbm.who.int.). It imposes a heavy constraint on economic and personal development: absenteeism, lost productivity, undeveloped industries and markets, together with expenditure on treatment and prevention of the disease contribute to slowed economic growth (Sachs and Malaney, 2002). From an individual point of view, loss of earnings through illness or support for sick family members, plus personal expenditures on insecticide-treated mosquito nets, transport to health facilities, doctors' fees and anti-malarial drugs, can be financially crippling. In the case of death, the indirect cost includes the discounted future lifetime earnings of those who die (http://www.rbm.who.int.). The chronic pain and suffering brought by malaria can also be emotionally unbearable.

Young children and pregnant women are most at risk from malaria. Many children who survive an episode of severe malaria may suffer impaired educational and social development as a result of learning difficulties and permanent neurological damage. Pregnant women and their unborn children are especially vulnerable; malaria is a major cause of perinatal mortality, premature delivery, low birth weight and maternal anaemia (Warrell, 1993).

Though malaria is a major public health problem in parts of Central and South America, Eastern Europe, the Middle East, South East Asia and the Western Pacific, approximately 90% of the malaria burden occurs in Africa, south of the Sahara (Sachs and Malaney, 2002). Through much of this area malaria has a high endemicity, particularly in the coastlands and low-lying regions (Gilles, 1993). Epidemics do occur but these are largely limited to the highlands of East Africa and in the periphery of malaria distribution in the south of the continent (Lindsay and Martens, 1998). In Africa malaria represents 10% of the overall disease burden and is the leading cause of mortality in those under 5 years old; anaemia and cerebral malaria cause over 500,000 child deaths each year (Snow *et al.*, 1999). A plethora of socio-economic and biological reasons lie behind this striking incidence of malaria. The parasite *Plasmodium*

falciparum, the most severe form of the disease, is responsible for most malaria infections in Africa. Resistance in this parasite to drugs such as chloroquine and sulfadoxine-pyrimethamine continues to spread across southern and eastern areas of the continent (Brasseur *et al.*, 1987; Heymann *et al.*, 1990; Wongsrichanalai *et al.*, 2002), while poor infrastructure and resources prevent effective disease management, despite heavy investment (up to 40% of public health expenditure) in malaria control by many countries. However the single most important reason is the presence of the long-lived and largely anthropophilic vector complex, *Anopheles gambiae sensu lato* (Greenwood and Mutabingwa, 2002), though the importance of *An. funestus*, which breeds year round in streams, swamps and rice fields, should not be overlooked during periods of seasonal change (Mendis *et al.*, 2000).

Over 80% of malaria deaths are caused by parasites transmitted by members of the *An. gambiae* complex, chiefly *An. gambiae sensu stricto* and *An. arabiensis*. Both mosquito species are efficient vectors of malaria although *An. gambiae s.s.* is considered the better vector as it is more strongly attracted to humans than *An. arabiensis*, which also feeds readily from cattle (Coluzzi, 1984). Although the species are sympatric, it is known that *An. arabiensis* predominates in arid environments and *An. gambiae s.s.* is more common in humid regions (Coz, 1973; Lindsay *et al.*, 1998). Mechanisms underlying these climatic preferences have up to now remained unidentified. My experiments have been designed to elucidate the behavioural and physiological reasons behind the distribution of these two species, and therefore to resolve one of the fundamental unanswered questions in medical entomology.

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CHAPTER I

Introduction:

Thermotolerance, desiccation resistance and the *Anopheles gambiae* species complex

Insects and climate

Most insects are able to exploit thermally heterogeneous microhabitats unavailable to larger animals (Casey, 1981). Insects can also be found in some of the harshest environmental conditions on the planet. Many of the insect species that exist in the presence of high temperature extremes are Diptera (Gullan and Cranston, 2000). Ephydrid, stratiomyiid and chironomid larvae are tolerant of nearly 50°C hot-water springs in Iceland, New Zealand and South America (Foote, 1995). The silverleaf whitefly *Bemisia argentifolii* undergoes peak population outbreaks in arid regions where air temperatures can exceed 45°C (Salvucci, 2000), and cactophilic *Drosophila mojavensis* survive in the Sonoran desert, Mexico, where air temperatures can reach 49°C (Gibbs *et al.*, 1997). At the other extreme, adults of the lady beetle *Harmonia axyridis* are capable of surviving temperatures as low as -5°C (Watanabe, 2002), while the antarctic springtail *Gomphiocephalus hodgsoni* can exist at –39°C in the Antarctic by biochemically controlling its supercooling point so as to avoid freezing (Sinclair and Sjursen, 2001).

Temperature and humidity are the most important environmental factors influencing the physiology, and therefore the distribution of insects (Wigglesworth, 1972). These two elements are closely interrelated and as such their individual effects are often difficult to distinguish. The putative mechanisms by which insects tolerate temperature extremes and desiccating conditions (summarised in fig1.1) are dealt with in this chapter.

Insects, thermoregulation and thermotolerance

Most insects are unable to maintain a constant internal temperature independent of fluctuations in surrounding environmental conditions. This is known as poikilothermy. In the absence of mechanisms of temperature control, insects must develop methods of

tolerating unfavourable temperatures. However most insects do have some degree of control over their body temperature. Such methods of thermoregulation can be either physiological or behavioural.

Physiological thermoregulation

Physiological mechanisms of thermoregulation involve either the control of heat production or the control of heat loss (Wigglesworth, 1972). Larger insects such as moths, bees and beetles are capable of maintaining a constant (and slightly higher than ambient) thoracic temperature. Heinrich (1975) demonstrated that bumblebees are capable of maintaining a thoracic temperature of 38°C over an ambient temperature range of 2-36°C. This physiological regulation of body heat, known as endothermy, is usually associated with the production and dissipation of heat generated by large flight muscles. As a consequence wing beat frequency and wing loading may affect the amount of heat generated by flight and the resultant thoracic temperature (Kammer, 1981). Variation in metabolic heat production during flight is the major mechanism of thermoregulation in the anthophorid bee *Centris pallida* (Roberts *et al.*, 1998). Other energetic activities may generate enough heat to maintain high body temperature. The African dung beetle *Kheper nigroaeneus* maintains an elevated thoracic temperature whilst constructing and rolling dung balls (Ybarrondo and Heinrich, 1996).

Insulation, body shape and heat circulation influence rates of heat loss. Thoracic heat is well maintained in insects with insulating scales, hairs or pile. In order to lose heat, bumble-bees (*Bombus spp*) increase their heartbeat to improve haemolymph circulation to the more poorly insulated abdomen. The head, legs and wings may serve as additional sites for radiative heat loss (Bishop and Armbruster, 1999). Evaporative cooling is used to avoid lethal overheating. Mechanisms of evaporative cooling include changes in ventillatory patterns and tight spiracular regulation. Such respiratory control is exhibited by the tsetse fly *Glossina morsitans* (Edney and Barrass, 1962), and the desert grasshopper *Calliptamus barbarus* (Roxburgh *et al.*, 1996).

Wingless insects and those of a smaller size are likely to have less physiological control over their internal body heat. There is a heat management disadvantage to being small; the physiology of these insects is not adapted to generate or conserve enough heat from activity (Gullan and Cranston, 2000). However smaller insects are able to rapidly increase body temperature through exposure to even the slightest external heat source. Such insects are more likely to depend on behavioural thermoregulation.

Behavioural thermoregulation

Behavioural regulation of body heat is known as ectothermy, and is widely used by insects. Behavioural mechanisms all revolve around aspect adoption, the extremes of which are basking (to increase internal temperatures) and shade seeking (to decrease internal temperatures). The posture and orientation to the sun adopted by a diurnal insect directly influences the uptake of heat from the environment. When body temperature is low, dragonflies, cicadas and butterflies are known to orient the long axis of the body so as to maximise radiative heat gain through increased surface area exposure (Casey, 1981). Orientation to wind velocity is also known to occur in some insects (Waloff, 1963). Winged insects fold and unfold the wings over the body to restrict or increase heat gain respectively (Mazer and Appel, 2001), while the setae of some caterpillars are arranged to enhance radiant heat uptake whilst also insulating against convective heat loss (Gullan and Cranston, 2000). Arid-zone ant genera such as *Cataglyphis* and *Ocymyrmex,* which live in the Sahara and Namib deserts respectively, exploit the buffering effect of the desert sands by burrowing in stable temperatures a few centimetres below the exposed surface (Johnson, 2000). Desert ants are also fast movers and good navigators, minimising time spent foraging away from the burrow (Johnson, 2000). Even the whitefly *B. argentifolii*, which exhibits a strong physiological tolerance of heat, uses shade-seeking behaviour. They are mainly found feeding on lower surfaces of leaves in a microenvironment of higher humidity and lower heat than the surrounding air (Salvucci *et al.*, 2000).

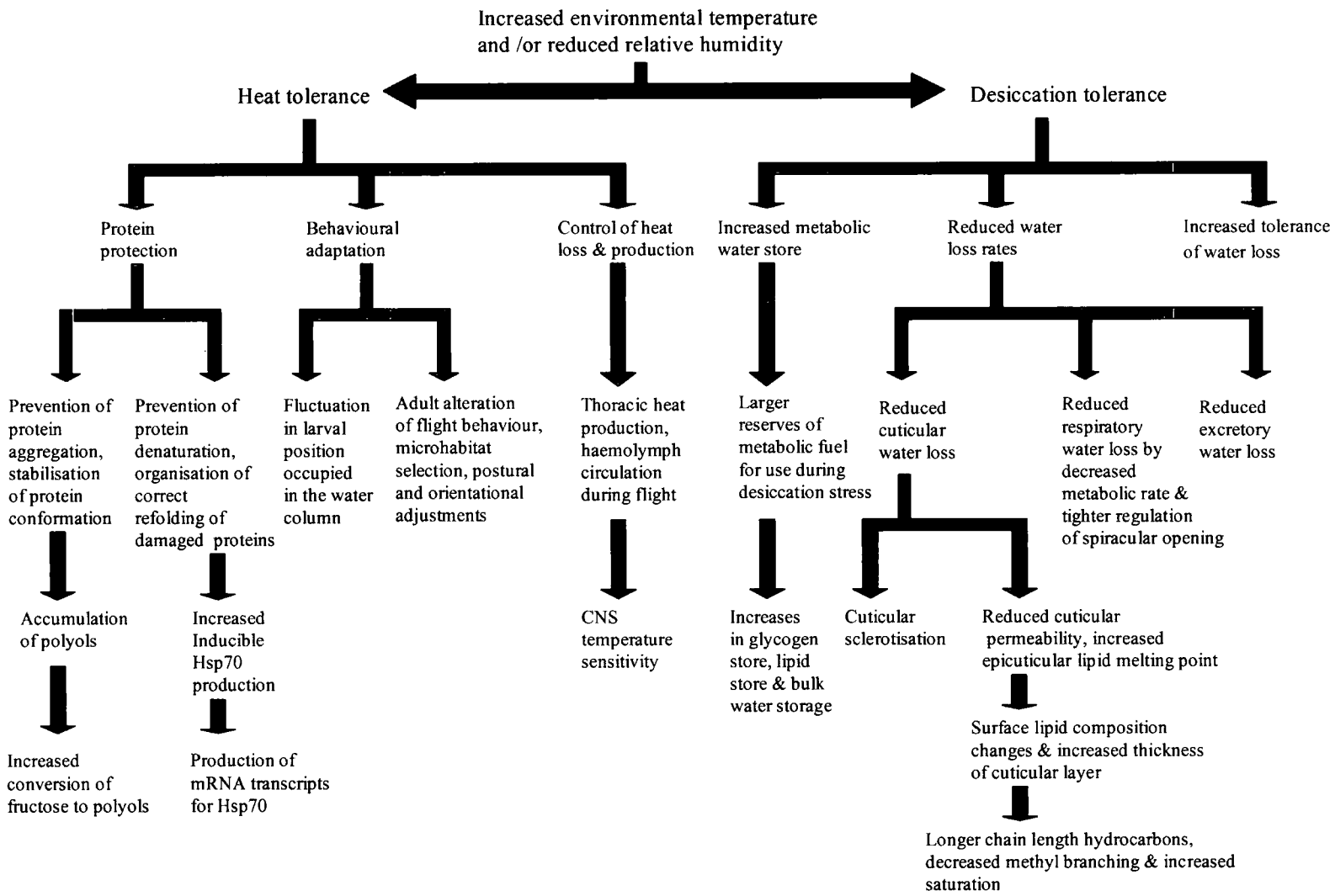

Fig 1.1 Putative mechanisms of tolerance of environmental stressors in insects

Thermotolerance

Thermotolerance in insects varies across and within life stages (Dahlgaard and Loeschcke, 1997). Wild-type strains of *D. melanogaster* develop a higher level of thermotolerance during the first 12 hours of embryogenesis than at any other time (Welte *et al.*, 1993 in (Feder *et al.*, 1996). Krebs and Bettencourt (1999) argue that fully grown larvae, young pupae and young adults have most to gain in fitness terms from increased thermotolerance as these stages show low investment in division or enlargement of cells. Higher selection intensities for heat tolerance demonstrated in the embryos and pupae of *D. buzzatii* may reflect the lack of behavioural and physiological mechanisms of internal temperature regulation (Dahlgaard and Loeschcke, 1997).

The cellular stress response has been described in nearly all studied organisms (Iwama *et al.*, 1999). The heat shock proteins (Hsps) are a common feature of this response. These can be constitutively expressed, constitutively expressed but increasing during stress or exclusively inducible (Feder and Hofmann, 1999). The Hsp70 group are some of the most important stress-related inducible proteins found in animals, and their expression in Dipteran insects is often high (Krebs and Bettencourt, 1999). Their most important function is to minimise the aggregation of non-native proteins and target aggregated proteins for degradation and removal from the cell; in fact most Hsp70 expression appears to correlate closely with stress-induced denaturation of other proteins (Feder and Hofmann, 1999). Several Hsp groups respond to a variety of stresses, most notably temperature extremes (Krebs and Feder, 1997; Lansing *et al.*, 2000), cellular energy depletion, toxic waste products (Fry, 2001) and nutritional limitations (Sorensen and Loeschcke, 2001). All these stresses may be experienced in crowded larval conditions (Schneider *et al.*, 2000).

Both natural (Krebs and Feder, 1997) and engineered (Feder *et al.*, 1996) Hsp70 variation correlates well with intrapopulation variation in *D. melanogaster* larval tolerance of temperature stress. This and other similar evidence from larval and adult studies (Dahlgaard *et al.*, 1998; Lansing *et al.*, 2000) appears to support a causal relationship between Hsp70 expression after exposure to heat and increased thermotolerance in natural

populations. Importantly it has been shown that induced Hsp70 expression in larvae can lead to increased longevity and stress resistance in the resulting adults (Sorensen and Loeschcke, 2001).

However, increased thermotolerance can occur independently of Hsp expression. Zatsepina *et al.* (2001) found that an exceptionally thermotolerant strain of *D. melanogaster*, originating from a semi-arid region of Chad, exhibited slower and lower Hsp70 and Hsp68 expression than a less tolerant strain. Similarly Sorensen *et al.* (2001) demonstrated a down-regulation of Hsp70 in a low altitude (warmer) population of *D. buzzatii* compared with high expression in a conspecific population at higher altitude (cooler). The low altitude population was more knock-down resistant at stressful but sub-lethal temperatures, yet had a lower survival after exposure to a potentially lethal heat shock temperature. It therefore seems logical that species from high-temperature climates can become thermally acclimated and not rely on inducible Hsps. Acclimation leading to enhanced thermotolerance can occur within an individual's lifetime or evolve over generations (Huey and Kingsolver, 1993). Genetic differences in thermal tolerance were detected between the *D. buzzatti* populations described above (Sorensen *et al.*, 2001). Acclimated species might have higher levels of constitutive Hsps than related species occupying cooler environments (Zatsepina *et al.*, 2001), but they are unlikely to rely on inducible Hsps because of associated costs. Hsp70 expression is strongly repressed in the absence of heat shock (Feder and Krebs, 1998) because it can result in restricted larval growth, lower survival to adulthood (Krebs and Bettencourt, 1999) and reduced fecundity (Krebs and Loeschcke, 1994; Hoffmann, 1995).

There is also evidence that Hsp induction does not always improve heat shock tolerance. The induction of Hsps at 40°C in both fed and unfed whiteflies could not explain the substantially improved survival of the fed group after heat shock at 46°C (Salvucci *et al.*, 2000). Inducible Hsps are therefore only one of several molecular mechanisms that result in thermotolerance (Feder and Hofmann, 1999) and are probably not an underlying mechanism of acclimation or basal thermotolerance.

Metabolic arrest and the synthesis of osmotic stress protectants such as polyols and trehalose are probably the most well known other potential stress tolerance mechanisms. Diapause, and the equivalent summer hibernation, aestivation, are important physiological survival techniques under cold and heat stress respectively. Depression of metabolism is the most obvious characteristic in these states of existence. In the grasshopper *Melanoplus differentialis* respiration occurs in diapausing eggs at 25% of the rate exhibited by developing eggs of the same age (Wigglesworth, 1972). A similarly low oxygen consumption has been measured in the Mediterranean tiger moth *Cymbalophora pudica* (Kostal *et al.*, 1998). Prior to the onset of dormancy in both diapause and aestivation, increased accumulation of lipids, polyols such as glycerol, sorbitol and myo-inositol, and low molecular weight sugars such as glucose (in the form of glycogen) and trehalose is often observed in insects (Clements, 1992). Female *Aedes taeniorhynchus* and *Ae. aegypti* have increased optimal survival and speed of flight when fed on carbohydrates which supported the accumulation of glycogen (Nayar and Sauerman, 1971). Fungi and bacteria are known to accumulate trehalose after exposure to a heat stress (Ribeiro *et al.*, 1997) where it plays an important role in the stabilisation of protein structure. Trehalose and other sugars may have a similar function in insects. The synthesis of polyols is thought to reduce the potential melting and supercooling of body fluids. By acting as cryoprotectants, these alcohols may have facilitated insect colonisation of colder climates (Pullin, 1996).

The relationship between biochemical stockpiling and thermotolerance does not always exist. *C. pudica* increases its percentage body content of lipids and glycogen up to 6-fold before undergoing obligatory prepupal aestivation during the unfavourably hot summer period (Kostal *et al.*, 1998). However neither of these components seem to be associated with increased thermotolerance; glycogen was used up as a metabolic fuel while the lipids were conserved for larval-pupal metamorphosis. Additionally an increase in cold hardiness in the final instar larvae after cold acclimation was not associated with a build up of polyols. The accumulation of protective biochemicals is not limited to the juvenile life stages of insects. Salvucci (2000) found that whiteflies, *B. argentifolii*, fed on cotton leaves at 40°C accrued sorbitol at a concentration 20 times higher than those at 25°C. Those at the higher temperature also contained slightly higher levels of trehalose. The author also

demonstrated *in vitro* that sorbitol prevents the heat inactivation of sucrase and glucokinase and also delays heat-induced aggregation of soluble whitefly proteins. Another polyol, mannitol, appears to increase the thermotolerance of adult aphids in a similar manner (Hendrix and Salvucci, 1998).

A role for radical scavengers (such as superoxide dismutase, cytochrome P450 and the glutathione system) in inducible stress tolerance has also been suggested (Feder and Hofmann, 1999). Glutathione S-transferase has been shown to protect cells from toxins and oxidative stress in the spruce budworm *Choristoneura fumiferana* (Feng *et al.*, 2001), but as yet no links with thermotolerance have been discovered.

Insects, humidity and water loss

Desiccation is a critical stress for insects living in arid zones, and thus the water vapour content of air is an important environmental factor for insects (Uvarov, 1931). It can be measured as vapour pressure divided by that of saturated air, and as such is commonly expressed in terms of relative humidity. Significantly the vapour content of air is dependent on temperature because saturation vapour pressure is a function of temperature (Unwin and Corbet, 1991).

The significance of the large surface to volume ratio of most insects is not limited to temperature regulation. Oxygen-demanding insect activities require a large surface area to support an extensive tracheal system, in order that gaseous exchange with the environment occurs efficiently (Unwin and Corbet, 1991). Disadvantageously this increases the likelihood that body water content will equilibriate with the water content of the surrounding air. Water loss is especially significant in Lepidoptera, due to their high level of diurnal activity and their elevated surface area to volume ratio compared to other insects (Mazer and Appel, 2001). However, just like temperature, insects exercise a certain amount of control over their body water content through physiological and behavioural mechanisms.

Physiological water regulation

The greatest water loss by most insects occurs as transpiration through the cuticle (Gibbs, 1998). To minimise this passive movement of water between their body fluids and the environment, most insects have developed an external covering of low permeability. This ubiquitous outer layer is the epicuticle; it consists of an inner homogeneous layer perforated by wax filaments from which the external wax layer (and sometimes a further 'cement' layer) is secreted (Noble-Nesbitt, 1991). The water-repelling nature of the epicuticle derives from the presence of hydrophobic lipids such as *n*-alkanes and *n*-alkenes, internally branched monomethylakanes, dimethylalkanes and wax esters (Renobales *et al.*, 1991).

Just like thermotolerance, water loss rate and therefore desiccation resistance varies with age (Gibbs and Markow, 2001) and life stage (Yoder *et al.*, 1997). Populations of *D. melanogaster* selected for postponed ageing and desiccation resistance exhibited consistently lower rates of water loss than controls (Nghiem *et al.*, 2000). Both groups had similar initial percentage water content and water content at death. Haematophagous insects and arthropods have a particular problem with water balance when processing a bloodmeal. The elimination of water helps to concentrate the bloodmeal and allows for more blood to be consumed (Clements, 1992). The loss of water from feeding Lone Star Ticks (*Amblyomma americanum*) is much more rapid than when off the host (Yoder *et al.*, 1997). Post-feeding, a three-fold boost in surface wax deposition and subsequent extreme water retention was observed. Several authors have claimed that the waterproofing quality of the epicuticular layers is the single most important adaptation enabling the success of insects on land (Gullan and Cranston, 2000, Noble-Nesbitt, 1991). Yet apparent adaptive changes in cuticular lipids do not always result in reduced water loss rates. Significant increases in hydrocarbon chain length and surface lipid quantity with age had no effect on water loss rates in *D. mojavensis* (Gibbs *et al.*, 1997). In species occupying warmer, drier habitats the positive effects of longer chain-length hydrocarbons on surface lipid melting temperature are often negated by internal methyl branching and saturation changes (Gibbs and Pomonis, 1995). Thus other water regulatory mechanisms may also be important in preventing water loss. Avenues of non-cuticular water loss are the mouth, anus and

spiracles. In *D. melanogaster* selected for resistance to desiccation, approximately 25% of evaporative water loss is respiratory (Williams and Bradley, 1998). Many insects are known to open and close their spiracles in a periodic fashion; this discontinuous gas exchange cycle may reduce respiratory water loss. When dehydrated the tenebrionid beetle *Pimelia grandis* releases CO_2 in discrete bursts but when given access to food and water the beetles show a form of continuous CO_2 release demonstrating that some insects can control water loss when stressed (Duncan *et al.*, 2002). However in *D. melanogaster* periodic spiracular CO_2 release was not associated with reduced spiracular H_2O release (Williams *et al.*, 1998).

Aside from prevention of water loss, initial total body water content and tolerance of water loss have been identified as two of the most important factors preventing or limiting the effects of desiccation (Gibbs *et al.*, 1997). The percentage contribution of water to total body weight ranges from less than 50% to more than 90% (Wigglesworth, 1972). Total body water content may be affected by the water content of the food consumed, the ability to store water in various forms, and the physiological state of the insect (Mazer and Appel, 2001). The water content of ingested food meets the water demand of most insects, though for phytophagous insects this itself is often dependent on precipitation and evaporation affecting the quantity of water available from host plants (Uvarov, 1931).

Ae. aegypti adults are more resistant to desiccation than two smaller species *Ae. albopictus* and *Ae. paullusi* (Mogi *et al.*, 1996). However in intraspecific studies there was little evidence of a relationship between wing length and survival under desiccation stress. Glycogen and lipid content per unit body weight were higher in *Ae. aegypti* and the most desiccation-resistant strain of *Ae. albopictus* suggesting an involvement of metabolic water storage in the prevention of water loss (Sawabe and Mogi, 1999). Accumulation of glycogen also occurs in the eggs of the silk moth *Bombyx mori* before entering diapause, a life stage requiring protective mechanisms against desiccation (Chino, 1958).

As an explanation of desiccation resistance, tolerance of water loss should not be overlooked. The eggs of *Aedes* (*Stegomyia*) mosquitoes are tolerant of desiccation, with

some capable of surviving dry conditions for several months (Sota and Mogi, 1992). Tree hole and container breeding species are especially vulnerable to desiccation during overwintering periods. The eggs of *Ae. albopictus* survive longer than others at low humidity, possibly a result of thicker chorions and waxy coatings and related to adaptation to their habitat selection: open containers in disturbed areas experience particularly high evaporation rates (Sota and Mogi, 1992). Diapausing eggs survive longer at low humidities than non-diapausing eggs possibly because of their low metabolism, yet *Ae. aegypti* resists desiccation but has no diapause. In adult *Ae. aegypti* and *Ae. albopictus*, urban strains appear more resistant to desiccation than conspecific rural strains (Mogi *et al.*, 1996). High desiccation resistance is necessary for diurnal activity and for dispersal through urban regions where water may be scarce. Access to water will vary with climate, vegetation type and human activity and thus the degree of adult desiccation resistance could influence the distribution of mosquito species.

The Anopheles gambiae complex and climate

A species complex is a group of genetically distinct but morphologically cryptic species (known as sibling species) which may vary in their distribution and behaviour (Coetzee *et al.*, 2000). The *An. gambiae* complex consists of at least seven species: *An. gambiae s.s.*, *An. arabiensis*, *An. bwambae*, *An. melas*, *An. merus*, *An. quadriannulatus* (A), and an unnamed species (*An. quadriannulatus* B) recently identified in Ethiopia (Hunt *et al.*, 1998). Identification of each of the sibling species has been achieved through the application of several methods, including cross-mating techniques (Hunt *et al.*, 1998), chromosomal banding arrangements (Coluzzi *et al.*, 1979), gas-chromatography of cuticular hydrocarbons (Milligan *et al.*, 1993) and PCR (Scott *et al.*, 1993).

The MARA (Mapping Malaria Risk in Africa) database (www.mara.org.za) is a regularly updated source for recorded collections of members of the *Anopheles gambiae* species complex that accurately describes their distribution. *An. bwambae* is restricted to humid forest foothills within a 10km radius of the geothermal springs at Mongiro in Bwamba County, Bundibugyo District, Uganda (Harbach *et al.*, 1997), where it exists sympatrically with *An. gambiae s.s.* and *An. arabiensis*, and breeds in warm saline spring waters. As a

vector of human malaria it is probably of local importance (White, 1973). *An. melas* is limited in its distribution to the brackish water conditions of mangrove swamps in West Africa (White, 1974). Populations peak in the rainy season when flooding and rainfall combine with tidal sea-water to create ideal breeding conditions (Gillies and DeMeillon, 1968). In the dry season there may also be a population explosion 7-11 days after the spring tide (Muirhead-Thompson, 1951). It is more abundant in animal shelters than houses (Bryan *et al.*, 1987) reflecting its preference for feeding from cattle and other domestic animals. However where it occurs in isolation from the other members of the *An. gambiae* complex, *An. melas* may be an important malaria vector, especially if humans are the only available host species. *An. merus* is also a saltwater breeder, limited in distribution to brackish lagoons and swamps in Kenya, Mauritius, Mozambique, Somalia and Tanzania in East Africa (Gillies and DeMeillon, 1968). *An. merus* is probably more zoophilic and exophilic than *An. melas* (Coluzzi, 1984); both species are more zoophilic than *An. bwambae* (Costantini *et al.*, 1999).

An. quadriannulatus occurs in three foci characterised by low annual rainfall – Zanzibar and southern Africa where it is strongly exophilic, and at high altitudes in Ethiopia where it is more endophilic; in all locations it is markedly zoophagic and as such of little importance to malaria transmission (White, 1974). Recently identified as separate species, *An. quadriannulatus* (A) from South Africa and *An. quadriannulatus* B from Ethiopia have homosequential banding patterns and thus have only been distinguished by crossing experiments between the two. The crosses give rise to sterile males, a marked sex ratio distortion, and extensive asynapsis in the ovarian polytene chromosomes of hybrid females common to interspecific crosses within the complex (Hunt *et al.*, 1998). *An. melas*, *An. merus*, *An. bwambae* and *An. quadriannulatus* are mutually allopatric but are frequently sympatric with *An. gambiae s.s.* and/or *An. arabiensis* (Coluzzi, 1984).

An. gambiae s.s. and *An. arabiensis* are the most widely distributed members of the complex (Coluzzi *et al.*, 1979; Rogers *et al.*, 2002). Over extensive areas of Africa they occur in sympatry (Coetzee *et al.*, 2000), and in some areas such as Kisumu, Kenya the two vectors are the only representatives of the complex (Petrarca *et al.*, 1991). *An. arabiensis* is

the more widespread (White, 1974; Coetzee *et al.*, 2000; Rogers *et al.*, 2002). It is endemic in Afrotropical regions, in the drier savanna areas and steppes, including parts of the Arabian peninsula (Coluzzi *et al.*, 1979), and it sometimes occurs as isolated populations in urban centres (Trape *et al.*, 1992). By contrast, *An. gambiae s.s.* is more common in humid savanna and forest zones although its distribution does extend into some arid savanna regions (Faye *et al.*, 1997). For example in West Africa, *An. gambiae s.s.* prevails across a wide range of climatic zones, from Sahel to forest areas (Petrarca *et al.*, 1987). The ability of both of these vectors to exploit such a range of conditions is associated with adaptive chromosomal forms resulting from fixed and polymorphic paracentric inversions, largely on chromosome-2 (Coluzzi *et al.*, 1985). Some of the inversion karyotypes have distinct geographic variation in distribution and frequency related to climatic conditions and vegetation zones (Coluzzi *et al.*, 1979). In fact climate variables have been used successfully to predict the distribution of the *An. gambiae s.s.* chromosomal forms Mopti, Bissau, Forest and Savanna in West Africa, an area of high inversion polymorphism (Bayoh *et al.*, 2001). Mopti is associated with dry season irrigation and is limited to the upper Niger river basin (Touré *et al.*, 1994), Bissau is found in wetter forest areas in the west, the Forest form is found in the wet forests of the south and east (Bayoh *et al.*, 2001) while the Savanna form persists across the savanna belts (Coluzzi *et al.*, 1985). Another form, Bamako, has been identified in the Sudan savanna, though in drier seasons it is inferior to Mopti (Touré *et al.*, 1998). Where there is overlap in their distribution e.g. in Mali, the chromosomal forms exhibit different seasonal fluctuations (Touré *et al.*, 1998).

In Sudan *An. gambiae s.s.* populations have low inversion polymorphism and resemble intergrading savanna/forest forms; there is no evidence of the arid-adapted 2Rbc chromosomal variant Mopti which perhaps explains the absence of *An. gambiae s.s.* from all areas of Sudan except the more humid south (Petrarca *et al.*, 2000). In contrast *An. arabiensis* is found in almost all ecological situations in Sudan and exhibits high inversion polymorphism, incorporating novel polymorphisms as well as inversion systems recorded in West Africa (Petrarca *et al.*, 2000). *An. arabiensis* resembles *An. gambiae s.s.* in West Africa (Coluzzi *et al.*, 1985); there is a high frequency of inversion polymorphism which may reflect the well-defined ecoclimatic zones in the west (Bayoh *et al.*, 2001). West

African populations of *An. arabiensis* and forms of *An. gambiae s.s.* with similar climatic preferences, e.g. *An. arabiensis* of the Sudano-Sahelian belt and Mopti in arids regions of Mali (Touré *et al.*, 1994), often also share inverted chromosome arrangements (Coluzzi *et al.*, 1979). This may have resulted from genetic transfer between the two by introgressive hybridisation (Coluzzi, 1982). In contrast there is little chromosomal variation in *An. arabiensis* in the rest of Africa (Petrarca *et al.*, 2000). The chromosomal constitution of *An. arabiensis* in Madagascar most closely resembles populations in East Africa; both are more zoophilic and exophilic than *An. arabiensis* from West Africa (Rallisoa Randrianasolo and Coluzzi, 1987).

The chromosomal forms of both vectors may in fact represent different species; in *An. gambiae s.s.* there is evidence of complete absence of interbreeding between particular inversion karyotypes (Bryan *et al.*, 1982). Gene flow is especially limited between Mopti and Savanna forms (Della Torre *et al.*, 2001), and Mopti and Bamako forms (Coluzzi, 1984). It is possible that these forms represent divergent incipient species (Coluzzi *et al.*, 1985). The development of a PCR-based method to distinguish between the chromosomal forms should facilitate the resolution of this issue (Favia *et al.*, 1994).

Just as climatic parameters can be used to predict the distribution of the chromosomal forms of *An. gambiae s.s.*, they can also be used to map the range and relative abundance of *An. gambiae s.s.* and *An. arabiensis* on a wider scale. Annual precipitation and temperature data were used by Lindsay *et al.* (1998) to generate a predictive model based on an index of saturation. Where five-monthly maximum precipitation exceeds five-monthly maximum potential evapotranspiration, and therefore the air is moist, *An. gambiae s.s.* is the common species. When the relationship is reversed and thus the air is dry, *An. arabiensis* dominates. More specifically *An. arabiensis* is predominant in regions which experience a low average annual precipitation of less than 500mm (Rishikesh *et al.*, 1985; Petrarca *et al.*, 2000). In Matola, a coastal suburb of Maputo, Mozambique, which experiences an annual rainfall of 450-650mm, *An. arabiensis* is the only member of the *An. gambiae* complex present (Mendis *et al.*, 2000). Here it was found to breed all year round in swamps dominated by reed-grass, though peak population densities occurred early in the rainy season when

numerous rainwater pools developed. In northern and western Sudan, which receives very little or no rain, only *An. arabiensis* occurs, breeding in wells and irrigation ditches (Dukeen and Omer, 1986; Petrarca *et al.*, 2000). In similarly dry conditions in the Rift Valley in Ethiopia commonly only *An. arabiensis* can be found, breeding in peri-domestic situations where drainage is disturbed (Mekuria *et al.*, 1982; Ye-Ebiyo *et al.*, 2000). In The Gambia *An. arabiensis* is rarely found in coastal regions where *An. gambiae s.s.* prevails, but is more common inland in the dry season, and dominant only in the northernmost extremes of the country (Bryan *et al.*, 1987).

However *An. arabiensis* does not always require environmental conditions of high temperatures and/or low humidity to dominate indoor resting samples. In the Senegal River basin, where *An. gambiae s.s.* and *An. arabiensis* are sympatric, the latter is found in large numbers only in coastal areas characterised by lower mean annual temperatures and higher mean humidities than inland areas (Petrarca *et al.*, 1987). Interestingly *An. arabiensis* breeds throughout the dry season in the coastal regions while the breeding of *An. gambiae s.s.*, the prevailing inland species, is strictly seasonal occurring only during the rainy season. This is despite the creation of breeding sites by irrigation during the dry season. Climate has such an impact on the relative abundance of these two species in the river basin that in 1990, a wet and humid year, *An. gambiae s.s.* dominated indoor spray catches whilst *An. arabiensis* was the more common species in 1991, a significantly more arid year (Faye *et al.*, 1997). Thus it can be seen that *An. arabiensis* and *An. gambiae s.s.* differ in their temporal distribution as well as their spatial distribution. In the Kisumu area of Western Kenya there are significant and progressive temporal differences in the relative percentage of *An. gambiae s.s.* caught resting indoors compared with *An. arabiensis* (Petrarca *et al.*, 1991). Initial sampling in September revealed a 50:50 ratio between the two species but by late November *An. gambiae s.s.* represented 92% of the total catch. This increase correlates with an increase in night temperatures and a decrease in overall temperature fluctuation, perhaps indicating that *An. arabiensis* is more tolerant of microclimatic instability (Petrarca *et al.*, 1991). In Saruja, The Gambia, there are two annual peaks in mosquito numbers: in the hot dry season, characterised by maximum temperatures in excess of 40°C and relative humidity as low as 20%, *An. arabiensis* predominates over *An. gambiae s.s.*, but in the wet

season when relative humidity rarely drops below 60% and maximum temperatures do not reach 35°C, the situation is reversed (Lindsay *et al.*, 1991). Similarly in Segera, Tanzania, where both species are well represented, indoor densities of *An. arabiensis* rise sharply coinciding with the start of the rains, but *An. gambiae s.s.* gradually replaces *An. arabiensis* as the dominant species as the rainy season progresses reaching a ratio of 50:1 at the end of the cool long rainy season (White *et al.*, 1972). *An. arabiensis* was also found to dominate when day-time humidities were below 60%RH and during the months of highest maximum temperatures (White *et al.*, 1972). In the dry savanna zones of Mali and Burkina Faso where both species are found, mosquito numbers also show marked seasonal dynamics (Taylor *et al.*, 1993). Population densities are extremely low until the start of the rainy season when numbers dramatically increase. Both species are largely dependent on the creation of temporary rain-filled pools, though in irrigated areas population densities remain high for a longer period of the year around seepage pools and drainage ditches (Petrarca *et al.*, 2000). *An. arabiensis* and the Mopti chromosomal form of *An. gambiae s.s.* have been identified in even drier desert regions, though their presence here is dependent on a close association with river systems (Touré *et al.*, 1994).

There is limited evidence to suggest that *An. arabiensis* is capable of surviving throughout the dry season in arid regions as adults, perhaps with physiological alterations such as gonotrophic dissociation (Taylor *et al.*, 1993) and behavioural adaptations such as seeking out shaded spots in dry wells and rodent burrows (Omer and Cloudsley-Thomson, 1968). This may explain situations where *An. arabiensis* is the first species to take advantage of the rains in the early rainy season but is then later out-competed by *An. gambiae s.s.*, such as in Western Kenya (Petrarca *et al.*, 1991) and Tanzania (White *et al.*, 1972).

Understanding the evolutionary relationship between the members of the *An. gambiae* complex, let alone the relationship between *An. gambiae s.s.* and *An. arabiensis*, is a difficult process because the evolutionary history of the complex has been short (Besansky *et al.*, 1994). Chromosomal relationships (particularly species-specific inversions on the X chromosome) and marked zoophily suggest *An. quadriannulatus* as a likely ancestral link between *An. gambiae s.s.* and *An. arabiensis*, which have been placed on distant branches

of the phylogenetic tree (Pape, 1992). If this is the case then the relationship with man and habitat exhibited by both species is probably a result of evolutionary convergence (Coluzzi *et al.*, 1979). However the chromosomal phylogeny could be misguiding because, as has already been suggested, the paracentric inversions on which it is based may have passed between species by introgressive hybridisation. Hybridisation and therefore incomplete reproductive isolation of the species has been demonstrated both in the laboratory and in nature (Coluzzi, 1982).

Because of the behavioural and ecological similarities between *An. gambiae s.s.* and *An. arabiensis,* (as well as between *An. merus* and *An. melas*) it would seem more parsimonious to suggest that these are sister taxa. Recent molecular phylogenies based on rDNA, mtDNA and an esterase gene of five taxa of the complex support these suggestions, with *An. quadriannulatus* more closely associated with the more zoophilic *An. merus-An. melas* clade (Besansky *et al.*, 1994). There appears to be no geographic structure to the mtDNA variation within *An. gambiae s.s.* and *An. arabiensis* (Besansky *et al.*, 1997), unlike that underlying inversion polymorphism. That there is more inversion polymorphism in both species in West than East Africa might suggest that the divergence of *An. arabiensis* from *An. gambiae s.s.* is a more recent event there (Petrarca *et al.*, 2000), though this could equally be a response to a harsher or more variable environment. The information taken from both techniques may reflect a rapid general range expansion of both species across much of the African continent followed by recent localised adaptation events. It is tempting to link the former to human activities. The evolution of settled communities and compact villages (and thus an increase in human density) occurred within the last 10,000 years, suggesting that the anthropophilic and endophilic behaviour of both species also developed during this time period (Costantini *et al.*, 1999).

But the question remains - when did *Anopheles gambiae* and *An. arabiensis* diverge? *An. arabiensis* is the more diverse in behaviour and this added to its wider distribution across Africa would suggest it has had longer to evolve and adapt than *An. gambiae s.s.* In addition, several populations of *An. arabiensis* that exist outside the range of *An. gambiae s.s.* and feed from large ungulates may represent an early form of *An. arabiensis* that

predates the expansion of both species. The other species of the complex also generally feed from ungulates, particularly bovids, suggesting this is the more primitive feeding habit of *Anopheles* in Africa. Alternatively it seems equally possible that *An. gambiae s.s.* is the ancestral species and *An. arabiensis* represents an arid-adapted form of *An. gambiae s.s.*.

Adaptation to climatic extremes in An. gambiae s.s. and An. arabiensis

Possible survival strategies that allow the persistence of *An. arabiensis* through the dry season have already been identified e.g. laying eggs on damp surfaces, delayed egg hatching and reproductive quiescence, in which a single gonotrophic cycle is experienced over the whole period (Taylor *et al.*, 1993). However some of these strategies have also been demonstrated for *An. gambiae s.s.*. Minakawa *et al.* (2001) found that *An. gambiae s.s.* exhibited no oviposition preference between moist soil and open water and laid similar numbers of eggs on each. In addition, viable eggs of *An. gambiae s.s.* have been found in soil samples prior to the onset of the rainy season in Western Kenya (Beier *et al.*, 1990). To determine why *An. arabiensis* out-competes *An. gambiae s.s.* in drier and hotter conditions, clear and consistent physiological and behavioural differences between the two species must be identified.

There are consistent differences between the cuticular hydrocarbon composition of adult *An. arabiensis* and *An. gambiae s.s.* (Hamilton and Service, 1983), between Gambian, Nigerian and Tanzanian strains of *An. gambiae s.s.* (Anyanwu *et al.*, 2000) and between the different chromosomal forms of *An. gambiae s.s.* (Milligan *et al.*, 1993). Hydrocarbon differences are also detectable between individual larvae of the two species, though in this case distinction relies on the relative concentrations of the hydrocarbons rather than mere presence or absence (Anyanwu *et al.*, 1994). Extraction of cuticular hydrocarbons in hexane or light petroleum followed by injection onto a gas-liquid chromatography (GLC) capillary column produces a range of peaks corresponding to C15 through C44 hydrocarbons (Milligan *et al.*, 1993). The hydrocarbon peaks selected for the recognition of *An. gambiae s.s.* strains are not those used to distinguish between the two species and this may reflect strain interrelatedness. Two strains from Nigeria are harder to separate from each other than from Tanzanian or Gambian strains (Anyanwu *et al.*, 2000) and it is

tempting to relate this to environmental selection on the waterproofing qualities of the hydrocarbons. However although there is geographical variation in hydrocarbon profiles, sympatric forms are usually more distinguishable than allopatric forms (Milligan *et al.*, 1993). Therefore this variation may not be the result of adaptive waterproofing under different environmental conditions (unless the species respond to the environment in opposite ways) and may in fact be part of the mate recognition system in locations where sibling species coexist (Howard and Blomquist, 1982). The relationship between water loss and cuticular structure for *An. arabiensis* and *An. gambiae s.s.* has not been examined.

Do these mosquitoes exhibit differences in their behavioural avoidance of heat and desiccation stress? Differences in exposure to stresses may have led to the development of alternative strategies for resistance in the two vectors. By retreating to cooler, fairly constant microhabitats *An. gambiae s.s.* adults may have developed less physiological capacity to cope with long periods of exposure to stress. In contrast *An. arabiensis* may have evolved physiological strategies (e.g. high water retention, tolerance of water loss, improved control of heat loss or other unidentified mechanisms) as a result of long term exposure to environmental extremes. If this is the case then it should be more competitive under such conditions. Therefore survival and competition between the two species need to be assessed under a range of conditions. Any differences in survival rates must then be related to differences in the mechanisms of protein protection and water conservation in these mosquitoes, hitherto unexplored areas.

Hypothesis

The larval and adult life stages of *An. arabiensis* have behavioural and physiological adaptations that allow for out-competition of *An. gambiae s.s.* in hot and dry conditions.

Research objectives

To determine if:

1. Adult survival of *An. arabiensis* is greater than that of *An. gambiae s.s.* at high temperatures and low humidity (chapter III);
2. Adults of both species actively avoid exposure to high temperatures (chapter IV);

3. Flight activity of *An. arabiensis* is greater at higher temperatures than that of *An. gambiae s.s.* (chapter V);
4. *An. arabiensis* larvae out-compete those of *An. gambiae s.s.* when reared separately and together at high water temperatures (chapter VI);
5. Larvae avoid high surface water temperatures by diving to cooler water (chapter VII);
6. Temperature can predict the spatial distribution of adult and larval *An. gambiae s.s.* and *An. arabiensis* during the dry season in The Gambia (chapter VIII);
7. Adults of *An. arabiensis* have a greater ability to resist water loss and tolerate desiccation than *An. gambiae s.s.* (chapter IX);
8. Adults of *An. arabiensis* express higher levels of Hsp70 than *An. gambiae s.s.* when thermally stressed (chapter X).

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CHAPTER II

Methods for maintenance of *Anopheles gambiae sensu stricto* and *Anopheles arabiensis* under laboratory conditions

Abstract

In order to supply sufficient numbers of each mosquito life stage for experimentation, it was necessary to maintain *Anopheles gambiae sensu stricto* and *An. arabiensis* in an insectary. The rearing procedures outlined in this chapter were rigorously followed throughout the course of the experiments, ensuring reproducibility of mosquito size and physiological condition. Apart from their blood feeding, both species were treated in an identical fashion. Protocols for wing length measurement and the PCR identification of the species are given as these were standard practices followed in several of the experiments.

Introduction

Maintaining mosquito populations in a laboratory situation is extremely important to the mosquito research community. In order to successfully manage mosquito populations in the wild, knowledge of physiology, genetics and behaviour must be acquired from laboratory studies. Mosquitoes have been colonised for the purpose of species identification (Davidson, 1964), assessment of vectorial capacity (Blandin *et al.*, 2004) and vector-parasite interactions (Krishnamoorthy *et al.*, 2004), and as subjects for control technique evaluation (Rey *et al.*, 2004; Zahiri *et al.*, 2004). Complete control over breeding populations has allowed the creation of trait-specific iso-female lines to assay the mechanisms of insecticide resistance (Brogdon *et al.*, 1999) and parasite refractoriness (Olson *et al.*, 2002; Kim *et al.*, 2004). The mass production of genetic and field strains in laboratories is essential to the success of genetic control techniques such as population replacement of present vectors with genetically-engineered benign mosquitoes (Gerberg *et al.*, 1994).

A lack of uniformity of methods and the absence of a standardised food for larvae are possibly the two most important causes of high variability in laboratory-maintained mosquito populations (Asahina, 1964). A high production of mosquitoes with similar size and physiological condition was required for the experiments described in this

thesis. As such the following method was devised and followed. Larval rearing media were assessed for their ability to produce consistently a high percentage of pupae from first instar stages.

An. gambiae s.s. and *An. arabiensis* belong to a group of morphologically indistinguishable yet genetically and behaviourally distinct mosquito species. Genetically they differ significantly in the intergenic spacer (IGS) regions of the ribosomal DNA. The rDNA genes are present in hundreds of tandem copies per cell nucleus; they are highly conserved yet consistently different between closely related species (Paskewitz and Collins, 1990). The rDNA PCR technique described here has a very high specificity (~100%) for all tested members of the *An. gambiae* complex throughout their distribution, and has good sensitivity using relatively small amounts of DNA generated from either an extraction process or from triturated tissue (Scott *et al.*, 1993). The PCR method is the most suitable species-identification technique for mass diagnostic use as it is compatible with a number of preservation techniques and efficient in diagnosing all life stages (Paskewitz *et al.*, 1993), as well as being less time consuming and less labour-intensive than polytene chromosome analysis and isoenzyme diagnosis.

Wing length is an appropriate measure of mosquito body weight and size (Briegel, 1990). In this thesis the effect of mosquito size on survival at high temperature and low humidity (chapter III), temperature avoidance behaviour (IV), flight activity (V) and water loss rates (IX) are explored. In chapter IX weight is measured directly but in all other cases wing length is used to estimate mosquito weight. A standard procedure presented here was adopted for measuring wing length.

Materials and Methods

The insectary

All the laboratory experiments reported in this document used mosquitoes raised in the insectary of the School of Biological and Biomedical Sciences, University of Durham. The insectary consisted of a preparation room and an anteroom leading to two windowless culture rooms, one of which was used for mosquito stock maintenance and the other for experiments. The stock room contained extensive shelving units for cages and larval rearing bowls to rest on. It was maintained at 26 ± 2°C and 30 ± 10% relative

humidity (RH) on a 12:12 light/dark cycle. The experimental room contained five programmable LMS cooled incubators 45 x 30 x 100cm (S.H. Scientific, Kent, UK). Temperature and illumination were controlled within the chambers to examine insect responses to a range of conditions. Space was also available in the room for other experimental equipment to be set up.

The mosquitoes

The Durham colonies of both species were established in 2001 from eggs from the London School of Hygiene and Tropical Medicine. The London colony of *Anopheles gambiae sensu stricto* KWA was established in 1975 from eggs collected in the field at Kwale, 35km north of Tanga, Tanzania. *An. arabiensis* KGB was colonised in London in 1998. That population was raised from a colony held at the Medical Research Council's laboratory, Cape Town, South Africa, and was originally derived from wild material from the Kanyema Zambesi Valley and maintained in Harare, Zimbabwe.

Adult maintenance

The adults of both species were confined in separate $30cm^3$ aluminium-wire frame cages covered with fine nylon of mesh size $1mm^2$. These were fitted with a 25cm long mesh sleeve attached to a 15cm opening, allowing easy access to the investigator while limiting escape of mosquitoes. 50% ± 10%RH was maintained in the cages by placing a water-soaked sponge in a petri dish on top of the cage, and by putting a plastic cover over the cage. Water was added to the petri dish on a daily basis. The bottom of each cage was lined with white paper to facilitate the periodic removal of dead adults, and between generations the cages were sanitised using boiling water. Adults used in experiments were removed from the cages with a simple sucking-type aspirator.

Both sexes of both species require a source of carbohydrates. A 10% glucose solution was supplied *ad libitum* on a cotton lint wick feeder based on the design of Porter *et al.* (1961). It consisted of a 75mm long x 25mm diameter tube lined with the lint and filled with glucose solution, inverted into a 50mm long x 35mm diameter holding tube. Additional glucose was then added to the lint until saturation. The lint was washed in distilled water and replaced regularly to prevent sugar fermentation and fungal growth.

Most female mosquitoes require a bloodmeal for vitellogenesis (Clements, 1963). Though the use of vertebrate animals as hosts for adult mosquitoes is still a common practice (Zerpa *et al.*, 1998), many species of mosquitoes will feed on warmed blood through a membrane stretched over a holding tube (Fritz *et al.*, 1989; Gerberg *et al.*, 1994). In the insectary both *An. gambiae s.s.* and *An. arabiensis* were initially offered defibrinated horse blood (Oxoid, Basingstoke, UK) through a Parafilm (Parafilm American National Can™, Chicago, USA) membrane using the Hemotek Membrane Feeding System (Discovery Workshops, Accrington, UK). Blood stored at -5°C was shaken gently then pipetted into the feeding pot (35mm diameter, 5mm deep). Parafilm was stretched over the top of the feeder and secured with a rubber ring; the feeder was then screwed into the heating device. 5 minutes were allowed for the blood to warm to 37°C and then the feeder was placed on top of the cage so that the membrane was in contact with the mesh of the cage. Although most *An. gambiae s.s.* females were successfully fed on horse blood, very few *An. arabiensis* females took a bloodmeal and as a result egg production was low. *An. arabiensis* fed more successfully on defibrinated sheep blood (Oxoid, Basingstoke, U.K.) but the eggs produced were of low viability, and so this species was subsequently fed from the author's arm. Female *An. arabiensis* were attracted to feed by lightly exhaling air at the cage entrance. Once several females were airborne, the right forearm was pushed into the cage. Mosquito feeding was limited to one area of the arm from the wrist to the upper forearm. The tunnel entrance of the cage was tied around the upper arm to prevent the escape of mosquitoes feeding near the cage entrance. Many more *An. arabiensis* females took a bloodmeal from the author's arm than fed from the artificial system, egg production was greater and egg viability higher. Females of both species were offered a blood meal on the day after emergence, and once in every three days after that. 10-15 minutes were allowed for feeding in all situations.

Mating and egg laying

Often newly-established colonies of both *An. gambiae s.s.* and *An. arabiensis* do not mate successfully under laboratory conditions, and the creation of an artificial dusk is necessary to increase insemination rates (Marchand, 1985). Though neither species were directly observed mating in the insectary, the relatively high production of viable eggs rendered a simulated crepuscular period unnecessary.

Mosquitoes usually lay their eggs on the surface of water bodies or on nearby surfaces subject to inundation. A small oviposition bowl 70mm diameter x 60mm deep was added to each of the cages after each blood-feeding session. The bowls were lined with Whatman No.1 filter paper to provide a surface for the eggs to be deposited on, and then filled to a depth of 50mm with dechlorinated water. In accordance with Gerberg *et al.* (1994) the container was removed every 24 hours if eggs were deposited, covered, and the larvae left to hatch. This normally happened within two days. During this period additional water was added to the bowl if necessary, and on the third day a drop of Liquifry No.2 (Interpet Ltd, Surrey, UK) was added to provide food for the newly hatched larvae. Once the larvae were 1-2 days old they were transferred into 300mm diameter x 130mm deep polythene rearing bowls.

Larval Rearing

De-chlorinated water plus Liquifry was chosen as the larval rearing medium. Four drops of Liquifry in two litres of de-chlorinated water was added to all larval rearing bowls used. The bowls were covered with netting weighed down with beads. This prevented oviposition of stray adults and exit of any emerging adults not collected as pupae.
A fixed feeding schedule was determined and followed, and the type and amount of food administered to the larvae was consistent. The larvae were fed approximately 15mg of ground fish food daily.

Pupal collection

Pupae were collected daily from the rearing bowls using a large-bore pipette and added to plastic cups 50mm diameter x 60mm deep filled with dechlorinated water. These cups were placed into the appropriate cages under zinc mesh funnels. The cone-shaped funnels were 210mm high with a 25mm diameter opening at the top and a 130mm diameter opening at the bottom, allowing emerging adults to fly up and out into the cage but preventing established gravid adults from laying eggs in the pupal bowls.

Mosquito size and wing length

Where it was necessary to estimate the size of *An. gambiae s.s.* and *An. arabiensis* mosquitoes, the right wing of all specimens was removed, immersed in alcohol, and mounted onto a clean glass slide under a stereo microscope. Wing length was measured from the distal end of the alula (a 'notch' in the wing near the point of attachment) to

the tip (fig 2.1), excluding the fringe scales, using a graticule and a graduated eyepiece (Lanciani and Le, 1995). All wing measurements are given in millimetres.

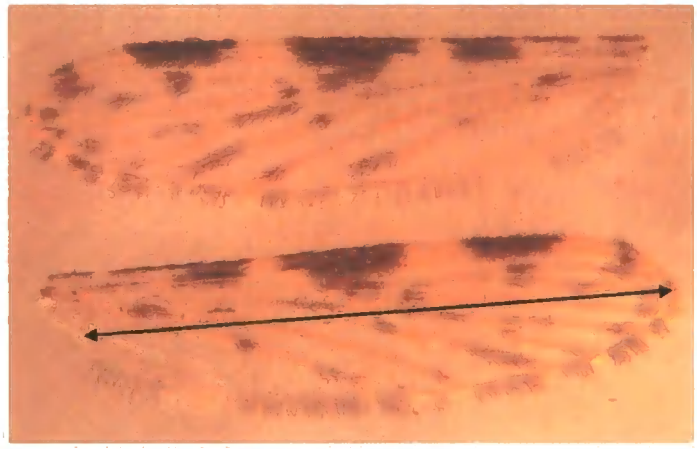

Fig 2.1 Wing length measurement.

PCR identification of sibling species

The identification procedure comprised three parts. Firstly DNA was extracted from individual mosquitoes using the InstaGene matrix kit (BIO-RAD). This utilises Chelex resin that removes cell lysis products, which would otherwise interfere with PCR. This leaves only the genomic DNA template in the supernatant.

Using sterilised forceps the specimen was placed into a sterile 1.5ml microfuge tube. 200µl of InstaGene matrix was added and vortexed. The mosquito was triturated using a sterile grinder (Treff pellet mixer, Anachem Ltd, UK). The microfuge tube was incubated in a water bath at 56°C for 45 minutes and then placed in dry heating block at 95°C for 10 minutes. The reaction mix was again vortexed briefly and then centrifuged at 17,500g (model 5415C, Eppendorf AG, Germany) for 7 minutes. The resulting supernatant was removed and stored at –20°C until needed.

The scalpel and forceps used to manipulate adults were sterilised over a flame before each specimen was handled. This prevented DNA cross-contamination of Eppendorf tubes and allowed subsequent PCR results to be treated with confidence. Secondly, PCR reagents (Promega, USA) were added to the sample at the following concentrations

modified from Scott *et al.* (1993): 0.5μL of mosquito DNA (1/400th of extracted DNA), 2.5μL of 10x reaction buffer, 1.5μL of 25mM $MgCl_2$, 0.625 units of Taq DNA polymerase in storage buffer B, 6.25ng of primer GA, 12.5ng of primer UN, 18.75ng of primer AR (table 2.1), 0.125mg of bovine serum albumin and sufficient sterile water to give a total volume of 25μL. This mixture was overlaid with a drop of mineral oil (Sigma ®) and the PCR programme carried out as follows:

Step 1. 94°C for 5 minutes
Step 2. 94°C for 1 minute
Step 3. 50°C for 1 minute
Step 4. 72°C for 1 minute
Go to Step 2 30x
Step 5. 72°C for 5 minutes

Finally on completion of the PCR programme, 10μl of the DNA plus 2μl of a standard agarose gel loading buffer was loaded onto a 1.5% agarose gel and run at 125V for 50-60 minutes. Both the gel and gel tank buffer contained ethidium bromide at 0.5μg/ml enabling amplified fragment visualisation using a UV transilluminator. Gel images were generated using the Molecular Analyst® Software (BIO-RAD) computer package (fig 2.2).

Table 2.1 Characters of universal- and species-specific primers

Species	Primer Code	Primer Nucleotide Sequence (5'→3')	Length of specific PCR Product (bp)
Complex (universal)	UN	GTG TGC CCC TTC CTC GAT GT	-
An. arabiensis	AR	AAG TGT CCT TCT CCA TCC TA	315
An. gambiae s.s.	GA	CTG GTT TGG TCG GCA CGT TT	390

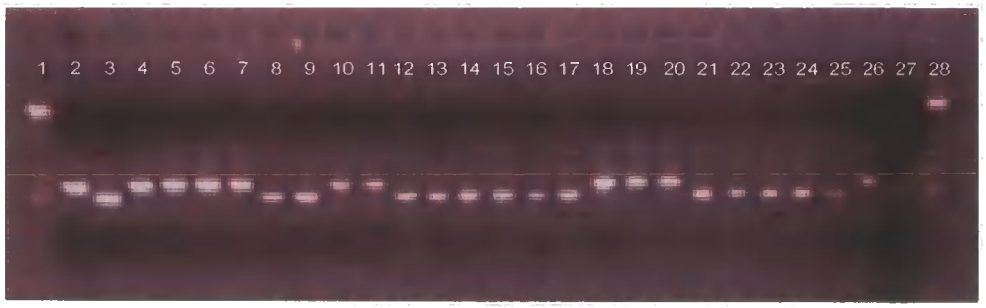

Fig 2.2 DNA bands produced by ribosomal DNA PCR amplification of DNA from *Anopheles gambiae s.s.* (lanes 2, 4 -7, 10-11, 18-20) and *An. arabiensis* (lanes 3, 8-9, 12-17, 21-24). Lanes 25 & 26 are positive controls, 27 = negative control, 1 and 28 = EZ load™ 100bp Molecular Ruler (BIO-RAD).

Conclusions

The maintenance techniques described in this chapter produced sufficient mosquitoes of both *An. gambiae s.s.* and *An. arabiensis*. These reproducible conditions were necessary to ensure comparisons between and within replicates could be treated with confidence.

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CHAPTER III

The effect of low and high humidity at high temperature on the survival of adult *Anopheles gambiae sensu stricto* and *An. arabiensis*

Abstract

Understanding the relationship between climatic conditions and vector mortality is fundamental to producing accurate maps of vector distribution, and ultimately disease transmission. Here I examined the relative contribution of temperature and relative humidity (%RH) to the adult mortality and knock-down resistance of *Anopheles gambiae sensu stricto* and *An. arabiensis* at high temperatures. To determine the importance of humidity at high temperature, batches of insects were exposed to 40°C and either 30% or 80%RH for different periods. The relative importance of temperature was examined under conditions of constant saturation deficit, a convenient way of representing the degree of moistness of the environment (14.8mb); cohorts of both species were exposed to 30°C and 65%RH and survival compared with that at 40°C and 80%RH. At 40°C *An. arabiensis* survived significantly longer than *An. gambiae s.s.* at both 30 and 80% RH. At 40°C and 30%RH it took nearly twice as long to kill 50% of *An. arabiensis* (112 min) than it did *An. gambiae s.s.* (67 min). Significantly longer survival was experienced by both species at 80%RH compared to 30%RH. There was no difference in survival between the species at 30°C, and both survived significantly longer at the lower temperature. Survival under all test conditions was not dependent on mosquito size in either species. However, little intraspecific variation in size existed in the laboratory populations. These data show that adult *An. arabiensis* are better adapted to survive hotter and drier conditions than *An. gambiae s.s.*, a characteristic that is reflected in their dominance in hotter and more arid regions in Africa.

Introduction

Insects, like all living organisms, are adapted to survive and reproduce within specific niches described by biotic and abiotic factors (Gullan and Cranston, 2000). Temperature and humidity are probably the two most important contributors to the abiotic environment, and even used alone they can often predict the distribution and abundance

of insects with reasonable accuracy (Platt *et al.*, 1958; Dow and Gerrish, 1970; Baylis *et al.*, 1999; Bryant *et al.*, 2002). Expressed simply, temperature and humidity combine to provide suitable conditions for survival. However the relative importance of each factor to the survival of any given individual can be difficult to establish, and varies depending on the actual temperature and humidity values operating, and with the level of stress resistance expressed by that individual.

High temperatures have a lethal effect independent of humidity. As insect body temperature increases, metabolism and respiration increase concomitantly up to a critical thermal limit, at which point these processes become progressively more affected by the debilitating effects of temperature (Neven, 2000). High temperatures can also denature proteins, cell membranes and enzyme structures (Feder and Hofmann, 1999). However, heat death is not directly a consequence of protein instability or general breakdown of specific metabolic processes but more often the result of a loss of integration between these processes (Cossins and Bowler, 1987). Nervous and endocrine systems are also known to malfunction at extreme temperatures with resulting changes in behaviour and development (Neven, 2000). Loss of nervous integration may in fact be an early indication of thermal damage as it is known that activity of some central neurons declines rapidly with small temperature changes above critical thermal maxima (Cossins and Bowler, 1987).

High temperatures alone can also result in cell damage and death due to desiccation, though greater desiccation risk is more usually associated with conditions of low humidity at moderate temperatures. Humidity is commonly expressed in terms of relative humidity, that is, the amount of water vapour in the air divided by the amount that would be contained in the same volume of saturated air at the same temperature (Unwin and Corbet, 1991). *Aedes aegypti* held at 28°C, a temperature conducive to high longevity, survive twice as long at 45%RH than at 0%RH (Bar-Zeev, 1958). Similarly 50% of *Anopheles quadrimaculatus* females at 23-26°C survive for 65 hours at 73%RH but only 17 hours at 20%RH (Platt *et al.*, 1957). These small insects are particularly susceptible to respiratory and cuticular water loss due to their relatively large surface area and extensive tracheal system. Though the immediate causes of death by dehydration are not well understood, it is at least apparent during periods of water stress

that tissue homeostasis and ion concentrations become difficult to maintain (Folk and Bradley, 2003).

For most insects it can be said that temperatures of 40-45°C, humidities below 50%, and especially a combination of the two, have a rapidly detrimental effect on survival. Exposure at 41°C and 50%RH is sufficient to kill 50% of adults of the blowfly *Calliphora erythrocephala* within 40 minutes (Davison, 1969) while *An. minimus* females held at 25%RH and heated at 5°C/hr become highly active at 36°C, then inactive with further increases and die after less than 5 minutes at 40°C (Muirhead-Thompson, 1951). However it is often difficult to predict the consequences of temperature and humidity on survival as the responses can vary considerably even between closely-related species. For example, the reduviid bugs *Triatoma infestans* and *Rhodnius prolixus* differ extensively in their environmental preferences. *T. infestans* exhibits preferential selection of refuges at 34°C and 20%RH (Barrozo *et al.*, 2003), while *R. prolixus* survives longest at temperatures in the range of 24-26°C (Schilman and Lazzari, 2004) and more elevated humidities (Luz *et al.*, 1998). Similar differences exist between sympatric species of tortoise beetles (Hull-Sanders *et al.*, 2003) and tiger beetles (Hadley and Schultz, 1987), and between populations of *Drosophila buzzatii* (Sorensen *et al.*, 2001). Here the effects of temperature and humidity on the survival and knock-down resistance of two sibling species with spatial and temporal differences in distribution, *Anopheles gambiae s.s.* and *An. arabiensis*, are assessed. *An. arabiensis* is the more abundant species in hotter and drier parts of Africa, therefore it was hypothesized that in the laboratory *An. arabiensis* would survive better at high temperatures and low humdities than *An. gambiae s.s.*

The influence of body mass (as estimated by wing size) on life-cycle parameters has been established in both species. Larger *An. arabiensis* in Ethiopia have a greater probability of survival, insemination and parousness than smaller individuals (Ameneshewa and Service, 1996). Similarly there is a positive correlation between fecundity and body size (wing length) in *An. gambiae s.s.* in Tanzania (Lyimo and Takken, 1993). Here it was hypothesized that larger individuals of both species would show a greater tolerance of high temperature and low humidity. Larger mosquitoes should have a smaller surface to volume ratio and larger water reserves.

Materials and Methods

Three- to six-day old female mosquitoes of both species were produced as described in chapter II. Ten mosquitoes were removed from the stock cages and aspirated into $15cm^3$ wire-framed net-covered cages. These test cages were placed into rectangular glass chambers 39x20x22cm with fitted lids, which in turn were put onto shelves within programmable LMS incubators (S.H. Scientific, Kent, U.K.). Each glass chamber housed two experimental cages. Because of slight differences in conditions within the incubators dependent on proximity to the fan, only one chamber per incubator was used. A drop of approximately 2-3°C occurred in the incubator air temperature when the mosquito cages were added. As a result, 5 minutes were allowed for the temperature to return to the required level before the recording time was started for each run. Suitable test conditions within the incubators were determined by pilot experiments with males and females of both species.

Mosquitoes were subjected to one of three experimental environments for 30, 60, 90, 120, 240, 480 or 1440 minutes:

i) 40°C and 30%RH (52.0mb Saturation Deficit, SD)
ii) 40°C and 80%RH (14.8mb SD)
iii) 30°C and 65%RH (14.8mb SD)

SD values were derived from temperature and relative humidity data, and calculated from saturation vapour pressure (SVP) and vapour pressure (VP), following the method of Unwin and Corbet (1991):

$$\text{Log}_{10}\,\text{SVP} = 9.24349 - (2305/t) - (500/t^2) - (100{,}000/t^3)$$

$$\text{VP} = \text{SVP} \times \text{RH}/100$$

$$\text{SD} = \text{SVP} - \text{VP}$$

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

Using saturation deficit allowed assessment of the importance of temperature and humidity as independent factors. The results of experiments i) and ii) were compared to

examine the effects of humidity on survival at constant high temperature, while ii) and iii) were compared to examine the effects of temperature on survival at constant saturation deficit.

Experiments requiring low humidity were run at the ambient humidity of the incubators (~30%RH). To create higher humidity conditions, two 15x10x8cm plastic bowls, containing either 500ml of dechlorinated water (80%RH) or a solution of potassium hydroxide (65%RH) (Solomon, 1951), were placed in the bottom of the glass chambers. Humidity was monitored using a pen-type digital humidity meter (RS Components Ltd, Northamptonshire, U.K.). Temperature within the glass chambers was monitored using a digital thermometer (probe type T, Digitron Instruments, U.K.).

At the conclusion of the experimental run, the cages were removed from the incubators and the number of mosquitoes knocked-down determined. Mosquitoes unable to right themselves after probing with the tip of a pipette were scored as knocked down. The cages were then transferred to another glass chamber held at ~27°C and >80%RH to allow mosquito recovery. Mortality was determined 24 hours later. Ten repeats at each time period were carried out.

Size and survival

Separate cohorts of both species were used to assess the effect of size on survival. 140-180 specimens of *A. gambiae s.s.* and *An. arabiensis*, in groups of 20, were subjected to each of the experimental environments for the length of time required to kill 50% of the cohort as established in the main analysis. The mosquitoes were then transferred to a recovery chamber for 24 hours. All mosquitoes (dead or alive) were removed from the test cages at the end of the recovery period. Those still alive were killed by exposure to ethyl acetate. The wings were removed and wing length measured as described in chapter II.

Analysis

Mantel-Haenzel χ^2 test was used to test for differences in mortality between the two species for all conditions investigated adjusting for period of exposure. Probit regression analysis generated LT_{50} and kdT_{50} values (time in minutes for 50% lethality/50% knock-down at a particular temperature), which were used as a parameter

for comparing species tolerance. Mantel-Haenzel χ^2 tests were also used to assess the independent effects of humidity and temperature on survival rate. Two-sample independent t-tests were used to compare interspecific size differences and mean wing length between survivors and non-survivors within species

Results

Survival

Anopheles arabiensis exhibited a three-fold greater survival than *An. gambiae s.s.* at 40°C and 30%RH ($\chi^2_{M\text{-}H}$, adjusted for different time periods = 58.3, df = 5, p<0.001, Mantel-Haenzel weighted odds ratio = 3.3, 95% confidence intervals (C.I.) = 2.4-4.7, fig 3.1), and a two-fold greater survival than *An. gambiae s.s.* at 40°C and 80%RH ($\chi^2_{M\text{-}H}$ = 23.0, df = 5, p<0.001, O.R. = 2.1, 95% C.I. = 1.5-2.8). There was no significant difference in survival between the two species at 30°C and 65%RH (fig 3.1). Table 3.1 summarises the LT_{50} values under the experimental conditions.

Table 3.1 LT_{50} values for *An. gambiae s.s. and An. arabiensis* exposed to experimental conditions.

Species	Temperature (°C)	Humidity (%RH)	LT_{50} (95% C.I.) (min)	Probit (P) model
An. gambiae s.s.	40	30	66.7 (51.3-82.3)	P = -6.441 + 1.534 x ln time
	40	80	106.4 (85.2-133.5)	P = -6.506 + 1.394 x ln time
	30	65	342.1 (304.4-391.4)	P = -1.27 + 0.0037 time
An. arabiensis	40	30	112.2 (90.4-139.4)	P = -5.472 + 1.159 x ln time
	40	80	152.6 (116.5-206.6)	P = -5.548 + 1.103 x ln time
	30	65	374.7 (305.3-494.5)	P = -1.28 + 0.0034 time

Both humidity and temperature contribute to survivorship for both species. At 40°C *An. gambiae s.s.* and *An. arabiensis* survived for significantly longer at high humidity than at low humidity (*An. gambiae s.s.*, $\chi^2_{M\text{-}H}$ = 52.9, df = 5, p<0.001; *An. arabiensis*, $\chi^2_{M\text{-}H}$ = 15.8, df = 5, p<0.001). At a saturation deficit of 14.8mb survival in both species was significantly longer at 30°C than at 40°C (*An. gambiae s.s.*, $\chi^2_{M\text{-}H}$ = 90.6, df = 5, p<0.001; *An. arabiensis*, $\chi^2_{M\text{-}H}$ = 34.9, df = 5, p<0.001). In both species humidity and temperature are equal contributors to survival length; there are no differences between the odds ratios produced when humidity and temperature are considered independently (*An. gambiae s.s.*, RH% OR = 3.28 (95% C.I.= 2.35-4.62), T°C OR = 3.99 (3.05-5.7); *An. arabiensis*, RH% OR = 1.81 (1.34-2.44), T°C OR = 2.34 (1.77-3.2)).

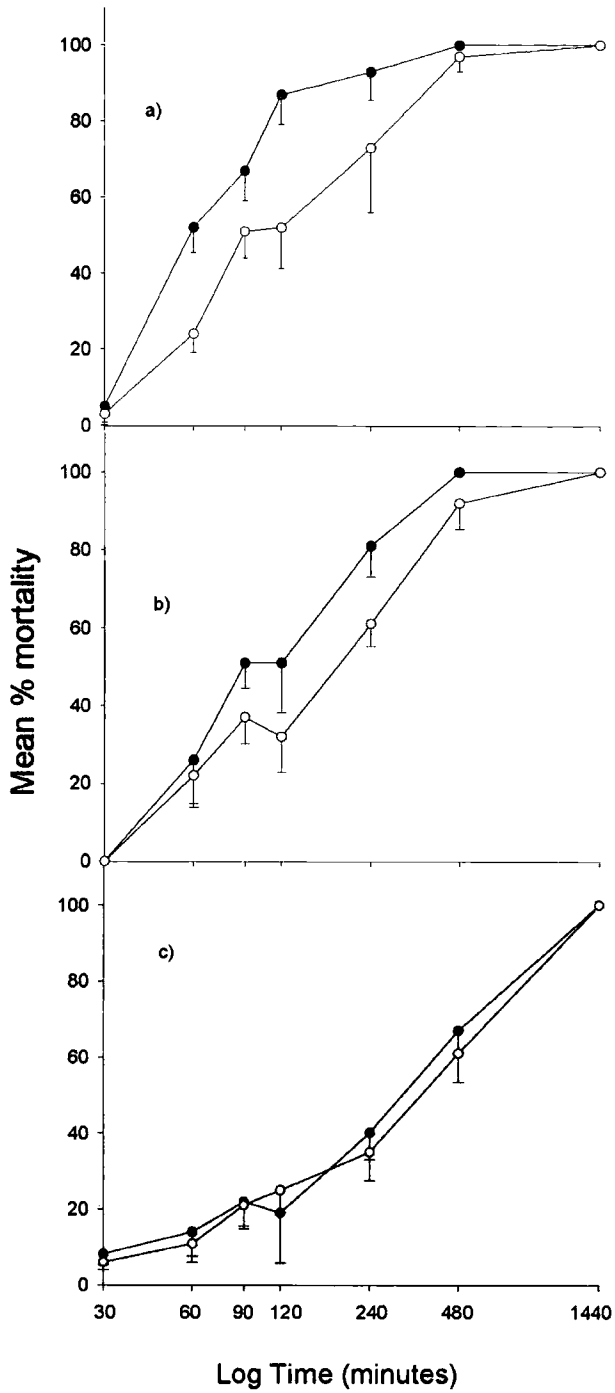

Fig 3.1 Mean percentage mortality 24 hours post-treatment for *An. gambiae s.s.* and *An. arabiensis* exposed to a) 40°C and a saturation deficit of 52.0mb (30%RH), b) 40°C and a saturation deficit of 14.8mb (80%RH) c) 30°C and a saturation deficit of 14.8mb (65%RH). Bars represent 95% C.I. X axis starts at 30 minutes.

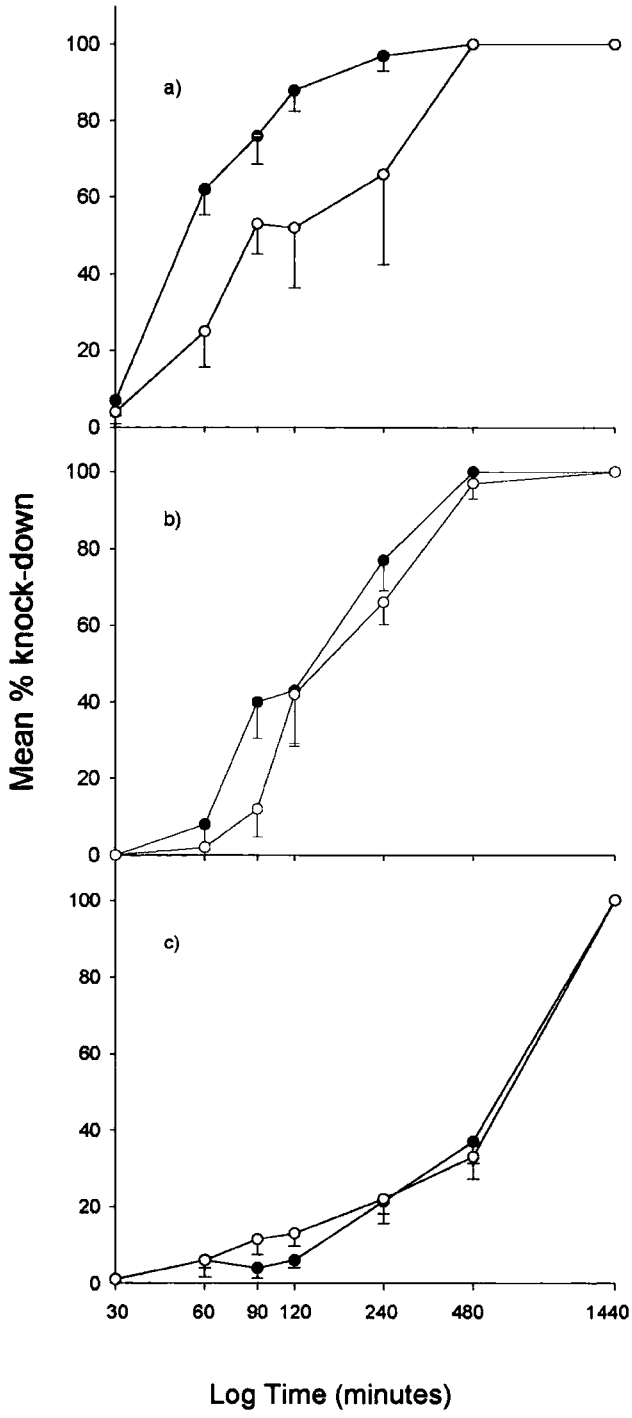

Fig 3.2 Mean percentage knock-down post-treatment for *An. gambiae s.s.* and *An. arabiensis* exposed to a) 40°C and a saturation deficit of 52.0mb (30%RH), b) 40°C and a saturation deficit of 14.8mb (80%RH) c) 30°C and a saturation deficit of 14.8mb (65%RH). Bars represent 95% C.I. X axis starts at 30 minutes.

Knock-down

Anopheles arabiensis exhibited a significantly higher knock-down resistance than *An. gambiae s.s.* at 40°C and 30%RH ($\chi^2_{M\text{-}H}$, adjusted for different time periods = 94.0, df = 5, p<0.001; fig 3.2), and at 40°C and 80%RH ($\chi^2_{M\text{-}H}$ = 16.3, df = 5, p<0.001; fig 3.2). There was no significant difference in time taken to knock-down between the two species at 30°C and 65%RH. Table 3.2 summarises the kdT_{50} values under all experimental conditions.

Table 3.2 kdT_{50} values for *An. gambiae s.s. and An. arabiensis* exposed to experimental conditions.

Species	Temperature (°C)	Humidity (%RH)	kdT_{50} (95% C.I.) (min.)	Probit (P) model
An. gambiae s.s.	40	30	58.9 (47.5-69.9)	P = -6.4798 + 1.668 x ln time
	40	80	129.3 (107.4-158.9)	P = -7.75 + 1.594 x ln time
	30	65	544.4 (481.8-633.8)	P = -1.91 + 0.0035 time
An. arabiensis	40	30	111.8 (78.9-159.1)	P = -5.33 + 1.13 x ln time
	40	80	166.4 (139.6-203.4)	P = -8.621 + 1.686 x ln time
	30	65	567.4 (449.7-793.3)	P = -1.65 + 0.0029 time

Size and survival

An. arabiensis adults were on average 0.34 mm bigger than *An. gambiae s.s.* (*An. arabiensis* mean wing length and 95% confidence intervals = 2.83 (2.80-2.85), *An. gambiae s.s.* = 2.49 (2.47-2.52); t = 23.0, df = 673, p <0.001) suggesting size may influence survival. However survival under all conditions was independent of mosquito size in both species (table 3.3). Survival rates replicated those of the main analysis.

Table 3.3 Mean wing lengths of survivors and non-survivors of *An. gambiae s.s.* and *An. arabiensis* exposed to their respective LT_{50} times at 40°C and either 30% or 80%RH.

Conditions	Exposure (min)	% Alive	Mean wing length (mm) + 95% C.I. of survivors	Mean wing length (mm) + 95% C.I. of non-survivors	t	df	p
An. gambiae s.s.							
40°C, 30%RH	67	47	2.48 (2.45-2.53)	2.49 (2.45-2.53)	-0.1	178	n.s.
40°C, 80%RH	106	46	2.53 (2.49-2.56)	2.49(2.46-2.53)	1.4	164	n.s.
30°C, 65%RH	342	54	2.55 (2.50-2.60)	2.50 (2.46-2.55)	1.3	138	n.s.
An. arabiensis							
40°C, 30%RH	112	56	2.84 (2.79-2.89)	2.86 (2.82-2.90)	-0.4	158	n.s
40°C, 80%RH	153	55	2.83 (2.78-2.87)	2.78 (2.75-2.82)	1.6	167	n.s.
30°C, 65%RH	375	55	2.85 (2.80-2.90)	2.90 (2.84-2.95)	-1.1	138	n.s.

Discussion

These findings demonstrate that *An. arabiensis* is capable of surviving stressful conditions of high temperatures and low humidities for longer than *An. gambiae s.s.* under experimental conditions. *An. arabiensis* also survives for longer at high temperature when humidity is conducive to survival. However, the most significant difference in survival between the two species is observed under the most stressful conditions, namely 40°C and 30%RH. *An. arabiensis* is also more able to resist being knocked-down under these conditions than its sibling species. These differences in survival and knock-down rates reflect what is known of distributional and behavioural differences between the two species.

Though the distribution of both species overlaps throughout much of sub-Saharan Africa, *An. arabiensis* is more common at the fringes of the range, being endemic in the hotter and drier savanna areas of the African continent and steppes of the Arabian peninsula (Coluzzi *et al.*, 1979; Lindsay *et al.*, 1998; Coetzee *et al.*, 2000). More specifically, *An. arabiensis* is predominant in regions which experience a low average annual precipitation of less than 500mm (Rishikesh *et al.*, 1985; Petrarca *et al.*, 2000) and is the only species present in arid areas of Sudan (Dukeen and Omer, 1986), Ethiopia (Ye-Ebiyo *et al.*, 2000) and Mozambique (Mendis *et al.*, 2000). In contrast *An. gambiae s.s.* is more common in humid savanna and forest zones (White, 1974; Rishikesh *et al.*, 1985; Lindsay *et al.*, 1998; Rogers *et al.*, 2002). *An. gambiae s.s.* is predicted to dominate where five monthly maximum precipitation exceeds 800mm (Lindsay *et al.*, 1998). Where the two species are sympatric, they differ in their temporal distribution. For example, *An. arabiensis* predominates only in the hot dry season in The Gambia, during which average weekly maximum temperatures reach 40°C or more and relative humidity can fall below 20% (Lindsay *et al.*, 1991). Similarly around Kisumu, Kenya, *An. arabiensis* only dominates mosquito catches in the dry season, when minimum relative humidity does not exceed 20%, though at 27°C average daily temperatures are only 2°C higher than in the wet season, when *An. gambiae s.s.* dominates (Rishikesh *et al.*, 1985).

Although both species exhibit a high degree of anthropophily throughout much of their distribution (Costantini *et al.*, 1999), *An. arabiensis* is more exophilic than *An. gambiae s.s.* (White, 1974; Coluzzi, 1984; Mnzava *et al.*, 1995; Githeko *et al.*, 1996). Because of this, *An. arabiensis* may experience more extreme climatic conditions more frequently than *An. gambiae s.s.*, especially in the dry season when mean maximum outdoor air temperatures are consistently 1-6°C greater than indoor temperatures in mud and thatch houses (De Meillon, 1934; Yohannes, 2002) and humidity outside is frequently 10-20% lower than that indoors (personal field data). *An. arabiensis* is more catholic in its feeding habits than *An. gambiae s.s.*, taking bloodmeals from a range of animals. This flexibility in feeding preference may promote adaptation to more extreme temperatures and also rapid temperature fluctuations encountered outdoors, and has promoted the idea that *An. arabiensis* is more tolerant of microclimatic instability, including extremely hot and dry conditions (Petrarca *et al.*, 1991).

In both species, temperature and relative humidity seem to be of approximately equal importance to survival period. Both contribute to the dehydration risk, thus water loss may be more important than the independent effects of temperature or humidity in determining the distribution of these two species. It is important to realize that dehydration is not an all or nothing process, and sub-lethal dehydration is known to affect a range of biological processes, particularly during developmental periods (Edney, 1977). Heat injury by contrast often occurs only after a specific length of exposure, with insects exposed for a sub-lethal length of time making a full recovery after knock-down. For example, *An. quadrimaculatus* knocked-down after 2 minutes exposure at 41°C and then moved to lower temperature have a high survival rate compared to those exposed to 5 minutes, of which very few survive (Platt *et al.*, 1957). A positive correlation of distribution and abundance with relative humidity has been demonstrated for several mosquito species including *Aedes vexans* (Platt *et al.*, 1958), *Culex nigripalpus* (Dow and Gerrish, 1970), *Ae. serratus*, *Ae. scapularis*, *Coquillettidia venezuelensis* and *Runchomyia reversa* (Guimaraes *et al.*, 2000) and humidity is also an important influence on host-seeking behaviour. *An. gambiae s.s.* females did not respond to host odours and seek out a bloodmeal when relative humidity was less than 40% (Takken *et al.*, 1997). A

similar behaviour is observed in tsetse flies, for which only moist (82%RH) heated air elicits upwind anemotaxis (Evans and Gooding, 2002). With this in mind, it is important to realise that conditions suitable for survival of an individual mosquito may not equate to conditions suitable for reproduction and therefore species survival in a given location. For example, *Ae. krombeini* adults are capable of surviving at constant 33.5°C but females do not take blood-meals and males do not attempt to mate at this temperature (Joshi, 1996). If there is a difference in the host-seeking ability of *An. gambiae s.s.* and *An. arabiensis* at low humidity, this will exacerbate the difference in the ability of these species to survive in such conditions.

This study finds that *An. arabiensis* is on average larger than *An. gambiae s.s.*. This is consistent with data from the field (Petrarca *et al.*, 1998) and may underlie the superior tolerance of low humidity exhibited by *An. arabiensis*. The smaller *An. gambiae s.s.* individuals have very little excess water to lose and so might respond to desiccation caused by low humidity by seeking out a high relative humidity site. This behaviour is evident in other arthropods with small water reserves (Bruce *et al.*, 1997; Yoder *et al.*, 1997). *An. gambiae s.s.* is perhaps more likely to avoid low humidity sites in the first place; the endophilic nature of this species protects the mosquito from the highly variable and more extreme external climate. *An. quadrimaculatus*, a species which readily enters houses, has been shown to exhibit sharp avoidance of humidities lower than 70-80%RH (Platt *et al.*, 1957). Survivorship has been shown to increase with size in several mosquito species (Haramis, 1983; Kitthawee *et al.*, 1990) including *An. arabiensis* (Ameneshewa & Service, 1996). That there are no intra-specific differences in size between survivors and non-survivors in the present study does not weaken this argument to any great extent when one considers the narrow range of size variation within the species when laboratory-reared. In natural populations of mosquitoes, the size of adults can be much more variable (Lyimo and Takken, 1993; Yuval *et al.*, 1993), though where seasonal variation in wing length occurs it is apparent that mosquito wing size decreases with increasing air temperature (Day *et al.*, 1990; Le Sueur and Sharp, 1991; Lyimo and Takken, 1993). However these studies only examined wing length of emerging adults and

so are not truly representative of the mosquito population which survives to reproductive age in the dry season.

This study helps confirm that the geographical and temporal distribution of *An. arabiensis* and *An. gambiae s.s.* across Africa is characterised by species-specific adaptations to climate. This study demonstrates that subtle adaptations to local climatic parameters can be a major cause of distributional differences between closely related species. From a disease management perspective it is especially important to determine how climatic parameters affect mosquito species as those that are capable of surviving a period of high heat or desiccation stress and subsequently reproducing, will be primary candidates for disease vectors under such conditions.

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CHAPTER IV

Temperature avoidance behaviour of adult *Anopheles gambiae sensu stricto* and *An. arabiensis*

Abstract

It is well known that amongst the sibling species of the *Anopheles gambiae* complex, *An. arabiensis* predominates over *An. gambiae sensu stricto* in hotter, drier parts of Africa, but limited information is available about the niche adoption of these species at a microclimatic level. Avoidance of high temperatures may allow adult *An. gambiae s.s.* to persist at the fringes of the *An. arabiensis* distribution, but a better tolerance of high resting temperature in *An. arabiensis* may result in longer-term improvements in survival rates. I tested for differences in the behavioural avoidance activity of these mosquitoes in the presence of increasing temperature. Female mosquitoes were introduced into a heated holding tube from which they could escape into a cage through a one-way funnel. From a starting temperature of 28°C they were exposed to a 2°C rise in temperature every 30 minutes until all mosquitoes had escaped or been knocked down. The effects of adult age and feeding status were investigated. As temperature increased, 3-6 day old unfed *An. arabiensis* left the holding tube at higher temperatures than *An. gambiae s.s.* (*An. arabiensis* mean activation temperature = 35.7°C, 95% CIs = 35.4-36.1°C; *An. gambiae s.s.* = 33.0°C, 32.5-33.5°C). Bloodfed mosquitoes were activated at higher temperatures than any other group (*An. arabiensis* 36.83°C (36.47-37.18°C); *An. gambiae s.s.* 36.05°C (35.63-36.47°C). Mosquitoes aged ≥ 14 days were activated at lower temperatures than any other group (*An. arabiensis* 33.73°C (33.28-34.18); *An. gambiae s.s.* 32.35°C (31.86-32.84). This work suggests that inherent differences in thermotolerance do exist between adult *An. arabiensis* and *An. gambiae s.s.*, and may help to build a more predictive model of their distribution in a changing climatic environment.

Introduction

Environmental temperature is an important factor governing the behaviour of insects, not least because many find it difficult to maintain a constant internal temperature through physiological mechanisms alone (Uvarov, 1931; Denlinger *et al.*, 2001). At low temperatures activities such as flight often depend critically on muscles being warm enough to operate effectively (Unwin and Corbet, 1991; Foster and Robertson, 1992), while extremely high temperatures can lead to a loss of nervous integration and a breakdown of neuromuscular processes (Cossins and Bowler, 1987). Insects, in common with other organisms, are adapted to specific climatic niches outside of which they cannot survive. However insects do have one physiological advantage over larger organisms. Because they are small, insects are able to take advantage of small local variations in temperature and maintain an optimal body temperature by seeking out favourable microclimates in which to rest (Casey, 1981).

In mosquitoes (Diptera: Culicidae), temperature is important in a number of behaviours and physiological processes including host seeking (Petric *et al.*, 1995), blood feeding (Samish *et al.*, 1995; Crans *et al.*, 1996) and development (Lyimo *et al.*, 1992; Lanciani and Le, 1995), and is one of a number of climatic factors that affect their geographic distribution (Lindsay *et al.*, 1998; Bayoh *et al.*, 2001). The influence of temperature on mosquito flight activity is clearly important for quantifying the risk of mosquito-borne diseases, particularly at high temperatures, since in the absence of flight, pathogen transmission is unlikely to occur. In general mosquito activity is greater on warmer nights than colder ones, though activity is also low at temperature extremes. In the Upper Rhine Valley, catches of *Aedes vexans* increase by 6% for each degree of increase in average evening temperature (Petric *et al.*, 1995). Read and Adams (1980) demonstrated similar increases in flight activity with increasing temperature, up to a thermal threshold, for *Haemagogus lucifer* and *Mansonia dyari*.

The upper temperature threshold where mortality increases sharply appears to be between 38-45°C for most tropical mosquitoes, depending on the species and life-stage involved (Muirhead-Thompson, 1951; Benedict *et al.*, 1991). For *Anopheles gambiae s.s.* very few adults survive longer than one day above 40°C, at 40% relative humidity (Bayoh, 2001). Behavioural avoidance of extreme heat might be one of several putative

survival strategies used by adult mosquitoes encountering such conditions in the field. Though they have a limited flight range of a few kilometres, they are regularly exposed to a range of conditions from which they must select suitable feeding, resting and breeding sites. Thus they are capable of responding to temperature fluctuations that can be quite large. For example, in Tigray, Ethiopia, air temperature can fluctuate daily from 11 to 27°C (Yohannes, 2002), whilst in Kaduna, Nigeria, it can range from less than 15 to nearly 35°C (Rishikesh *et al.*, 1985) and in northern Sudan diurnal fluctuations from 24-49°C occur in the dry season (Dukeen and Omer, 1986).

Many previous behavioural studies of the *An. gambiae* complex, especially in the older literature, are difficult to interpret because the mosquitoes reported are a combination of different species (Muirhead-Thomson, 1951). *An. gambiae s.s.* and *An. arabiensis* are the two most efficient malaria vectors of the complex, and though they are morphologically identical, they express distinct behavioural characteristics (e.g. preferred host, feeding and resting sites) which may relate to their differential use of habitat (Coluzzi, 1984). *An. arabiensis* dominates in hot and dry conditions while *An. gambiae s.s.* thrives in cooler, wetter conditions (Lindsay *et al.*, 1998). *An. arabiensis* has a number of strategies allowing persistence in arid areas which include laying eggs on damp surfaces (Coluzzi, 1965), delayed egg hatching and reproductive quiescence, in which a single gonotrophic cycle is experienced over the whole period (Omer, 1970; White, 1974; Taylor *et al.*, 1993). However these processes operate over periods of several days, and are not concerned with shorter-term responses to changing climate. This present study set out to test whether there are inherent differences in the behavioural response to high temperatures between the two species.

Materials and methods

Mosquitoes

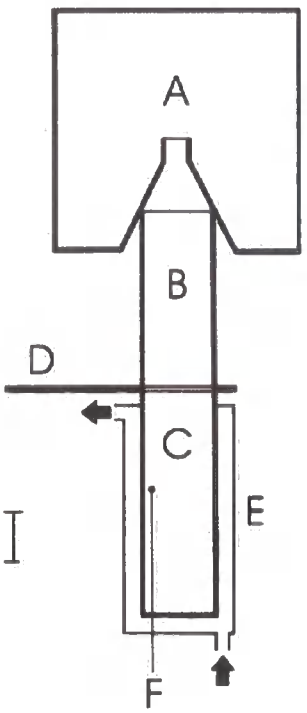
In addition to the *An. gambiae s.s.* KWA strain and the *An. gambiae* KGB strain (see chapter II for origins), this study used *An. gambiae s.s.* 16CSS, derived in 1974 from Lagos, Nigeria and *An. arabiensis* Dondotha, from Kwa Zulu-Natal in South Africa. These were supplied by the London School of Hygiene and Tropical Medicine. Adults of *An. gambiae s.s.* (KWA) and *An. arabiensis* (KGB) only were evaluated at different

ages and feeding conditions. To assess the effects of age, mosquitoes were tested within 24 hours of emergence, at 3-6 days old and at 14-17 days old. In the oldest group, in order to simulate conditions experienced in nature, mosquitoes were blood-fed every 3 days on the author, with the final feed 2 days before the assay. Cohorts of both species aged 3-6 days were allowed to blood-feed to repletion to examine the responses of newly-fed females to rising temperature. Fully engorged females were immediately transferred to the holding tubes. The 16CSS *An. gambiae s.s.* and Dondotha *An. arabiensis* strains were used to test whether differences observed were consistent between species.

Experimental system

The experimental system consisted of a plastic holding tube, heated by warm water, connected to an escape cage (fig 4.1). The plastic tubes used were WHO insecticide resistance testing-kit tubes measuring 125mm length x 44mm internal diameter (WHO, Geneva). In the holding tube, the mesh screen at the end of the tube was replaced with a solid clear plastic disc, 46mm in diameter, glued into place. The disc prevented the inflow of air at room temperature (28.0±1.0°C) affecting internal tube conditions. A 3mm diameter hole in the disc allowed a thermometer (probe type T, Digitron Instruments, U.K.) to be inserted into the tube. Humidity within the holding tube was monitored using a pen-type digital humidity meter (RS Components Ltd, Northamptonshire, U.K.). At the other end of the tube there was a sliding gate that allowed the introduction of mosquitoes into the holding tube through a 20mm diameter hole. A water bath fitted with an immersion thermostat with integral pump unit (Grant Instruments Ltd, Cambridge, UK) for external circulation was used to drive heated water around the holding tube, at a flow rate of 350ml min^{-1}. The water was circulated through translucent silicone tubing (4mm internal diameter, 1mm thick) coiled around the holding tube and taped into place in order to minimise internal thermal gradients.

A plastic funnel (70mm and 15mm diameter) was secured to the outside of a $15cm^3$ mesh cage. The wide end was fitted flush to the cage surface so that the small end protruded into the cage. This allowed easy entrance to the cage but prevented highly active mosquitoes returning into the escape tube, which linked the holding tube to the escape cage. The escape tube prevented mosquitoes returning to the holding tube if they did not enter the escape cage immediately.



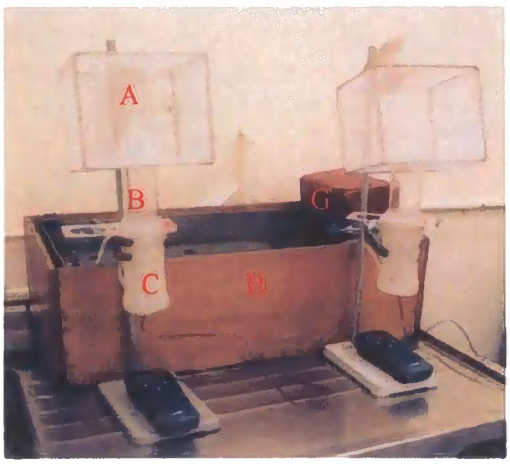


Fig 4.1 The experimental apparatus.

A = escape cage, B = escape tube, C = holding tube,
D = sliding gate, E = silicone tubing 'water jacket',
F = temperature probe, G = pump and heater unit,
H = water bath. Bar on schematic (left) represents 3cm.

Experimental procedure

Ten mosquitoes were introduced into the holding tube at the starting temperature of 28 ± 0.5°C. Mosquitoes were acclimated for 30 minutes, after which the gate was opened and the insects were free to move out of the holding tube. Any mosquitoes that left in the first 5 minutes were judged to have been disturbed by the gate opening and were not included in the final analysis. After 20 minutes, the numbers of mosquitoes in the holding tube and cage were recorded. In the next 10 minutes the water bath temperature was increased to create a holding tube temperature of 30 ± 0.5°C at a rate of 0.2°C min^{-1}. This procedure was repeated for all further temperature steps up to 42°C. Thus every 30 minutes the air temperature was increased by 2°C (fig 4.2). Cage temperature was recorded continuously at each temperature step. Females remaining in the tube at the end of the experiment were removed to see if they were still responsive. Those unable to right themselves after probing with a pipette tip were recorded as 'knocked-down' and excluded from the final analysis. A paper facemask was worn by the observer when counting mosquitoes so as to minimise the impact of breathing carbon dioxide near the set-up. Each treatment group had 10 runs, each with 10 mosquitoes.

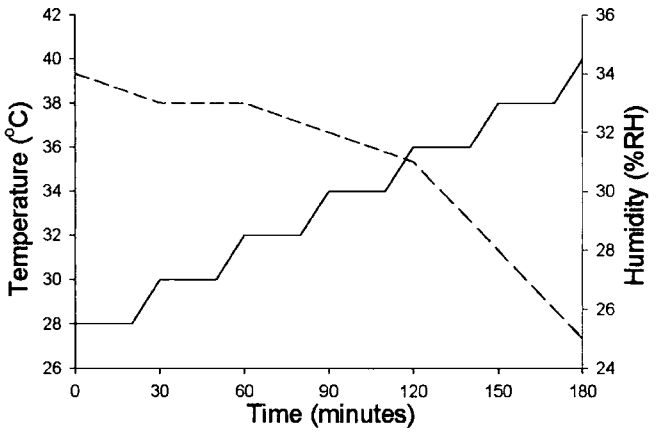


Fig 4.2 Step-wise increase in experiment air temperature and negative correlation with humidity (solid line = T°C, dashed line = %RH).

The experiment had two controls. Firstly, in order to demonstrate that mosquitoes responded to increasing temperature and not time, six runs each with 10 mosquitoes for each treatment group were carried out at a constant temperature of 28 ± 0.5°C. The number of mosquitoes left in the holding tube at the end of 3 hours was recorded. Secondly, it was necessary to show that the actual temperature values were important in dictating activity and not simply a 2°C change in ambient temperature. Therefore six runs each with 10 mosquitoes for each treatment group were introduced into tubes at 24°C for 20 minutes and increased to 26°C at a rate of 0.2°C min^{-1}. This process was repeated up to 28°C.

Climate data

The World Meteorological Organisation weather station data presented for the sites of origin of the mosquito strains was accessed from the International Centre of Insect Physiology and Ecology website (http://informatics.icipe.org/databank/wmo.htm).

Analysis

3-6 day old unfed female mosquitoes were used as the standard to which all other treatments were compared. The one-sample Kolmogorov-Smirnov test was used to test the normal distribution of the data. Normally-distributed data were analysed by standard parametric methods, using SPSS for Windows v10.0. F tests were used to compare variances between groups, t-tests were used to test for equality of means between two

groups and χ^2 was used to test for a trend in the data. Multivariate analysis of variance (general linear model) was used to identify the relative importance of species, adult age and feeding status as determinants of activation temperature. To assess the possibility that the activity of one mosquito stimulated the movement of others resulting in a non-independence of data points, Mann-Whitney U tests were employed to test for strain and species differences between the first mosquito to be activated in each run.

Results

5.9% of the mosquitoes observed (31 *An. gambiae s.s.* and 22 *An. arabiensis*) were either disturbed or trapped by the gate opening, and 4.6% were 'knocked down' at the end of the experiments (22 *An. gambiae s.s.* and 20 *An. arabiensis*). Thus the main analysis was based on observations of 458 *An. arabiensis* and 447 *An. gambiae s.s.* The variances of all groups tested were similar in all comparisons (table 4.1), and all data were normally distributed (Kolmorov-Smirnov Z, n.s.). *An. arabiensis* consistently left the holding tube at higher temperatures than did *An. gambiae s.s.* (table 4.2). The greatest inter-specific difference was observed between 3-6 day old unfed mosquitoes (*An. arabiensis* KGB, mean activation temperature = 35.7°C, 95% CIs = 35.4-36.1; *An. gambiae s.s.* KWA = 33.0°C, 32.5-33.5, fig 4.3). There was no difference between the mean activation temperatures for strains of the same species (*An. arabiensis*; KGB = 35.7, 95% CIs 35.4-36.1, Dondotha = 35.5, 35.2-36.0, t = 0.5, n.s., *An. gambiae s.s.*; KWA = 33.0, 32.5-33.5, 16CSS = 33.3, 32.8-33.8, t = -0.8, n.s.)

Table 4.1 Two-tailed F-test values for treatment groups.

Adult status		s^2 *An. gambiae s.s.*	s^2 *An. arabiensis*	F	p
Age	<24hr	4.19	5.71	1.36	n.s.
	3-6d*	5.14	3.35	1.53	n.s.
	≥14d	5.29	4.47	1.18	n.s.
Feeding status	Unfed*	5.14	3.35	1.53	n.s.
	Bloodfed	3.81	2.50	1.52	n.s.

* Drawn from identical data. 25°C-reared, 3-6d old unfed female mosquitoes were the standard to which all other conditions were compared. s^2 = sample variance. Critical value of F at $df_{90,90}$ = 1.625.

Table 4.2 Comparisons of mean activation temperatures for *An. gambiae s.s.* KWA and *An. arabiensis* KGB for all treatments.

		An. gambiae s.s.		*An. arabiensis*			
Adult status		n	Mean Temperature (°C) + 95% C.I.	n	Mean Temperature (°C) + 95% C.I.	t	p
Age	<24hr	91	34.9 (34.4-35.3)	94	35.5 (35.0-36.0)	-2.1	0.04
	3-6 days*	92	33.0 (32.5-33.5)	92	35.7 (35.4-36.1)	-9.2	<0.001
	≥14days	86	32.4 (31.9-32.8)	89	33.7 (33.3-34.9)	-4.1	<0.001
Feeding status	Unfed*	92	33.0 (32.5-33.5)	92	35.7 (35.4-36.1)	-9.2	<0.001
	Blood-fed	85	36.1 (35.6-36.5)	80	36.8 (36.5-37.2)	-2.8	<0.001

*Drawn from identical data. 25°C-reared, 3-6 day old unfed female mosquitoes were the standard to which all other conditions were compared.

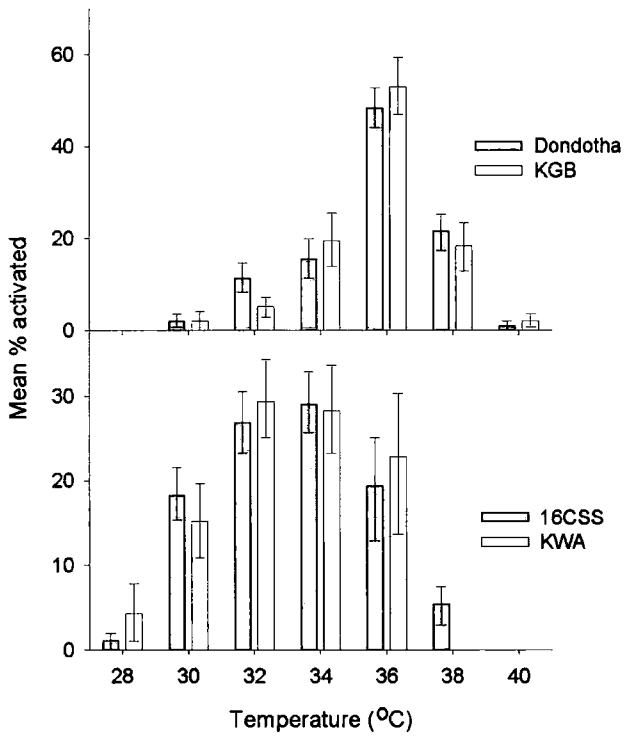


Fig 4.3 Temperature activation profiles for 25°C-reared, 3-6day unfed adults of a) *An. arabiensis* strains b) *An. gambiae s.s.* strains. Error bars represent 95% C.I.

In all age treatment comparisons, *An. arabiensis* left the tubes at higher temperatures than *An. gambiae s.s.* (<24hr, t = -2.06, p = 0.04; 3-6 days, t = -9.17, p<0.001; ≥14days, t = -4.14, p<0.001). The temperature at which mosquitoes were activated declined with age in *An. gambiae* s.s. (χ^2 for trend: *An. gambiae s.s.* at 36°C, χ^2 = 32.4, p = <0.001), but not *An. arabiensis* (at 36°C, χ^2 = 0.2, n.s., fig 4.4). In both species newly emerged mosquitoes were activated at higher mean temperatures than adults ≥ 14 days (*An. arabiensis* = 1.8°C higher, 95% CIs = 1.1-2.5°C, t = 5.4, p <0.001; *An. gambiae s.s.* = 2.5°C, 1.9-3.2°C, t = 7.7, p <0.001). Blood-fed mosquitoes were activated at higher temperatures than unfed mosquitoes for both *An. arabiensis* (1.1°C, 0.6-1.6°C, t = 4.2, p <0.001) and *An. gambiae s.s.* (3.1°C, 2.4-3.7°C, t = 9.5, p<0.001), though *An. arabiensis* was still activated at higher temperatures than *An. gambiae s.s.* (t = - 2.80, p <0.001, fig 4.5). 18% of blood-fed *An. arabiensis* and 15% of blood-fed *An. gambiae s.s.* were 'knocked down' at high temperatures and did not leave the holding tube, even when the temperature exceeded 40°C. 44% of the knocked down *An. arabiensis* and 53% of the *An. gambiae s.s.* died less than 24 hours after the bioassay.
Multivariate analysis revealed that all the treatment factors (feeding status, age and species) were significant predictors of the activation response (table 4.3).

Table 4.3 Univariate analysis of variance of treatment effects on activation temperature.

Factor	Type III Sum of Squares	df	F	p
Feeding Status	377.556	1	85.325	<0.001
Age	423.534	2	47.858	<0.001
Species	207.077	1	46.798	<0.001

In the control trials only 3.6% (17/466) of mosquitoes left the holding tube after 3 hours at constant 28°C, and only 5.4% (25/459) were activated by either of the step-wise temperature changes (24-26°C and 26-28°C). These findings are clearly different to the results under bioassay conditions (step-wise temperature changes between 28-40°C), where 100% of the mosquitoes capable of leaving the holding tube had done so within 3 hours.

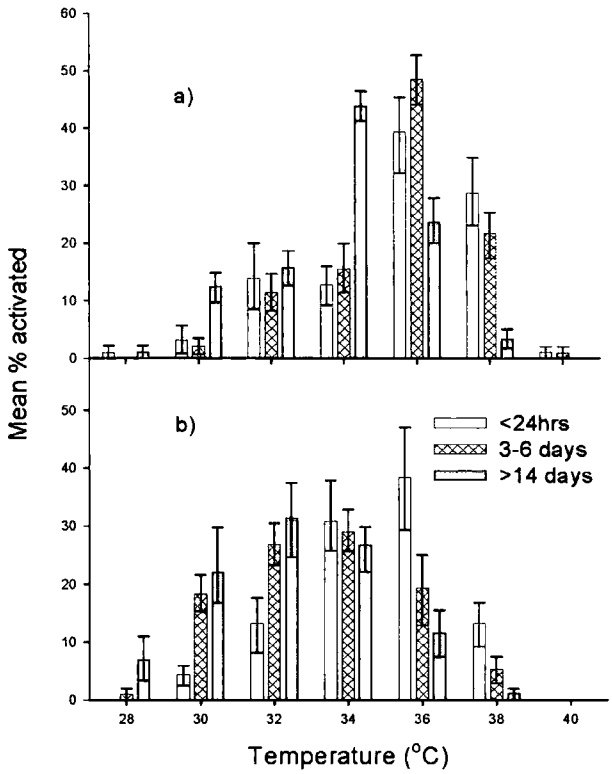


Fig 4.4 Temperature activation profiles at three adult ages for a) *Anopheles arabiensis* KGB and b) *An. gambiae s.s.* KWA. Error bars represent 95% C.I.

Observations of mosquitoes in the holding tube indicated that when one mosquito left the tube it did not cause others to leave at the same time. This is also reflected in the normal distributions seen in figs 4.3-4.5. If movement of the first mosquito had activated others, one would expect to see positively skewed distributions. Analysis of the movement of the first mosquito only in each test is also consistent with the main findings. There was a significant inter-specific difference between the temperatures at which the first mosquitoes were activated (*An. arabiensis* = 32°C, IQR = 32-34°C; *An. gambiae s.s.* = 30°C, IQR = 30-30°C, Z = -4.3, $p<0.001$), but again there were no intra-specific differences (*An. gambiae s.s.* KWA median activation temperature = 30°C, inter-quartile range = 29.5-32°C; 16CSS = 30°C, IQR = 30-30°C, Z = -0.9, p= 0.93: *An. arabiensis* KGB = 33°C, IQR = 32-34°C; Dondotha = 32°C, IQR = 31.5-32.5°C, Z = -1.3, p = 0.19).

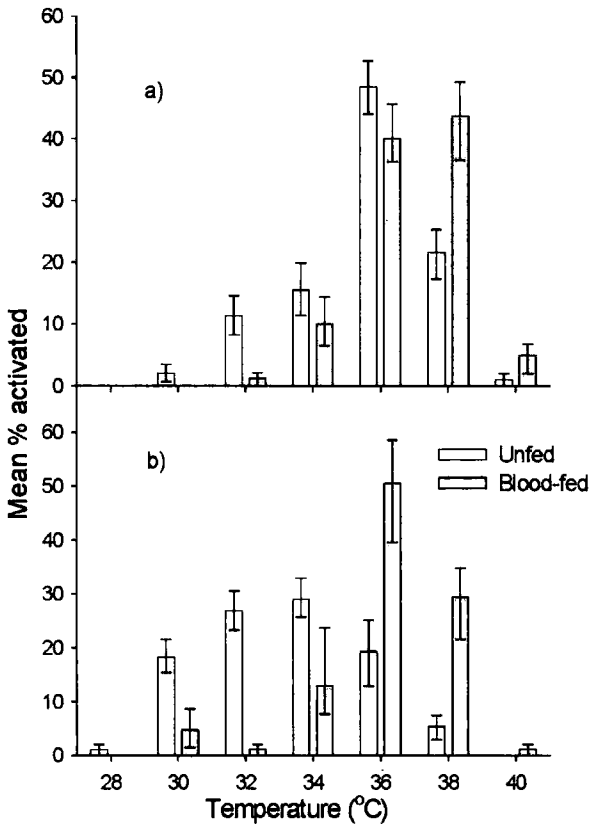


Fig 4.5 Temperature activation profiles for unfed and blood-fed a) *An. arabiensis* KGB and b) *An. gambiae s.s.* KWA. Error bars represent 95% C.I.

Climate data

The KGB *An. arabiensis* strain studied was originally collected from the Kanyemba Zambesi Valley, Zimbabwe where *An. gambiae s.s.* is not present. At Kanyemba the mean daily maximum temperature is 32.3°C and temperatures in September to November regularly exceed 34°C (World Meteorological Organisation, WMO, weather station 677670, data for September 1989 – October 1997). On the other hand the *An. gambiae s.s.* strain KWA is derived from wild stock near Tanga, Tanzania. At Tanga, the mean daily maximum is more than two degrees lower (29°C) and maximum temperatures above 34°C have only been recorded on 18 days in 17 years (WMO weather station 638440, data for Dec 1978 - May 1995). WMO climate data is very limited for Dondotha, South Africa, origin of the Dondotha strain of *A. arabiensis*, and for Lagos, Nigeria, origin of the 16CSS strain of *An. gambiae s.s.*, and was not evaluated.

Discussion

These findings show that adult *An. arabiensis* are more tolerant of high temperatures than *An. gambiae s.s.* When the holding tube temperature was progressively increased, unfed 3-6 day old *An. arabiensis* consistently tolerated temperatures 2.7°C higher than *An. gambiae s.s.* Nearly all *An. arabiensis* treatment groups avoided temperatures at or above 36.0°C, in contrast to *An. gambiae s.s.* of which almost 60% were activated at or before 34.0°C. This difference in response between these closely-related species is impressive considering the relative homogenous nature under which these laboratory-adapted strains have been maintained for many years. The fact that this behaviour is the same for different strains of the same species supports the notion that an innate difference in thermotolerance exists between the two species. This suggestion is supported by the findings of chapter III, namely that *An. arabiensis* is more capable than *An. gambiae s.s.* of surviving periods of exposure to extreme temperature (40°C).

These findings reflect what is known of the geographic and temporal distribution of the two sibling species. *Anopheles arabiensis* is more common on the fringes of the *An. gambiae sensu lato* range, being endemic in the hotter and drier savanna areas of the African continent (Coluzzi *et al.*, 1979; Lindsay *et al.*, 1998; Coetzee *et al.*, 2000). *An. gambiae s.s.* by contrast is more prevalent in humid savanna and forest regions (White, 1974; Rishikesh *et al.*, 1985; Lindsay *et al.*, 1998; Rogers *et al.*, 2002). Where the two species are sympatric *An. arabiensis* is more frequently found in the dry season, whilst *An. gambiae* predominates in the wet season (White *et al.*, 1972; Lindsay *et al.*, 1991). This situation is mirrored by the climate for the sites of origin of the studied strains; the KGB strain of *An. arabiensis* originates from the hot Kanyemba Zambesi Valley where *An. gambiae s.s.* is not present.

Both species exhibit a high degree of anthropophily, which dictates that they should spend a large proportion of their time in close human contact (Costantini *et al.*, 1999). Nevertheless it is apparent that even the most domestic mosquitoes spend time outdoors in daytime as well as at night (Githeko *et al.*, 1996). *An. gambiae s.l.* has been found resting in hollows under stones and in embankments (Muirhead-Thompson, 1951), and in Madagascar *An. arabiensis* is known to be exophagic (Rallisoa Randrianasolo and

Coluzzi, 1987). Of the two species, *An. arabiensis* is the more exophilic (White, 1974; Coluzzi, 1984; Mnzava *et al.*, 1995; Githeko *et al.*, 1996). *An. arabiensis* may therefore experience higher temperatures more frequently than *An. gambiae s.s.*, since mean maximum outdoor air temperatures are often greater than indoor temperatures (De Meillon, 1934; Yohannes, 2002). In Kisumu, Kenya, outdoor temperatures are 6°C higher than indoors in the dry season (Haddow, 1942). The highly endophilic nature of *An. gambiae s.s.* protects the mosquito from the highly variable and more extreme external climate. The endophily of this species may reflect its evolutionary past since it is thought to have originally developed from a forest dweller (Coluzzi *et al.*, 1985), adapted for a more benign and less extreme climate . The proliferation of settled human communities in Africa over the last 7,000 years (Roberts, 1998) created an extremely favourable environment for *An. gambiae s.s.*, namely a reliable food source and a stable thermal environment inside houses that resembled the forest climate (Costantini *et al.*, 1999). *An. arabiensis* by contrast is more catholic in its feeding habits, taking bloodmeals from a range of animals. This flexibility in feeding preference may promote adaptation to the more extreme temperatures encountered outdoors.

An. arabiensis and *An. gambiae s.s.* are able to detect and respond to increasing temperature by moving away from extreme heat. The fact that both species move away from temperatures several degrees lower than 40°C may seem premature, given that they are capable of surviving for short periods of time at such an extreme (see chapter III). However at temperatures over 38°C 96% of *An. gambiae* s.s. and 74% of *An. arabiensis* are knocked down and do not escape. The behavioural avoidance experiment tested a behaviour that mosquitoes are only likely to perform as a last resort – it is an escape mechanism. In nature this probably results in short distance flights to seek cooler spots, typically the shaded resting sites under vegetation outdoors or cool dark corners indoors. For *An. arabiensis*, being activated by rapidly rising temperatures outdoors will most likely expose the insect to an increased risk of desiccation exhaustion and higher predation risks. A higher thermal tolerance may therefore minimise risky shade-seeking behaviour in this species. For *An. gambiae s.s.*, becoming active at lower temperatures than *An. arabiensis* is less of a problem, provided the insect does not stray outdoors where it is liable to experience much hotter conditions. The ability to respond to microclimatic changes depends on the age of the insect and its stage of the gonotrophic cycle. Much higher temperatures were required to initiate

activation in recently-emerged mosquitoes than in older ones. Higher temperature tolerance in young adults is common in Diptera and has been demonstrated for blowflies (Davison, 1969) and *Drosophila* (Bowler and Hollingsworth, 1966) amongst others. In mosquitoes it probably occurs because there is little flight activity in the first few hours after eclosion (Gillett, 1971). Eclosion is an energy-expensive process, and rest after emergence is necessary to allow for the hardening of the cuticle (Gullan and Cranston, 2000). Blood-fed mosquitoes were the least responsive to increasing temperature. Whilst only 1.6% of unfed mosquitoes were knocked down as temperatures rose, 15.5% of bloodfed mosquitoes failed to escape and were knocked down. The reluctance of bloodfed mosquitoes to fly, even when temperatures became lethal, reflects the behaviour of this feeding stage in the wild. A blood-fed mosquito is not only typically 80-200% heavier when fed (Clements, 1992), its energy is also largely invested in utilising the blood to produce eggs. For *An. gambiae s.l.*, remaining indoors after a bloodmeal may reduce the exposure to attack by predators, parasites and parasitoids. Blood-fed mosquitoes are more visible and slower flying, increasing the likelihood of being preyed upon or killed by their host (Roitberg *et al.*, 2003). Muirhead-Thompson (1951) found similar trends in temperature sensitivity in *An. minimus*. Female temperature avoidance was most pronounced in hungry females (which avoid temperatures above 25°C), less strong in blood-feds (above 30°C) and least strong in newly emerged females (above 32°C).

This simple laboratory study illustrates pronounced differences in the way two closely related species of mosquito respond to increasing temperature. It is clearly not temperature alone that affects the behaviour or survival of the mosquitoes in these experiments or indeed in nature. As temperature rises relative humidity declines and the drying capacity of the air increases. Thus the responses of the mosquitoes to rising temperature should be seen as a response to the risk of desiccation, as well as temperature. Although these species have been reared under similar laboratory conditions for many generations, their responses to increasing temperature reflect the different environments these insects have adapted to in the wild. Thus this study helps confirm that the geographical and temporal distribution of *An. arabiensis* and *An. gambiae s.s.* across Africa is characterised by species and stage-specific adaptations to climate at the microclimatic level.

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CHAPTER V

Effect of temperature on the flight activity of *Anopheles gambiae sensu stricto* and *An. arabiensis*

Abstract

Accurate knowledge of the flight behaviour of mosquitoes is important for the prediction of their dispersal and distribution, and therefore for the targeting of control measures. *Anopheles gambiae sensu stricto* and *An. arabiensis*, two of the most important vectors of malaria, differ in their spatial and temporal distribution across Africa. Changes in the timing and degree of flight activity with temperature could be the result, or even a driving force, of adaptation to novel environments. Differences in the flight activity of these two species could therefore at least partly explain their present distribution. An electronic actograph able to continuously record the flight of *An. gambiae s.s.* and *An. arabiensis* was used to compare the flight activity of these two species over 24 hours under a 12:12 light:dark cycle at 10, 20 and 30°C. Although no differences were observed between the two species in total flight time, total number of flights made, or the timing of this activity within the 24-hour period, a trend of increasing activity with increasing temperature was apparent. Both species were largely inactive at 10°C. Mosquitoes flew on average 343 seconds longer at 30°C than at 20°C and 395 seconds longer at 20°C than at 10°C. Most flights were of short duration only, and there was no difference in the number of total flights lasting ≥ 1 second or ≥ 5 seconds made at 30°C compared with those made at 20°C. The similar flight activity of both species reflects the close phylogenetic relationship between these two members of the *An. gambiae* species complex.

Introduction

Flight plays an essential part in most mosquito behaviours, including dispersal (Thomson *et al.*, 1995), formation of mate-receptive male swarms (Charlwood and Jones, 1980; Yuval and Bouskila, 1993), host-seeking (Costantini *et al.*, 1999) and egg-

laying (Bidlingmayer *et al.*, 1974; Edgerly *et al.*, 1998). Mosquitoes are ideally adapted for flight: the elongate form with long body and long wings provides aerodynamic stability, whilst being small they have a high surface area to weight ratio and therefore do not require much wing power for propulsion and aerodynamic lift (Murty *et al.*, 1993).

In general the wing beat frequency of all mosquitoes remains remarkably steady throughout flight, irrespective of speed, varying from about 250 to 500 beats per second according to species (Gillett, 1971). The pitch, or audible flight tone, depends on the wing beat frequency and the wing harmonics (Clements, 1999) and can be used to discriminate between closely related species such as *Aedes aegypti* and *Ae. albopictus* (Brogdon, 1994). Flight tone can even characterise laboratory colonies of sympatric members of the *An. gambiae s.l.* complex (Brogdon, 1998), though field-caught specimens are less easily distinguished (Wekesa *et al.*, 1998). In the wild, flight tone may operate as a reproductive species-isolating mechanism. The acoustic and mechanical sensitivity of Johnston's sensory organ at the base of the antenna serves as a movement sound detector in male mosquitoes, sensing antennal vibrations induced by the flight sounds of conspecific females (Gopfert and Robert, 2000). This sensitivity may explain why natural hybridisation rates between different members of the *An. gambiae* complex are estimated at only 0.1% (Coluzzi *et al.*, 1979) despite the frequent formation of mixed species swarms in the field (Marchand, 1984). There is however a degree of intraspecific variation in wing beat frequency dependent on mosquito age and relative wing size. Older individuals of *An. gambiae s.s.* and *An. arabiensis* produce a lower and more varied flight tone and smaller mosquitoes have significantly lower wing beat frequencies than larger individuals (Wekesa *et al.*, 1998). Frequency, and therefore audible flight tone, is also higher in gravid and fully fed females (Ogawa and Kanda, 1986).

Flight tone has been recorded as an indicator of activity by acoustic actographs for over 40 years. In the original system first developed by Jones (1964), batches of 5 or10 female mosquitoes were kept in activity chambers, and flight sound was recorded by a microphone that created a signal which in turn controlled an event-recorder pen on a strip-chart (Jones *et al.*, 1966). Activity was scored as the number of minutes within each 30-minute period that contained at least one flight. More recent systems have been

refined to monitor the activity of individual mosquitoes simultaneously (Jones *et al.*, 1974), to record the total elapsed flying time per unit of time (Peterson, 1980) and to make use of computer technology to analyse data directly (Rowley *et al.*, 1987). Actographs have been used to study the flight activity of a range of mosquito species including *An. gambiae* s.s. (Jones *et al.*, 1967) and *Ae. aegypti* (Jones, 1981).

Photoperiod, insect age, degree of starvation and temperature are factors known to effect flight activity. Different mosquito species show different patterns of activity regulated endogenously and in phase with the cycle of light and darkness. In fact the activity of mosquito species can be broadly categorised as diurnal e.g. *Ae. aegypti* (Haddow and Gillett, 1957; Corbet and Chadee, 1990), *Ae. albopictus* (Trexler *et al.*, 1997), nocturnal e.g. *An. gambiae s.l.* (Jones *et al.*, 1967), *Culex quinquefasciatus* (Beehler *et al.*, 1993) and/or crepuscular e.g. *Ae. africanus* (Gillett, 1971). Some species exhibit just one daily activity maximum, such as *Haemagogus spegazzini* (Almeida and Gorla, 1995), while in others two peaks of activity are seen occurring around dusk and dawn such as *Ae. aegypti* and *An. stephensi* (Jones and Gubbins, 1979). These peaks usually represent oviposition-site seeking or mating followed by host-seeking behaviour.

Newly-emerged virgin *Ae. aegypti* females are relatively inactive, capable of flying only 0.5km before exhaustion. However activity increases substantially during the first few days of adult life with maximal flight performances often occurring within the first and third day of eclosion (Rowley and Graham, 1968; Jones, 1981; Briegel *et al.*, 2001). Starved females cannot sustain long periods of activity: the maximal flight distances of *An. gambiae s.s.* were 9 km when sugar-fed and 10 km when blood-fed, while in starved females it was below 3 km (Kaufmann and Briegel, 2004). Mosquito flight performance can also depend on the type of carbohydrate they are fed (Nayar and Sauerman, 1971).

Like photoperiod, environmental temperature behaves in a relatively predictable way and can be used to entrain the free-running, or circadian, rhythm of activity, though the relationship can be complex. For example, in *Cx. pipiens pallens* flight and locomotor activity may be controlled by two circadian oscillators moving in different directions in response to the temperature cycle (Chiba et al., 1993). In this way temperature affects the timing of flight activity. Temperature can also affect the degree of activity. The

flight muscles of many insects cannot function efficiently at low temperatures and so logically, species adapted to colder climates are more active at lower temperatures than those adapted to warmer conditions (Clements, 1999).

Changes in the timing and degree of flight activity could be the result of speciation and adaptation to novel environments or conditions. Differences in the flight activity between closely-related species may have even been a contributory force to the speciation process (Jones *et al.*, 1974). *An. gambiae s.s.* and *An. arabiensis*, two closely-related species, differ in their distribution as described by temperature and rainfall parameters. *An. arabiensis* predominates in arid regions while *An. gambiae s.s.* is more abundant in more humid locations (Lindsay *et al.*, 1998). Here I investigated the hypothesis that *An. arabiensis* is more active at higher temperature than *An. gambiae s.s.* The effect of temperature on the timing of activity is also explored.

Materials and Methods

The actograph consisted of 24 glass tubes each with a detection unit that routed activity signals generated by mosquito flight inside the tube to an interface card in a computer. A software programme logged activity over a 24 hour period.

Detection unit design

Chris Moore and Tom Jackson (University of Durham Physics Department) were largely responsible for the detection unit design described here. These units recorded flight activity of the mosquitoes whilst reducing the triggering of the data-logging software by sounds produced from other sources. To achieve this, a circuit was designed to filter out the audio frequencies outside of those produced by the wing-beat of both species. The circuit also restricted the number of logs by not responding to short periods of wing movement that may not represent flight.

The sound produced by the wing movements of *An. gambiae s.s.* and *An. arabiensis* was detected by an electret microphone cartridge (sensitivity 7.9mV/Pa/1kHz). The output of the microphone passed to a high gain microphone preamplifier (Maxim MAX 4465Exk). The resulting amplified signal passed through a clock tuneable, quad second

order filter building block (Linear Technology LTC1068-200) configured as a 10^{th} order elliptical bandpass filter, with a centre frequency of 900Hz, pass band width of 850Hz, stopband width of 1500Hz and a stopband attenuation of 40dB. The bandpass and stopband values were chosen to accommodate the range of frequencies produced by adult female mosquitoes of both species.

The filtered signal connected to a comparator (Maxim MAX999) with a trimable offset, allowing the sensitivity of the circuit to be adjusted. The output of the comparator produced a burst of oscillations in sympathy with the sound produced by a flying mosquito. This burst fed a variable integrator that stopped the detection units from responding to very short bursts of wing activity. If the activity is of sufficient duration the integrator triggers a timer (National Instruments LM555 Timer) configured as a re-triggerable monostable, with a minimum on-time of approximately 500 milliseconds. The output from the timer connected to one input of a twenty-four way, custom-made interface card (based on the Intel® 8255A), mounted inside a Pentium® Pro (Intel®) computer. The components for each detection unit were assembled onto a 45 x 50mm printed circuit board (Faraday Printed Circuits, Washington, U.K.) housed in a moisture-proof case and mounted on the top of the rubber bung containing the microphone (fig 5.2a). Each detection unit had its own on-board voltage regulation circuitry. Two 550mA switched mode plug-in power supplies provided the power to the 24 individual detection units via a common cable connection interface. The cable interface supplied power to each detection unit and routed the activity signals to the interface card via 24 individual screened cables. All electronic parts were supplied by Digikey® (Minnesota, USA), Summit Electronics (RF Components, Florida, USA) or Farnell Onecall (Premier Farnell UK Limited, Leeds, UK) unless otherwise stated.

In-house software using Labview 6.1 (National Instruments Corporation, Austin, USA) logged the start time and duration of flight, in multiples of 1 second, on each of the 24 channels. The software allowed the selection of a channel as a control to monitor unwanted noise and suppress erroneous logs on the remaining channels. At the end of a logging session the results were formatted and downloaded to a Microsoft® Excel spreadsheet. Data were analysed in SPSS for Windows v11.5 (SPSS Inc.).

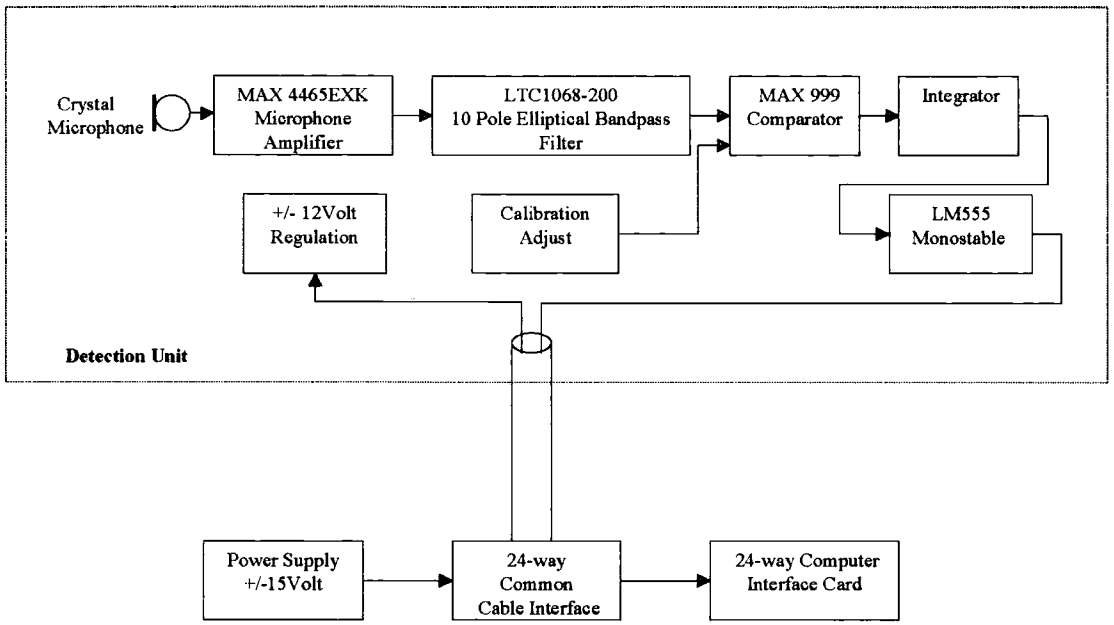

Fig 5.1 Schematic of actograph detection unit

Experimental set-up and procedure

The microphone cartridge was mounted flush into a rubber bung (diameter 43mm at bottom and 49mm at top, SLS, U.K.) pushed into the top of a 130mm long glass tube of 45mm internal diameter (fig 5.2a & b). Heat shrink material was fitted to the bung to reduce the gap between the bottom of the bung and the sides of the tube, preventing the mosquito resting in this gap above the microphone. The glass tube was fitted with a sintered glass filter of 10mm diameter that allowed the passage of air into the tube whilst acting as a baffle to any external sound. The tubes were mounted in 4 racks of 6 and stood inside programmable LMS incubators (S.H. Scientific, Kent, U.K.) in plastic trays approximately 35 long x 25cm (fig5.2d). 500ml of potassium hydroxide (KOH) solution was poured into each tray to maintain relative humidity in the tubes at 60%RH independent of tube temperature. Incubator temperature was set to 10, 20, 30 or 35°C and so 50, 45, or 40g KOH/100ml dH_2O was used respectively (Solomon, 1951).

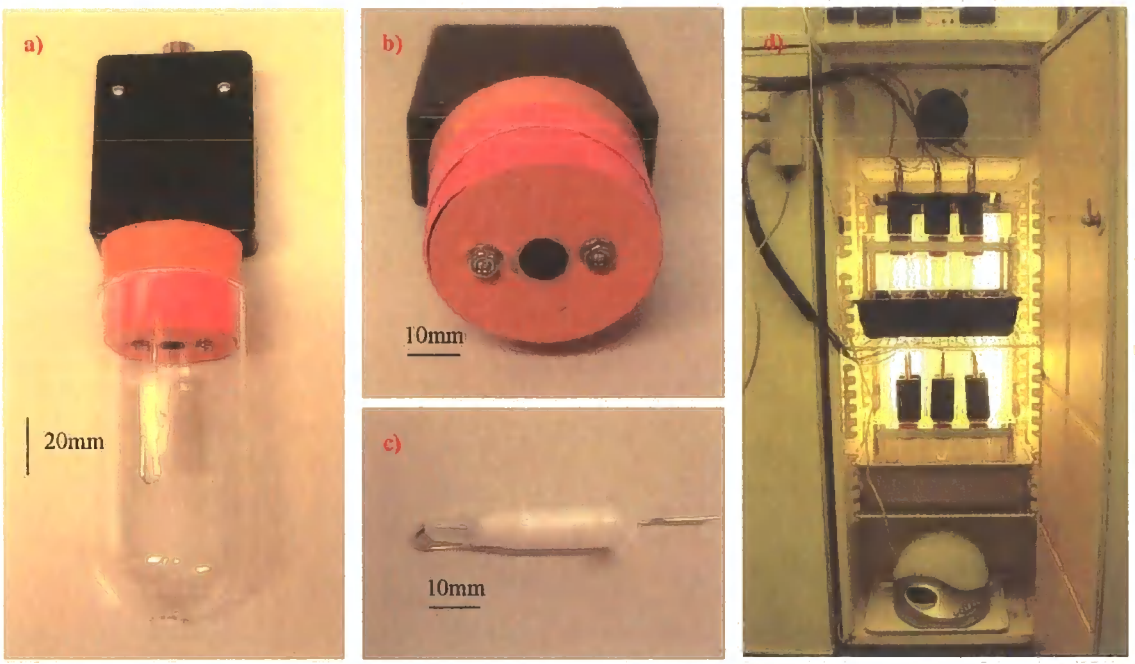

Fig 5.2a) Glass tube and detection unit, b) close-up of microphone, c) glucose feeder, d) actograph *in situ* (Lumie™ at bottom)

A 20mm layer of high-density foam was placed underneath each tray and also beneath the incubator fan to help reduce any noise interference from the lighting, fan or shelf vibrations within the incubator. The tube racks were lit with a cold light source from behind (fig5.2d), so the foam layers did not reduce the amount of light reaching each tube. A programmable dawn/dusk simulator (Lumie, Outside In Ltd, Cambridge, U.K.) produced a crepuscular period of 90 minutes before the incubator lights came on at 06.00hrs and immediately after incubator lights went off at 18.00h.

Mosquitoes were introduced with an aspirator into the glass tubes, one in each tube. Each rack contained six individuals of one species, and one rack of each species was put into each incubator. Three day old female mosquitoes were used because during the design process it was found that the wing-beat frequency produced by older mosquitoes was highly variable and subsequently less well detected. Activity was also maximal in 3 day old insects. One tube in each incubator was kept empty as a control, and the corresponding channel designated as a control by the software. Tube humidity and temperature were monitored using a pen-type digital humidity meter (RS Components

Ltd, Northamptonshire, U.K.) and a digital thermometer (probe type T, Digitron Instruments, U.K.) respectively. Measurements were taken at the beginning and end of each experiment. Cotton wool soaked in 1ml of 10% glucose solution was suspended in a 1.5ml microfuge tube ~1cm below the microphone by a small length of wire, to provide a food source for the mosquito (fig5.2c). One hour was allowed for the mosquitoes to adjust to their surroundings before recording commenced. The total recording time for each trial was 36 hours, though only the first 24 hours were analysed. Mosquitoes that died during the 36 hours were not included in the analysis because flight behaviour became erratic before death.

The wing length and insemination condition of all mosquitoes were determined. Wings were measured as outlined in chapter II. Insemination status was established by dissecting out the single spermatheca under a stereo microscope (x10 magnification), transferring it to a clean glass slide in a droplet of phosphate buffered saline (PBS) and squashing it under a cover slip. The presence of sperm in the spermatheca was established by observation under higher power (x40). Virgin females were excluded from analysis as inseminated *An. gambiae s.s.* have been shown to be much more active throughout the scotophase (period of darkness) than virgins of the same age (Jones and Gubbins, 1978).

Statistical analysis

Analysis was undertaken with SPSS software version 11.5. Over-dispersed data were corrected by log10 +1 transformation and analysed by Univariate statistics (general linear model) to establish differences in total flight time and total number of flights between species and temperatures. Individual flights of less than one second were not included in the analysis. Tukey's honestly significant difference (HSD) post-hoc test was used to explore the results of the univariate analysis. Results of these tests are displayed as geometric means. Analysis of the data by half-hourly period introduced a further degree of variability in the data that could not be normalized. Half-hourly data is therefore not analysed statistically and is displayed, as median median flight length, only to provide an image of the timing of flight activity. Night-time activity summarized by 6-hourly periods was normalized by log10 + 1 transformation and the activity of the two species during these periods compared using an independent samples t-test. A t-test

was also used to compare the mean wing length of the two species. The effect of wing length on transformed total flight time and transformed total number of flights was assessed using a linear regression.

Results

Only 20% (11/55) of *An. arabiensis* and 14.5% (8/55) of *An. gambiae s.s.* were alive in the actograph tubes after 36 hours at 35°C so data from experiments at this temperature were not analysed. Survival at all other temperatures exceeded 60% in both species.

Insemination rate

88% (206/235) of the surviving females were inseminated. The flight activity of these mosquitoes was analysed.

Flight activity

There were no significant differences between the flight activities of *An. gambiae s.s.* and *An. arabiensis* (table 5.1, 5.2). Temperature had a significant effect on the total time spent flying (table 5.1, fig 5.3) and the total number of flights made (table 5.2, fig 5.4) by both species during a 24-hour period. Both species were largely inactive at 10°C. A trend of increasing total flying time with increasing environmental temperature was observed. Mosquitoes flew on average 343 seconds longer at 30°C than at 20°C (Tukey HSD, $p = 0.04$) and 395 seconds longer at 20°C than at 10°C (Tukey HSD, $p<0.001$). Most flights were of short duration only, and there was no difference in the number of total flights lasting ≥ 1 second or ≥ 5 seconds made at 30°C compared with those made at 20°C (p = n.s.). Mosquitoes made on average 91 (63-132) more flights lasting ≥ 5 seconds at 30°C than at 10°C ($p <0.001$) and 87 (59-126) more flights at 20°C than at 10°C ($p<0.001$).

Table 5.1 Univariate analysis of variance (type III sum of squares) of the interactions of temperature and species on the total flight length of *An. gambiae s.s.* and *An. arabiensis* in a 24 hour period.

Factor	df	Type III sum of squares	F	p
Species	1	0.4	1.0	n.s.
Temperature	2	227.3	275.3	<0.001
Species x Temperature	2	0.07	0.1	n.s.

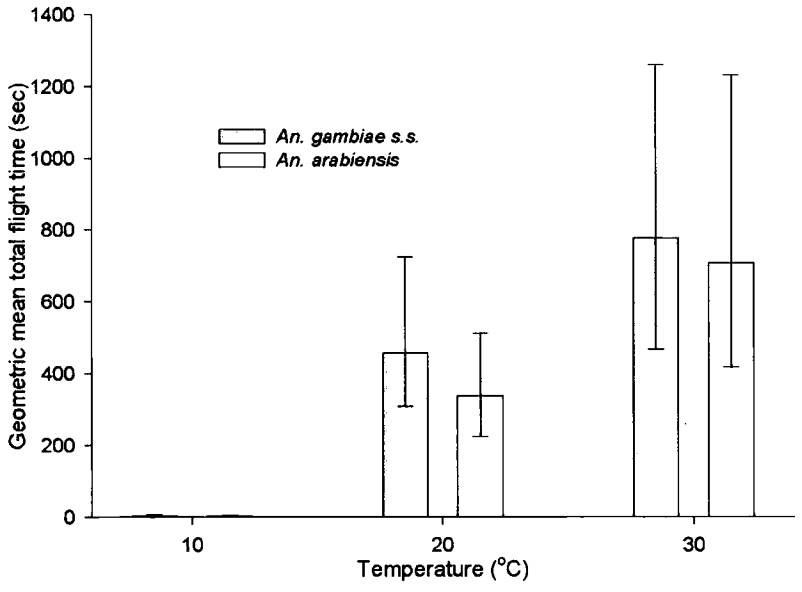

Fig 5.3 The effect of temperature on the total time spent flying by *An. gambiae s.s.* and *An. arabiensis* in a 24-hour period. Bars represent 95% Confidence Intervals.

Table 5.2 Univariate analysis of variance (type III sum of squares) of the interactions of temperature and species on the total number of flight events of *An. gambiae s.s.* and *An. arabiensis* in a 24 hour period.

Flight event length	Factor	df	Type III sum of squares	F	p
≥1 second	Species	1	0.01	0.05	n.s.
	Temperature	2	173.7	311.7	<0.001
	Species x Temperature	2	0.03	0.06	n.s.
≥5 seconds	Species	1	0.01	0.06	n.s.
	Temperature	2	157.8	569.1	<0.001
	Species x Temperature	2	0.02	0.08	n.s.

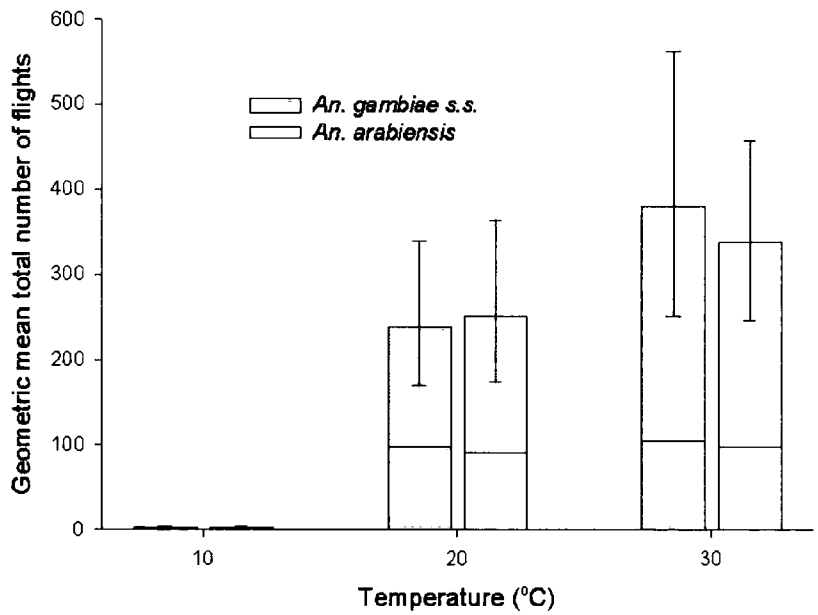

Fig 5.4 The effect of temperature on the total number of flights lasting ≥ 1 second of *An. gambiae s.s.* and *An. arabiensis* in a 24-hour period. The horizontal line across the bar designates the geometric mean number of flights lasting ≥ 5 seconds. Error bars are 95% Confidence Intervals of flights lasting ≥ 1 second.

Timing of flight activity

The main period of concerted flight activity occurred between 18.00h and 06.00h in both *An. gambiae s.s.* and *An. arabiensis* (fig 5.5). This was the case irrespective of temperature, although at 10°C activity was minimal throughout the 24 hours.

Mosquitoes were much less active during the light period, especially during the hours following a burst of activity close to light-on (06.00h). At 30°C if one examines the median median time flight time for each flight by half-hourly period, *An. gambiae s.s.* appears to be more active in the first 6 hours of the dark period and *An. arabiensis* more active in the second 6 hours. In *An. arabiensis* a secondary peak of activity can be seen 8-9 hours after light-off (fig 5.5).

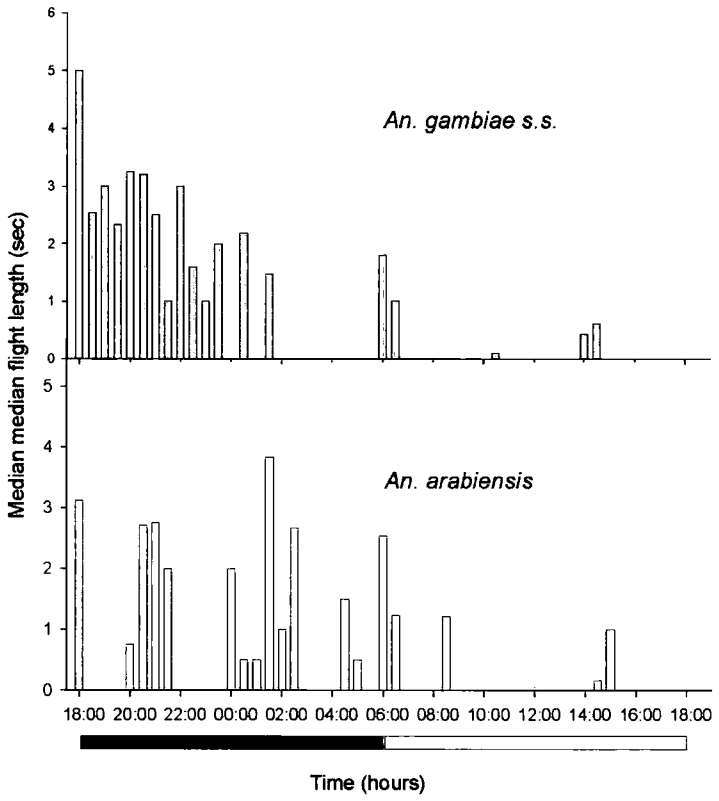

Fig 5.5 Median median flight length of *An. gambiae s.s.* and *An. arabiensis* by half-hourly period at 30°C. Bar under graph represents the dark and light periods.

There is however no significant difference in total flight time, a better measure of activity than time flown per flight, between the two species in either of these 6 hour periods (18.00-23.59h geometric mean *An. gambiae s.s.* total flight time = 343 (95% Confidence intervals, CIs = 231-635) seconds, *An. arabiensis* = 287 (160-435) seconds, t = 1.9, df = 68, p = n.s.; 00.00-05.59h *An. gambiae s.s.* total flight time = 313 (190-424) seconds, *An. arabiensis* = 405 (289-611) seconds, t = -1.6, df = 68, p = n.s.).

Wing length

The *An. arabiensis* cohort used in this experiment was significantly larger than that of *An. gambiae s.s.* (wing length for *An. arabiensis* = 2.90 (95% CIs = 2.87-2.94), *An. gambiae s.s.* = 2.81 (2.78-2.85); df = 204, t = 3.6, $p<0.001$). No inter- or intra-specific relationships between wing length and total flight time or total number of flights were significant (for all linear regressions $p>0.05$).

Discussion

Flight activity in small ectothermic insects such as mosquitoes is highly dependent on the effect of ambient temperature on core temperature (Foster and Robertson, 1992; Clements, 1999). In this study it has been shown that the flight activity, as measured by total flight length, of *An. gambiae s.s.* and *An. arabiensis* is maximal at 30°C, declines by 40-50% at 20°C and by 95% at 10°C. As adults both species have an optimal temperature range between 22-28°C (Gillies and De Meillon, 1968) and show optimal survival between 15-25°C (Bayoh, 2001).

The range of temperatures at which mosquitoes are active depends on the conditions to which they are adapted; Arctic species have a lower temperature threshold for activity than tropical species. *Ae. punctor* in Sweden is fully active at 4°C (Jaenson, 1988) and *Ae. impiger* and *Ae. nigripes* exhibit swarming behaviour at 6°C (Nielsen and Nielsen, 1962). However even these Arctic mosquito species are inactivated by temperatures below 5°C, which can prove lethal (Corbet and Danks, 1973). By contrast most tropical mosquitoes are not exposed to fatally low temperatures. However at temperatures below 15°C activity in several species is depressed greatly. Lowering the temperature from 25 to 20°C reduced the nocturnal activity of *Cx. pipiens pallens*, and at 15°C activity was all but eliminated (Chiba et al., 1982). In Florida *Cx. tarsalis* exhibits a similar behaviour, flying and feeding only when temperatures exceed 13°C (Bidlingmayer, 1974). In northern India winter room temperatures do not fall below 10°C and *An. culicifacies* has been found over-wintering indoors; however activity in these adults is extremely low (Muirhead-Thompson, 1951). At such temperatures the development and dispersal of insect populations may be slowed to such an extent to prevent population

survival and/or transmission of vector-borne diseases (Cox and Dolder, 1995). It seems likely that the activity at 10°C in this study is too low to allow dispersal or successful host-seeking, and that therefore neither species would survive for any length of time under such conditions. Activity at extreme high temperatures was not determined in this study as mortality was too high above 30°C to allow 24 hours of continuous recording from a large enough sample of mosquitoes. Evidence from the field suggests a relative inactivity of these species at high temperatures in the dry season (Omer, 1970) though this inactivity may be more of a result of the restrictive nature of low humidity at this time of year. The endophilic behaviour of *An. arabiensis*, and to a greater extent *An. gambiae s.s.*, will favour survival during periods of unusually cold or hot environmental temperatures, because the indoor climate is more stable than outdoor fluctuations in minimum and maximum temperatures (Muirhead-Thompson, 1951). By seeking out refugia with more suitable microclimates, both species can maintain the normal cycle of activity (Smith, 1958; White, 1974; Charlwood *et al.*, 2000).

Though the work of Jones *et al.* (1967; 1974) previously examined the pattern of activity in these two vector species, the effects of temperature were not investigated, all experiments being conducted at 25°C. It seems that the only other study of circadian activity at a range of temperatures in an actograph system involved the corn ear-worm moth *Heliothis zea* (Hsiao, 1978). However the results do bear similarity to those presented here. Hsiao (1978) found moth activity to be temperature dependent. Moths were active for only 4% of the time at 10°C but were almost 10x more active at 32°C. More frequent and longer lasting flights were responsible for this increase. That the activity of both *An. gambiae s.s.* and *An. arabiensis* increased with temperature is further supported by data from trapping studies in which day-to-day mosquito abundance of a range of species is positively correlated with air temperature (Almeida and Gorla, 1995; Guimaraes *et al.*, 2000).

The timing of activity in this study of both *An. gambiae s.s.* and *An. arabiensis* appears to be determined entirely by the circadian rhythm entrained to the light:dark cycle. Though temperature affected the intensity of flight activity, it does not seem to have had an effect on the timing of flight activity in either species. A similar effect of temperature has been recorded in the tsetse fly *Glossina morsitans* in which the amplitude of the rhythm of morning and evening activity changes according to temperature, but not the

bimodality (Brady and Crump, 1978). In the field, biting activity and oviposition in *An. gambiae s.s.* and *An. arabiensis* are essentially nocturnal (Haddow and Ssenkubuge, 1962), with activity peaking between midnight and 06.00 hours. The daily activity of many insect species occurs during this period, possibly because at these times relative humidity reaches its maximal daytime values (Barrozo et al., 2004). It would have been interesting to vary humidity levels in this study but it proved difficult to maintain constant levels within the tubes. There is evidence that humidity does not affect flight activity under suitable temperature conditions (Rowley and Graham, 1968).

In this study the cycle of activity for both species probably reflects host-seeking behaviour. Activity commences with a period of 30-60 minutes immediately after light-off, during which total activity and median flight length are high. Jones *et al.* (1974) found a similar pattern in individual mosquitoes of *An. gambiae s.s.* and *An. arabiensis* which were almost continually active for 15-20 minutes following light-off. The initiation of activity may be a startle response to the sudden change in light intensity, but the length of the activity and the evidence that this particular period of activity persists even after days in total dark (Jones et al., 1967) suggests that activity at this time is under circadian control. In experimental light regimes with gradual dusk and dawn transitions controlled with a dimmer switch, bursts of activity are also still seen at those times (Jones and Gubbins, 1979). It may instead represent a period of pre-feed activity, which can occur in the field as much as four hours before feeding takes place. Such activity has been recorded in *An. gambiae s.s.*, *An. funestus* and *Coquillettidia fuscopennata*, which move from often unidentified daytime resting sites to the outside and inside of house walls (Gillett, 1971; Gillies, 1988). This activity is usually performed in dusk and early hours of darkness in the field by *An. gambiae s.l.* (Smith, 1958).

After the initial burst of activity at dusk, flight activity drops off in *An. arabiensis* until 20.00 hours with further sporadic bursts of activity in the early hours of the morning. These mosquitoes were unfed and so no oviposition peak was anticipated. However a second peak occurs in inseminated *An. stephensi* females that have not been bloodfed (Rowland, 1989) and this may be true of *An. arabiensis* here. Activity in *An. gambiae s.s.* is more concentrated and continues more or less constantly after the light-off period until ceasing around 01.00 hours. Jones *et al.* (1974) found activity in *An. gambiae s.s.*

dropped off sharply after the initial activity and remained low for the rest of the scotophase. They found that activity in *An. arabiensis* was much more pronounced throughout the dark period, peaking 4-6 hours after light-off. There is a peak again in both species shortly after light-on at 06.00 hours. In this case a startle response is the most likely cause. Jones *et al.* (1967) found that in constant dark the activity following light-on is lost and that light otherwise appears to have an inhibitory effect on activity. Surprisingly the use of a transitional change in light in the present study did not remove the activity peak at light-on. It is possible that the startle response is to the noise generated by the incubator light coming on and not the light itself. An alternative explanation may be that the transitional light source used in this experiment is of a different intensity from that which was produced by the incubator lights. The occurrence of late afternoon activity, between approximately 15.00 and 17.00 hours, was not expected and may be the result of human activity in the insectary room housing the actograph. Routine maintenance of a locust colony in the room occurred daily at around this time.

Jones *et al.* (1974) report a large heterogeneity of variance between individuals in the onset of a period of sustained activity. A high degree of individual variation in activity throughout the recording period is apparent in this study. During periods of overall activity most mosquitoes flew for only some of the time and only a few were extremely active. Furthermore the flights of individual mosquitoes were of short duration in the main with a few significantly longer flights.

By presenting data as a score of 1 or 0 for activity in each minute of recording, Jones *et al* (1974, 1967) suppress this level of variation to a certain extent. However the advantage of the actograph system used here is the recording of flight length for every flight made. This allows data to be presented as total flight time, a better indicator of activity than either number of flights or time per flight, as it is a factor of both of those measures. The data handling system of Jones *et al.* may well be more biased towards startle response activity and also periods where mosquitoes were moving their wings but not flying, a criticism made by Powell *et al.* (1966) who suggested flight activity was over-estimated in the actograph system.

In recording any wing movement lasting longer than 0.5s, the actograph chamber system is biased towards recording trivial flights. Using a flight mill system, Kaufmann and Briegel (2004) recorded *An. gambiae s.s.* and *An. atroparvus* flight performances of 1-4 hour intervals of continuous flights, but mainly of bouts shorter than one hour, randomly distributed during a long flight trial. This is comparable to the activity of *Ae. aegypti* in which periods of continuous flights lasted on average 2.2 hours (Briegel *et al.*, 2001). By contrast the average flight lengths recorded in this study are of a few seconds only and the longest individual flight recorded was 220 seconds by *An. arabiensis* at 30°C. At a speed of 1.2km/h (Kaufmann and Briegel, 2004) this flight would cover just 73 metres. However it is important to realise that in flight mills mosquitoes respond to a loss of tarsal contact and thus flight activity is continuous until flight reserves are depleted (Rowley and Graham, 1968). Flight mills therefore have little meaning in behavioural, as opposed to physiological, terms (Clements, 1999). Activity and distances traveled by *An. gambiae s.s.* and *An. arabiensis* in the field may in fact occur in short bursts similar to those seen in the actograph. In Burkina Faso *An. gambiae s.l.* daily activity was estimated at 350-650m using mark-release-recapture techniques (Costantini *et al.*, 1996). Interestingly the amount of *An. gambiae s.s.* recaptured within 0.2km of the release site was similar to that of *An. arabiensis*, but at a greater distance almost three times as many *An. arabiensis* than *An. gambiae s.s.* were recovered (Costantini *et al.*, 1996). In the present study there is no difference in the flight distance; *An. arabiensis* flies on average roughly 240m and *An. gambiae s.s.* 260m in 24 hours at 30°C and at 1.2km h^{-1}.

An. gambiae s.s. and *An. arabiensis*, as phylogenetically closely related species that occupy different environmental habitats, offer a good model to assess daily rhythmic behaviour from an evolutionary standpoint. Shinkawa *et al.* (1994) found differences between the activity patterns of *Cx. pipiens molestus* strains from Iran, Egypt and Japan, some showing a continuous basal activity level during photophase and others only active during the dark period. It is tempting to suggest that this divergence represents adaptation to local climatic conditions. That this study has revealed no differences in intensity and periodicity of flight activity between *An. gambiae s.s.* and *An. arabiensis* hints at the propinquity of their speciation, and suggests that any differences which might exist in their natural flight activity are not responsible for present day distributional differences. Of course it is also possible that the uniform conditions of

their breeding and rearing have removed the traces of natural variability in activity that reflect prevalent climatic conditions.

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CHAPTER VI

The influence of temperature on the survival and development of larval *Anopheles gambiae sensu stricto* and *An. arabiensis* when reared as single- and mixed-species populations

Abstract

The two African malaria vectors *Anopheles gambiae sensu stricto* and *An. arabiensis* are sibling species that occupy different climate niches. Differences in the survival and development of the aquatic larval stages of these species at different temperatures could underpin adult distribution. The development time from first instar larva to adult at constant water temperatures was measured in *An. gambiae s.s.* and *An. arabiensis*. The two species were reared at three temperatures (25, 30 and 35°C) in the same container and also independently from one another. Larval density and the provision of food were kept constant throughout. Survival to adult is highest in both species at 25°C and decreases with increasing temperature. More adult *An. gambiae s.s.* developed at 25°C than *An. arabiensis* (80% I.Q.R.(78-88) vs 68% (63-78)) but this situation was reversed at 35°C (7% (3-17) vs 33% 27-32)). The survival of *An. gambiae s.s.* when reared alone is similar to that when reared in the presence of *An. arabiensis*. In marked contrast *An. arabiensis* suffers reduced survival when raised with *An. gambiae s.s.* at 30°C (20% (7-57)) than when reared independently (57% (45-72)) Mean age at eclosion and adult size decreased for both species with increasing water temperature: *An. arabiensis* larvae developed at a slower rate and resulted in larger adults than *An. gambiae s.s.* throughout. The apparent greater production of *An. arabiensis* at extreme water temperatures and *An. gambiae s.s.* at lower water temperatures may in part explain the spatial and temporal distribution of the two vectors in Africa.

Introduction

Water temperature is critical to key life-fitness parameters of the aquatic larval stages of many insect species (Baba and Takaoka, 1991; Joshi, 1996; Tun-Lin *et al.*, 2000). Behaviour, survival, rate of development, emergence time, adult size and gender are all to some degree affected by temperature (Ivanova, 1940; Pritchard and Mutch, 1985; Lyimo *et al.*, 1992; Hadi and Takaoka, 1995). Of these factors, survival to adult and development rate probably have the most influence on the recruitment of new individuals to insect populations, and are therefore the most important considerations in studies of insect population dynamics.

Insect larvae, like all organisms, survive within a range of temperatures (the critical thermal maximum and minimum) dependent on the evolutionary thermal experience of the species, and that of an individual during its lifetime (Cossins and Bowler, 1987). Tropical and sub-tropical mosquito larvae are necessarily warm-tolerant and cold-sensitive. Survival in such larvae tends to be highest between 20-30°C and declines rapidly either side of this range (Shelton, 1973; Tun-Lin *et al.*, 2000; Bayoh and Lindsay, 2003). By contrast larvae of the sub-Arctic mosquito species *Aedes impiger* and *Ae. punctor* only survive at temperatures between 3-16°C (Haufe and Burgess, 1956). It should be noted however that the thermal range for processes such as growth and reproduction is usually narrower than that for survival (Cossins and Bowler, 1987).

Insects must accumulate enough degree days to complete their life cycle (Stacey and Fellowes, 2002). A general trend of accelerated development and/or decreased time to emergence with increasing temperature, within minimum and maximum thermal limits, has been observed in blackflies (Baba and Takaoka, 1991; Hadi and Takaoka, 1995), stone flies (Hogg and Williams, 1996), midges (Frouz *et al.*, 2002) and in several mosquito species (Mottram *et al.*, 1994; Read and Moon, 1996; Tun-Lin *et al.*, 2000) including *Anopheles gambiae s.s.* (Lyimo *et al.*, 1992; Bayoh and Lindsay, 2003).

Anopheles gambiae and *An. arabiensis* are the principal vectors of malaria in Africa, though *An. gambiae s.s.* is considered the most important vector because of its greater

anthropophily. Their larvae are frequently found occupying the same habitats e.g. man-made holes, roadside ditches, transient puddles and footprints (Service, 1985; Minakawa *et al.*, 2002). Several studies have failed to show consistent differences in habitat use between the two species (Minakawa *et al.*, 1999; Gimnig *et al.*, 2001), though occasionally there are temporal differences in the relative abundance of the larvae of each species e.g. in Mali larval production of *An. gambiae s.s.* occurs predominantly in the rainy season whereas *An. arabiensis* breeds year round (Touré *et al.*, 1998).

Environmental stress is often associated with smaller habitats (Worthen *et al.*, 1998) since the habitats occupied by both species are less resistant to changes in environmental conditions than larger habitats (Williams, 1987), and they are at real risk from habitat drying. Typical habitats occupied by *An. gambiae s.s.* and *An. arabiensis* have a surface area of 1-4m^2, a mean daily temperature of 24-26°C and persist for 20-40 days (Gimnig *et al.*, 2001; Yohannes, 2002) A rapid development strategy coupled with mechanisms allowing survival at high and fluctuating temperature allow *An. gambiae s.s.* and *An. arabiensis* to utilise these habitats while other insects cannot. This life strategy has implications for vector control; rapid production leading to high adult numbers can result in increased disease transmission unless control methods are altered accordingly (Garett-Jones, 1964). In the absence of other species including predators, intra- and inter-specific competition are likely to be the most limiting density-dependent effects on the larvae of *An. gambiae s.s.* and *An. arabiensis*. Competition between these two species may be especially high because the larvae are often highly aggregated in their distribution within breeding sites (Service, 1985). Whilst it is known that *An. gambiae s.s.* survival to adult is greatest at 22-26°C and that development rate is fastest between 28-32°C (Bayoh and Lindsay, 2003), similar information is unavailable for *An. arabiensis*. The effects of the presence of one species upon the survival and development of the other is limited to one investigation at 27°C (Schneider *et al.*, 2000). This shortage of knowledge is surprising, especially when one considers the relative wealth of material on competition between species of *Aedes* mosquitoes which suggests that it has a major impact on the survival rates of the species involved (Black *et al.*, 1989; Livdahl and Willey, 1991; Nekrasova, 1997). The aim of the present study was to examine the survival and development of *An. gambiae s.s.* and *An.*

arabiensis when reared separately and together at a range of constant water temperatures, and to relate this to what is known of the spatial and temporal distribution of these vectors in the wild.

Materials and methods

Larvae were reared at a constant water temperature of 25, 30 or 35°C under a 12:12hr light and dark cycle in programmable growth chambers (LMS cooled incubators, S.H.Scientific, Kent, U.K.). Water temperatures within the incubators were monitored daily using a digital thermometer (probe type T, Digitron Instruments).

Cages of approximately 200 adult *An. arabiensis* and *An. gambiae s.s.* were fed simultaneously and the mosquitoes provided with 75mm diameter bowls, filled with 100ml dechlorinated water and lined with filter paper, in which to lay their eggs. When there were a large number of eggs (usually two days later) the bowls were removed from the cages and covered until the eggs hatched. Newly hatched larvae (<24 hours old) were transferred using a pipette into clear plastic sandwich boxes 15x10x8cm in volume, with an initial water volume of 500ml and 1 drop of Liquifry No.2 (Interpet®). 30 larvae were added to single-species containers, and 15 larvae of each species added to mixed-species containers. A magnifying glass was used to help ensure the correct numbers of larvae were added. A larval density of approximately 0.2 larvae/cm^2 surface area was therefore maintained in all experiments. Each egg batch provided larvae for two single species containers and two mixed species containers. In total, 12 single-species containers and 6 mixed-species containers were studied at each temperature.

Enough fresh dechlorinated water was added daily to each bowl to maintain a volume of 500ml. Two drops of Liquifry were stirred into this water before addition when larvae were 2-3 days old. From the 4^{th} day onwards the larvae were fed a constant amount of food, 10mg of Tetramin® tropical fish flakes (Tetra GmbH, Melle, Germany) daily. This is a greater quantity of food /larva than that given in the standard rearing procedure (chapter II) and so was considered non-limiting. When pupae appeared they were removed from the larval bowls and added to the eclosion set-up. This consisted of a plastic WHO insecticide

resistance testing-kit tube placed over a plastic beaker 60mm internal diameter filled with 100ml of dechlorinated water (fig 6.1). Emerging adults left the water surface and rested on the sides of the tube. Each day at the same time the tubes were checked for adults. If present, the gate of the tube was closed to trap them and a new tube placed over the beaker if it still contained live pupae. The number of development days required to reach the pupal stage and subsequently the adult stage were recorded.

Adult mosquitoes were killed by exposure to ethyl acetate. A few droplets of ethyl acetate were added to a piece of cotton wool and this was dropped into the holding tubes. Mosquitoes were immediately sexed, the wings removed and measured as described in chapter II, and the specimens stored individually in Eppendorf tubes at –20°C until required.

All adults emerging from mixed species bowls were identified to species by PCR as described in chapter II. 25% of emerged adults from each individual single species bowl were also identified to species in the same manner. This was done to demonstrate that accidental contamination of a single species bowl by the other species involved in the experiments was unlikely to have occurred.

Fig 6.1 Equipment used to trap emerging adults

Statistical Analyses

Non-parametric Kruskal Wallace tests were used to assess the effect of temperature on survival to adult. Mantel-Haenzel X^2 tests were used to examine differences in survival between species at each temperature, and for differences between single- and mixed-species populations. Independent samples t-tests were used to compare development rates. A one-way ANOVA was used to compare changes in stage duration with temperature. Wing length data were normalised by square-root transformation and analysed using independent samples t-tests. A X^2 test was used to look for variation from the expected 1:1 emerging adult sex ratio within groups and the Mantel-Haenzel summary X^2 test used to test for inter-specific sex ratio differences at each temperature.

Results

Survival

The percentage of first-instar larvae surviving through to the adult stage at each temperature is shown in figure 6.2. In both species, reared separately or together, survival rates to adult declined with increasing temperature (*An. gambiae s.s.* single-species population $X^2 = 13.3$, df = 2, p = 0.001; *An. gambiae s.s.* mixed-species $X^2 = 11.8$, df = 2, p = 0.003; *An. arabiensis* single-species $X^2 = 10.6$, df = 2, p = 0.005; *An. arabiensis* mixed-species $X^2 = 7.5$, df = 2, p = 0.03). At 25°C in both single- and mixed-species populations, the survival rate of *An. gambiae s.s.* was significantly higher than that of *An. arabiensis*. However at 35°C *An. arabiensis* exhibits greater survival to adult than does *An. gambiae s.s.* (table 6.1).

Survival of *An. arabiensis* in mixed populations was significantly lower than that of single species populations at 30°C (median and inter-quartile range for single species survival = 61.7 (51.0-71.0); mixed species survival = 33.3 (6.7-66.7), Z = -2.02, p = 0.043) but not 25°C (Z = -0.94, p = n.s.) or 35°C (Z = -0.27, p = n.s.). No difference in survival rate was found between single- and mixed-species larvae of *An. gambiae s.s.* at any temperature.

Table 6.1 Median percentage survival to adult in single-and mixed-species populations of *Anopheles gambiae s.s.* and *An. arabiensis*. IQR = inter-quartile range.

		An. gambiae s.s.		*An. arabiensis*			
Temperature	Interaction	Median (%)	I.Q.R.	Median (%)	I.Q.R.	X^2_{M-H}	p
25°C	Alone	80	77.5-87.5	68.3	62.5-77.5	4.84	0.03
	Mixed	83.3	73.3-86.6	66.6	55-75	5.39	0.02
35°C	Alone	6.6	2.5-16.6	33.3	26.6-36.6	30.67	<0.001
	Mixed	3.3	0.0-21.6	26.7	16.7-40.0	11.26	<0.001

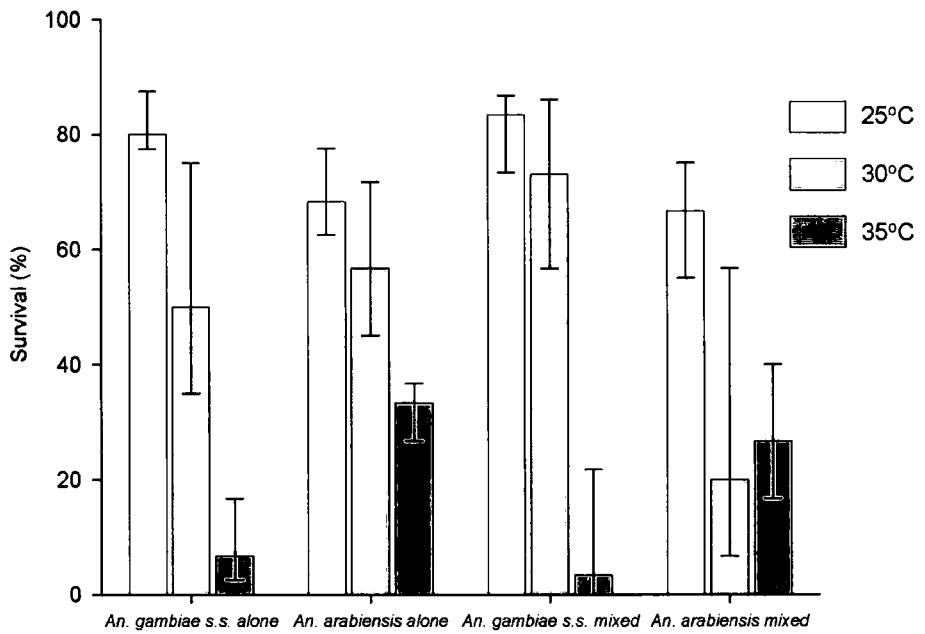

Fig 6.2 Survival rates (%) of *An. arabiensis* and *An. gambiae s.s.* from first instar to adult, reared as single- and mixed-species populations at different temperatures. Error bars represent inter-quartile range.

Development

All 1st instar larvae that developed to adults did so within 7 to 14 days (fig 6.3). Duration of the larval stage decreased with increasing temperature in both *An. gambiae s.s.* ($F = 85.3$, $df = 2$, $p<0.001$) and *An. arabiensis* ($F = 206.0$, $df = 2$, $p<0.001$). In single-species containers development to adult at 25°C was 0.75 (0.4-1.1) days quicker for *An. gambiae s.s.* than *An. arabiensis* (*An. gambiae s.s.* mean time to eclosion and 95% confidence intervals = 11.9 (11.6-12.1) days; *An. arabiensis* = 12.6 (12.4-12.8) days, $t = -4.7$, $df = 272$, $p<0.001$) and 0.9 (0.6-1.3) days quicker at 30°C (*An. gambiae s.s.* = 9.6 (9.4-9.9) days; *An. arabiensis* = 10.5 (10.3-10.8) days, $t = -5.0$, $df = 171$, $p<0.001$), but there was no difference between the two species at 35°C ($t = -1.9$, $df = 66$, $p = 0.07$). In mixed species bowls, *An. gambiae s.s.* developed comparatively quicker than *An. arabiensis* at 25°C (*An. gambiae s.s.* mean time to eclosion = 12.1 (11.8-12.4) days; *An. arabiensis* = 12.9 (12.6-13.3) days, $t = -3.3$, $df = 118$, $p = 0.001$), but not at 30°C ($t = -0.2$, $df = 94$, $p = 0.8$) or 35°C ($t = -1.6$, $df = 32$, $p = 0.1$).

Species differences in the duration of the larval stage to pupation mirrored the differences in development to adult in both single- and mixed-species containers. Pupation always occurred 1 or 2 days before eclosion. Higher temperatures reduced the duration of the pupal stage in both *An. gambiae s.s.* ($F = 24.0$, $df = 2$, $p<0.001$) and *An. arabiensis* ($F = 77.2$, $df = 2$, $p<0.001$). At 25°C the duration of the pupal stage was significantly shorter in *An. gambiae s.s.* than *An. arabiensis* (mean pupal duration and 95% confidence intervals for *An. gambiae s.s.* = 1.5 (1.4-1.5) days; *An. arabiensis* = 1.7 (1.6-1.8) days, $t = -4.5$, $df = 272$, $p<0.001$) but not at 30 or 35°C.

There were no developmental time differences between single- and mixed-species reared groups of either species with the exception of *An. gambiae s.s.* at 30°C; larvae in single-species containers at this temperature were quicker to develop than the respective mixed-species larvae (single-species mean time to eclosion and C.I.s = 9.6 (9.4-9.9) days; mixed-species = 10.3 (10.0-10.6) days, $t = -3.4$, $df = 146$, $p<0.001$).

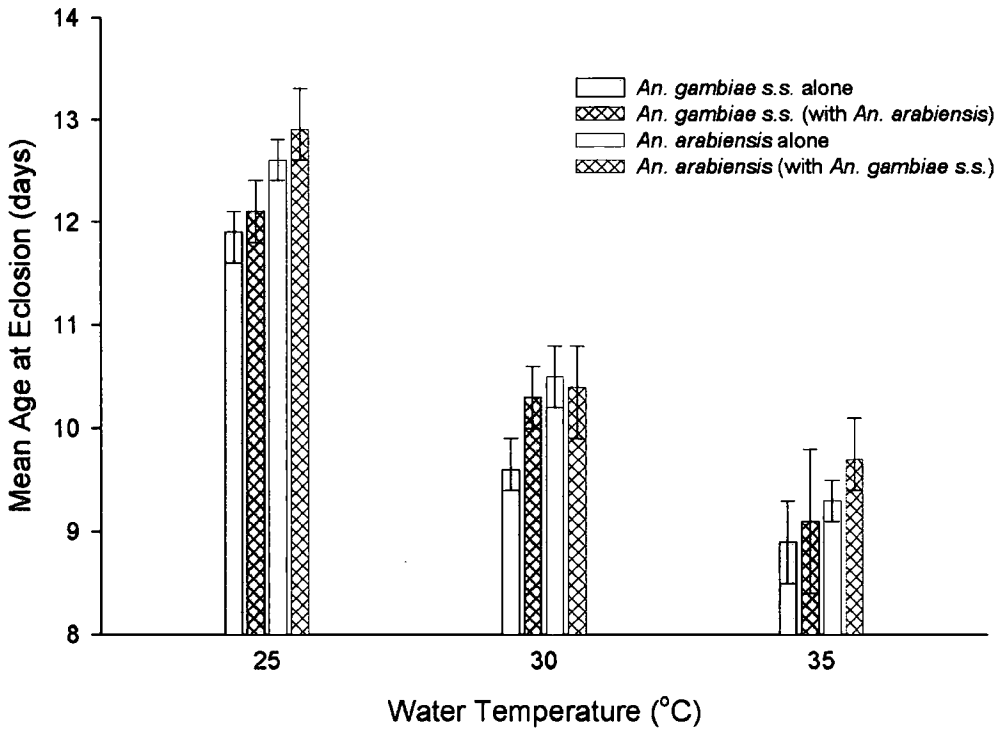

Fig 6.3 The effect of rearing temperature on the rate of development to adulthood. Note non-zero origin of y-axis.

Wing length

Univariate analysis of variance revealed temperature ($F = 54.2$, $p<0.001$) and species ($F = 105.2$, $p<0.001$), but not rearing status, i.e. single- or mixed-species ($F = 2.4$, $p = 0.1$), were important determinants of wing length. *An. arabiensis* adults from both single- and mixed-species containers were consistently larger than those of *An. gambiae s.s.* at all temperatures ($t = -13.1$, $df = 764$, $p<0.001$; fig 6.4).

Mixed-species rearing at 25°C, but not 30 or 35°C, reduced emerging adult size in both species when compared with their single-species counterparts (*An. gambiae s.s.* single-species mean wing length and 95% C.I.s at 25°C = 2.71mm (2.69-2.74); mixed-species = 2.64mm (2.60-2.68), $t = 3.3$, $df = 217$, $p = 0.001$: *An. arabiensis* single-species = 3.01mm (2.97-3.04); mixed-species = 2.86mm (2.82-2.90), $t = 4.7$, $df = 172$, $p<0.001$).

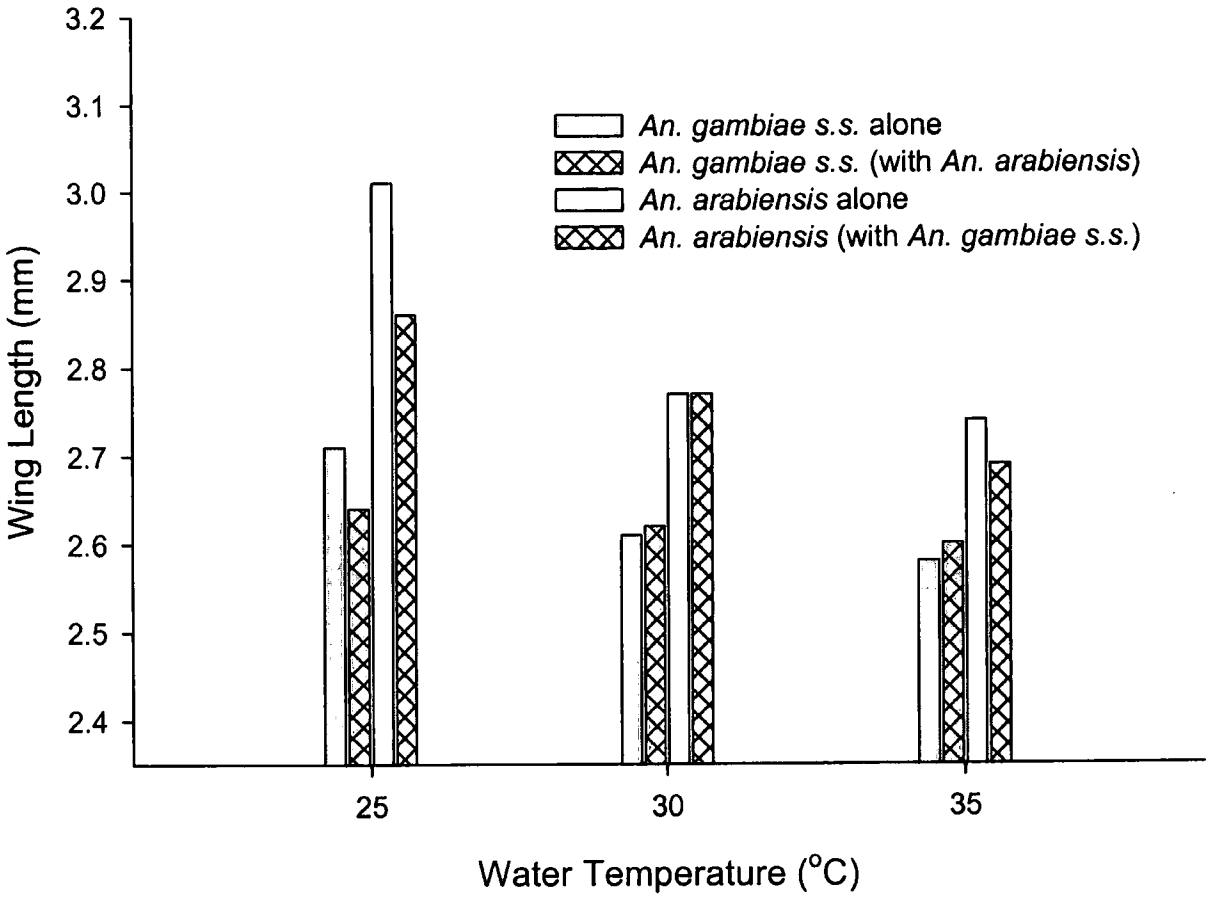

Fig 6.4 The effect of rearing temperature on emerging adult wing length. Note non-zero origin of y-axis.

Sex ratio

In all experimental groups the sex ratio of the emerging adults was not significantly different from 1:1 (table 6.2), nor was there any significant difference in emerging adult sex ratio between *An. gambiae s.s.* from single- or mixed-species containers and the respective *An. arabiensis* groups (table 6.3).

Table 6.2 Chi-squared values for variation from the expected 1:1 emerging adult sex ratio within groups

		25°C		30°C		35°C	
Species	Interaction	X^2	p	X^2	p	X^2.	p
An. gambiae s.s.	Alone	0.03	n.s.	0.05	n.s	0.6	n.s.
An. gambiae s.s.	Mixed	0.04	n.s.	2.5	n.s.	1.6	n.s.
An. arabiensis.	Alone	3.5	n.s	2.0	n.s	0.02	n.s
An. arabiensis	Mixed	0.2	n.s.	0.5	n.s.	0.6	n.s.

df = 1, critical value of 3.84 for p = 0.05

Table 6.3 Comparison of emerging adult *An. gambiae s.s* and *An. arabiensis* sex ratios at varying temperatures

Temperature	Single-species sex ratio F:M (n)		X^2	p	Mixed-species sex ratio F:M (n)		X^2	p
(°C)	*An. gambiae s.s.*	*An. arabiensis*			*An. gambiae s.s.*	*An. arabiensis*		
25	1:1.0 (146)	1:0.7 (127)	2.2	0.1	1:1.0 (73)	1:1.1 (47)	0.2	0.7
30	1:1.1 (82)	1:0.7 (96)	1.3	0.3	1:1.5 (67)	1:1.3 (32)	0.1	0.7
35	1:1.5 (15)	1:1.0 (51)	0.6	0.5	1:1.7 (8)	1:1.4 (26)	0.1	0.6*

*Fisher's exact one-tailed p value

Discussion

The survival and development rates of both *An. gambiae s.s.* and *An. arabiensis* larvae are dependent on water temperature, though there are clear differences in the responses of the species to each temperature regime. Furthermore, at least some of these responses are affected in the presence of the other species. Survival to adult is over 80% in *An. gambiae s.s.*, and over 60% in *An. arabiensis* reared as single- or mixed-species at 25°C. At 30°C survival is lower for single-species reared *An. gambiae s.s.* but not for the equivalent *An. arabiensis*, and at 35°C survival is considerably lower in both species, though *An. arabiensis* exhibits greater survival than *An. gambiae s.s.*. Larval development time decreased with increasing temperature in both species, taking on average 11.9 days in *An.*

gambiae s.s. and 12.6 days in *An. arabiensis* at 25°C but only 8.9 days in *An. gambiae s.s.* and 9.3 days in *An. arabiensis* at 35°C. Similar rates of survival and development have been reported for *An. gambiae s.s.* in comparable conditions (Lyimo *et al.*, 1992; Bayoh and Lindsay, 2003). Bayoh and Lindsay (2003) found 73% of larvae developed to adult at 26°C, taking an average 11.5 days, but only 35% at 32°C, taking 10.2 days, and no adults were produced at 35°C. Schneider (2000) reported 80% survival to pupal stage in *An. gambiae s.s.* and 60% in *An. arabiensis* reared in single-species bowls at 27°C. These patterns of increasing development rate but decreasing survival with increasing rearing temperature are also evident in other mosquito species including *Ae. aegypti* (Bar-Zeev, 1958; Tun-Lin *et al.*, 2000), *An. quadrimaculatus* and *Culex pipiens quinquefasciatus* (Shelton, 1973) and several other insect species (Petavy *et al.*, 2001; Stacey and Fellowes, 2002). It is important to realise that survival rates generated by this laboratory study probably overestimate survival rates in the field, where larval density may be higher, food availability lower, and risks of pathogens, predation and habitat desiccation much greater (Service, 1985). However this may not hold true for developmental rates, which may be faster under natural conditions with variable temperature than in a laboratory at comparable constant temperatures (Haufe and Burgess, 1956). Development time of wild populations may also be influenced by larval parasites. Koella and Offenberg (1999) found that infection by the microsporidian parasite *Edhazardia aedis* (and lower food availability) favoured earlier pupation in *Ae. aegypti*. Field studies of larval mortality are limited, but there is a suggestion that immature stage mortality in excess of 90% is not unusual for tree-hole breeders (Bown and Bang, 1980; Sempala, 1981), *Culiseta incidens* (Barr, 1985) and *An. arabiensis* in both temporary pools (Service, 1977) and rice fields (Service, 1985). In most of these studies survival rates varied monthly, suggesting an effect of temperature, though unfortunately no detailed temperature data was collected.

Despite the drawbacks of this laboratory study, it seems reasonable to suggest that there is greater production of *An. arabiensis* at extremely high water temperatures and *An. gambiae s.s.* at lower water temperatures. This reflects what is known of the relative abundance and distribution of the adults in Africa. *An. arabiensis* dominates during the months of highest maximum air temperatures (White *et al.*, 1972); water temperature usually shows a positive

correlation with air temperature so at such times it may be that *An. gambiae s.s.* production is restricted in the larval stage rather than the adult. Of the two species, only *An. arabiensis* is found in northern and western Sudan (Dukeen and Omer, 1986; Petrarca *et al.*, 2000), in Maputo, Mozambique (Mendis *et al.*, 2000) and in the Rift Valley, Ethiopia (Mekuria *et al.*, 1982; Ye-Ebiyo *et al.*, 2000). Water temperatures here are likely to reach extremes and so permit the development of *An. arabiensis* larvae but not *An. gambiae s.s.* for much of the year. Differences in larval survival may also help explain situations where *An. arabiensis* is the first species to take advantage of the rains in the early rainy season when water temperatures are likely to still be high, but is then later out-competed by *An. gambiae s.s.* e.g. in Western Kenya (Petrarca *et al.*, 1991) and Tanzania (White *et al.*, 1972). In these situations it is worth noting that populations experiencing mortalities over 90% only need a small change in survival rate, induced by a subtle rise in water temperature, to markedly increase population size (Service, 1985).

The current distribution of these two species may reflect subtle evolutionary changes in their metabolism over time. Changes in the temperature/growth rate relationship played a major role in the range expansion of mosquito species from an ancestral origin (Pritchard and Mutch, 1985). Mosquitoes appear to have undergone metabolic adaptations to their environmental surroundings, so that the optimal larval growth conditions vary considerably between species of the same genus, and even between populations of the same species, dependent on distribution. This is a very different strategy compared to that of other insects e,g, Odonata and Plecoptera, in which adaptation to latitudinal differences in environment involves synchronisation of temperature-sensitive life-history stages with warmer times of year (Pritchard, 1982).

Interspecific competition occurs between species with similar resource requirements and often results in the individuals of one species suffering reduced fecundity, survival or growth rate (Begon *et al.*, 1986). There is evidence of this occurring between tree-hole- and container-breeding mosquito species, e.g. *Ae. communis* and *Ae. flavescens* (Nekrasova, 1997), *Ae. aegypti* and *Ae. albopictus* (Black *et al.*, 1989), and *Ae. triseriatus* and *Ae. albopictus* (Livdahl and Willey, 1991). In the absence of predation, the typically small

breeding sites of the *An. gambiae s.s.* complex can support very high larval densities (Minakawa *et al.*, 1999; Gimnig *et al.*, 2001), thus promoting intra- and inter-specific competition. Data generated by this study and in a previous investigation (Schneider *et al.*, 2000) suggest that some level of inter-specific competition occurs between *An. gambiae s.s.* and *An. arabiensis*. Mixed-species rearing had a detrimental effect on the survival of *An. arabiensis* but not *An. gambiae s.s.* at 30°C. Schneider *et al.* (2000) found a similar effect at 27°C. They hypothesised that *An. arabiensis* may be larger than *An. gambiae s.s.*, and have greater food requirements and therefore a standard quantity of food might impose relatively more competition on *An. arabiensis* than *An. gambiae s.s.*. In this study a constant amount of food of similar quantity to that used by Schneider *et al.* (2000) was administered and so it is possible that this constraint on *An. arabiensis* also occurred here.

Anopheles arabiensis was consistently larger than *An. gambiae s.s.* in this study, and this difference in adult size between the two species appears to be intrinsic (Petrarca *et al.*, 1998). This may be reflected in the development rate data; *An. arabiensis* development may be slower than that of *An. gambiae s.s.* at 25°C and 30°C because their larvae require more time to acquire enough resources to reach optimal size. Temperature determines development rate, which in turn determines adult body size; smaller mosquitoes are produced at hotter times of year (Lyimo and Takken, 1993). If this is the case there does not need to be an actual shortage of resources; the emerging population numbers can be limited by the failure of the larvae to accumulate food in sufficient time. It is interesting to note that *An. gambiae s.s.* development rate was slower in the presence of *An. arabiensis* at 30°C; perhaps this is evidence of interference competition for food which benefits neither species. At 35°C on the other hand, the numbers of surviving *An. gambiae s.s.* are so low that little competition for food occurs and *An. arabiensis* larvae are able to rapidly accumulate enough resources.

Competition for food between the two species may also explain the production of smaller adults at 25°C from mixed-species containers than are produced from single-species containers at the same temperature. Adult size variation in the seaweed fly *Coelopa frigida* (Butlin and Day, 1984), the damselfly *Calopteryx splendens xanthostoma* (Plaistow and

Siva-Jothy, 1999) and the mosquito *Aedes triseriatus* (Fish, 1985) has been attributed to competition for limited food resources. Adult size may well be important to population growth, because there is evidence that larger mosquitoes survive for longer, show greater immunocompetence and therefore produce more offspring than smaller individuals (Haramis, 1985; Ameneshewa and Service, 1996; Koella and Boete, 2002), though in *Ae. aegypti* the positive relationship between adult size and fecundity only exists if larval food was abundant (Koella and Offenberg, 1999). A shortage of metabolic resources at emergence has repercussions for adult *An. gambiae s.s.*; small mosquitoes are more often pre-gravid, that is they require two blood meals to complete the first gonotrophic cycle, than larger mosquitoes (Lyimo and Takken, 1993).

There are other possible causes and effects of competition not likely to have been witnessed in this study. In insect populations with a wide range of co-occurring instars higher mortality rates are experienced than in populations with synchronous development (Buskirk, 1993; Fincke, 1994). This is manifest in the younger instars that suffer reduced growth rate and survival in the presence of larger larvae. In such situations, interference competition may result in size-dependent obligate killing of larvae. This has been witnessed in *Toxorhynchites* mosquitoes (Corbet and Griffiths, 1963) and in some Odonates (Fincke, 1994). Larval aggregation decreases with instar progression in *An. gambiae s.s.* and *An. arabiensis* (Service, 1985). Though this is probably a result of decreasing survival with age, the possibility of obligate-killing and even predation/cannibalism in these species has some empirical support (Koenraadt and Takken, 2003).

Density-independent effects may be more significant to mosquito survival than interspecific competition, especially in habitats such as ricefields, in which densities are unlikely to be high enough to cause limitation unless there is high heterogeneity (Service, 1985). The large natural variation in habitats due to flooding and desiccation may allow *An. gambiae s.s.* as the more rapid developing species the opportunity to out-compete *An. arabiensis* even at temperatures at which it suffers higher larval mortality.

At present we have only a very limited knowledge of the interaction of these two species at the larval stage, particularly in the field. It would be informative to study the effects of introducing the species separately, and at different age classes, and monitoring development at critical temperatures. The effects of daily temperature fluctuations around the critical thermal maximum on survival could reveal further differences between the two species. Dynamics of the inter-specific balance between the larval stages of *An. gambiae s.s.* and *An. arabiensis* will impact on the relative abundance, and therefore the vectorial capacity, of the combined adult population. This study has shown that variation in water temperature can produce differences in the productivity and competitiveness of *An. gambiae s.s.* and *An. arabiensis* which could in turn help to explain the difference in the distribution of these species.

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CHAPTER VII

The behavioural response of *Anopheles gambiae sensu stricto* and *An. arabiensis* larvae to high water surface temperatures

Abstract

The larvae of *Anopheles gambiae sensu stricto* and *An. arabiensis* are frequently exposed to water temperatures close to the critical thermal maximum of both species. The ability to avoid high surface temperatures by diving into the cooler depths and remaining submerged would, at least in part, explain how these species survive and thrive in habitats that experience temperatures at the upper end of the larval thermotolerance scale. The larval response was tested in 25cm deep Perspex tanks, in uniform thermal environments between 30 and 40°C, and in stratified thermal environments where the bottom of the water column was 5°C lower than the surface. Diving frequency and total submergence time increased in both species with increasing surface temperature, in both uniform and stratified thermal environments. At 40°C surface temperature, *An. gambiae s.s.* made on average 4.2 (95%C.I. = 3.8-4.8) more dives in a 15-minute experimental period in the uniform tank, and 3.0 (2.5-3.4) more dives in the stratified tank, than when the surface temperature was 30°C. A similar behaviour was also observed in *An. arabiensis* (4.6 (4.1-5.1) and 2.7 (2.3-3.1) more dives respectively). *An. gambiae s.s.* spent on average 104.2 (94.1-114.9) seconds longer away from the water surface in the uniform tank, and 99.2 (90.3-108.9) seconds longer in the stratified tank, when the surface temperature was 40°C compared with activity at 30°C. Again this pattern was mirrored in *An. arabiensis* (110.8 (76.6-129.4) seconds and 100.7 (88.6-113.9) seconds respectively). In both species, significantly greater time was spent diving at 40°C than at lower temperatures. Differences in larval behaviour between thermally stratified and uniform environments were also only observed at 40°C. At this temperature, larvae dived more frequently (*An. gambiae s.s.* larvae dived on average 30% more, *An. arabiensis* dived 45% more) but spent less time away from the surface during each dive (*An. gambiae s.s.* spent on average 58% less time away from the surface during each dive, *An. arabiensis* 64% less) in thermally uniform compared to thermally stratified environments. These findings suggest larvae can detect differences in water temperature within their habitats and dive to cooler water to avoid potentially lethal surface temperatures. Larvae of both species spent only a

fraction of the 15 minutes away from the surface yet they were capable of surviving much longer periods submerged (~ 6hr). No significant differences were detected between the diving behaviour of these species, suggesting that behavioural mechanisms do not underpin the greater survival to adulthood of *An. arabiensis* larvae over those of *An. gambiae s.s.* at high temperature.

Introduction

The larvae of *Anopheles gambiae sensu lato* characteristically occupy ephemeral puddles and semi-permanent water bodies such as rice fields and marshy-edged ponds (Laird, 1988), although *An. bwambae* larvae have up to now only been recovered from springwater pools in Uganda (White, 1985; Harbach *et al.*, 1997). *An. gambiae s.s.* and *An. arabiensis* larvae are frequently found in the same habitat (White and Rosen, 1973; Minakawa *et al.*, 1999), though they may be segregated temporally in some locations (Gimnig *et al.*, 2001). Both species are common in a diverse range of habitats including large permanent shoreline marshes, pluvial and riparian ponds, shallow market-garden wells and most typically, small temporary sunlit pools such as hoof prints and tyre ruts (Gillies and DeMeillon, 1968; Beier *et al.*, 1990; Robert *et al.*, 1998).

Within these breeding sites, larvae of the *An. gambiae* complex, in common with most mosquito larvae, spend much of their time at rest near the water surface. This position provides access both to the air for respiratory exchange of gases, and to the water, the surface layers of which provide rich sources of food. Mosquito feeding behaviour is restricted by the necessity to breathe air, and therefore to an extent by the surface resting position (Gillett, 1971). Anopheline larvae hang parallel to the water surface, maintaining this position through use of the hydrofuge respiratory apparatus, a series of abdominal and thoracic float-hairs, and a pair of specialised thoracic organs (Clements, 1999). In this position they feed predominantly on surface bacteria and algae; indeed the growth of anopheline larvae is retarded in the absence of these microlayer microorganisms (Wotton *et al.*, 1997). Particles landing on the water surface, such as maize pollen grains, are also an important source of food (Ye-Ebiyo *et al.*, 2000). In contrast the larvae of most culicine mosquitoes tend to hang down from the water surface with only the respiratory siphon in contact with the surface film of the water. As

a result, most feeding is done beneath the surface layers in these species (Clements, 1999).

Water temperature influences the rate of larval development, and also, by affecting survival to adult, species distribution. The way in which temperature influences these factors varies between mosquito species (Shelton, 1973). In general larval development is accelerated at higher temperatures until the upper lethal temperature is reached (Lyimo *et al.*, 1992; Bayoh, 2001). Survival to adulthood also increases with temperature, though the highest survival rates usually occur at lower temperatures than the highest development rates (Tun-Lin *et al.*, 2000; Bayoh and Lindsay, 2003). Thermal death points for most tropical anopheline mosquito larvae are reached between 30-45°C: less than 1% of *An. albimanus* and *An. quadrimaculatus* survive to adulthood at 35°C (Shelton, 1973), all fourth instar *An. minimus* die at 41°C (Muirhead-Thomson, 1940), while no *An. gambiae s.s.* larvae survive a prolonged exposure at 38-40°C (Bayoh, 2001).

As a consequence of their habit of resting at the surface of open water bodies in tropical Africa, *An. gambiae s.s.* and *An. arabiensis* larvae are frequently exposed to high temperatures, often reaching 35-40°C (De Meillon, 1934; Haddow, 1943; Bayoh, 2001). However this is not always uniform throughout the habitat. The sun provides a directional radiative heat source, warming the water surface throughout the day. The surface heat then disperses by convection into the deeper reaches of the water body. This means that whilst the surface water temperature rises quickly as solar radiation increases, reaching a maximum when the sun reaches its highest point, less heat reaches the substrate, resulting in a top-to-bottom temperature gradient (Williams, 1987). The turbid nature of many temporary mosquito habitats accentuates this thermal stratification; at 180ppm turbidity most absorption of solar heat occurs in the upper five centimetres (Butler, 1963). Additionally, because these habitats are usually small and shallow, they have low thermal inertia, and therefore respond rapidly to changes in exposure to the sun by heating up and cooling down.

With no opportunity for dispersal, mosquito larvae are restricted to the microclimate of their water environment. Because these habitats can quickly reach temperatures approaching upper lethal limits, larvae must have some mechanism of heat resistance

capable of protecting them, at least for the few hours when water temperature is at a maximum. Temperature avoidance through diving away from the surface is one method of surviving exposure to sub-lethal temperatures. This behaviour has been described in other aquatic insects. The predaceous diving beetles *Laccophilus fasciatus* and *L. maculosus*, and the water scavenger beetle *Tropisternus quadristriatus*, typically surface feeders, dive down and accumulate under debris or burrow in the bottom mud when surface temperatures approach 40°C (Young and Zimmerman, 1956). Diving behaviour in mosquito larvae has long been recognised as a startle response. Miall (1895) described how, when disturbed, some mosquito larvae sink slowly to the bottom, remaining below briefly before rising by a jerking movement with tail uppermost. The diving motion is affected by the buoyancy of the larvae; *An. gambiae s.s.* and *An. arabiensis* have a relative density greater than 1.0 so they can dive passively but must swim actively to re-surface (Clements, 1999). Mosquito larvae will respond to quickly changing conditions of light intensity or slight movement by diving immediately from the water surface (Christophers, 1960; Dukeen and Omer, 1986). Diving by mosquito larvae as a response to increasing surface temperatures has not been recognised, though it has been suggested that they can avoid high temperatures by vertical or horizontal migration in large or deep pools (Haufe and Burgess, 1956). Diving would bring larvae into contact with deeper and therefore potentially cooler water. The larvae would need to remain submerged until surface temperatures dropped. The diving behaviour of *An. gambiae s.s.* and *An. arabiensis* under a range of water temperatures and their survival without atmospheric oxygen was assessed here to see whether the larvae of these species actively avoid extreme temperatures and to observe whether any differences in behaviour between the two species reflected the temporal segregation observed in the field.

Materials and methods

Diving behaviour

Larval diving behaviour was observed in thermally uniform environments at 30, 35 or 40°C, and in stratified environments at the same surface temperatures but 5°C cooler at the base of the water column. A 5°C gradient was used as it was easily achievable and maintainable, regardless of surface temperature, using simple equipment alone (fig 7.1).

A transparent plastic cuboid tank measuring 27x15x30cm^3 was filled with de-chlorinated water to a depth of 25cm. An adjustable spotlight with a 60W bulb, positioned directly over the tank to mimic the directional radiation of the sun, heated the water surface and created the thermal gradient through the water column. In order to monitor the temperature gradient, two digital thermometers (probe type T, Digitron, Sifam Instruments Ltd, Torquay, UK) were positioned just below the water surface at 1cm depth and just above the tank bottom, at 24cm depth. The proximity of the bulb to the water surface dictated the surface temperature. At 40°C the heat source was so close to the water surface that it was concentrated on the centre of the surface and consequently did not warm the water at the tank edges to the same degree. To restrict larval movement to the area of uniform surface temperature, two sheets of transparent Perspex were inserted 5cm in from the cooler edges.

The tank set-up was identical for the thermally uniform experiments except that a small fishtank heater (Ecotherm® 50W heaterstat) was used to heat the water and the adjustable spotlight was substituted for an cold light source (Olympus Highlight 2100, Olympus Optical Co., Ltd). Light intensity, recorded by a universal exposure meter (Sangamo-Weston Ltd., U.K.), was a similar level in both environments.

Fig 7.1 The experimental set-up

Each trial run was videotaped using a Sony™ Handycam™ camera. This allowed for retrospective observation of the behaviour of individual larvae, and also prevented unwanted incidences of larval diving as a result of disturbance by the observer. Larvae were filmed from the side, with the camera set at an angle of 30° to create a depth of field that allowed a clear view of all larvae at the surface.

Larvae

Larvae of both species were reared in the manner described in chapter II. Only fourth instars were used because other larvae were too small to easily visualize with the camera.

Procedure

Five larvae were introduced into the tank and allowed 15 minutes to adjust to their new environment, after which the camera was switched on. After 15 minutes the larvae were removed, and another group of five larvae introduced. The procedure was repeated twelve times for both species in each of the six thermal environments.

Diving behaviour was analysed by watching the video playback on a standard VHS video recorder and television. Larvae were observed individually; the total number of dives and total submergence time (i.e. time spent away from the surface) for each larva were recorded using a hand counter and stopwatch. A dive was recorded when the larva descended to a depth of 1cm or more; submergence time ended when contact with the surface layer was re-established. A 1cm depth line was marked immediately below the water surface on one side of the tank to facilitate the recording of a dive. At the end of the 15-minute recording period the film was rewound and the next larva observed.

Survival of submerged larvae

In a separate experiment larval siphonal respiration was prevented and survival length measured using simple equipment alone. A perforated metallic plate of 31mm diameter, with mesh size of $1mm^2$, was fitted approximately half-way inside a 160mm long clear plastic tube of 31mm internal diameter. A solid rubber bung (diameter bottom and top 28x35mm) sealed the bottom of the tube which was then filled with 60mm of de-chlorinated water at 25°C to a level above the plate. A group of 10 fourth instar larvae were pipetted into the tube which was sealed with another bung and then gently inverted

(fig 7.2). Larvae were denied access to the water surface by the presence of the plate. Any air bubbles attached to the plate were removed with a pipette tip. As a control, a separate tube with another 10 larvae was set up in identical fashion but not inverted. The tubes were placed in an incubator to maintain the temperature of the water contained. Larvae were examined during the first 5 minutes in every 30 minute period following their introduction. Larvae were recorded as dead in a given period if they were observed resting on the bottom and motionless throughout both that period and the following one. The procedure was repeated three times for both species.

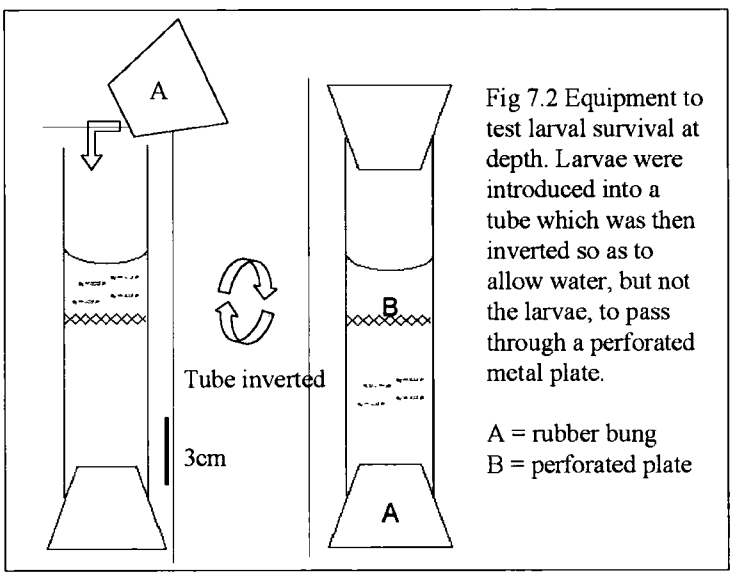

Fig 7.2 Equipment to test larval survival at depth. Larvae were introduced into a tube which was then inverted so as to allow water, but not the larvae, to pass through a perforated metal plate.

A = rubber bung
B = perforated plate

Analysis

Multivariate analysis of variance type III sum of squares (general linear model) was used to identify the importance of species, repetition number and environment as determinants of the number of dives, submergence time and time spent submerged per dive. Differences between environments were subsequently analysed using independent samples t-tests after data were normalised by log10 transformation. Univariate analysis was used to test the importance of species and repetition number to submergence survival length. All statistical analysis was performed with SPSS for Windows v10.0. The significance level for all statistics was 0.05.

Results

Diving behaviour

The diving behaviour of 360 *An. gambiae s.s.* and 360 *An. arabiensis* larvae was observed and analysed. Repetition number did not significantly affect diving behaviour and so the data for each experiment block were pooled. Species was also not found to be a determining factor of the number of dives performed ($df = 1$, $F = 0.8$, $p = 0.4$), the mean total submergence time ($df = 1$, $F = 0.5$, $p = 0.8$) nor the time spent submerged per dive ($df = 1$, $F = 1.5$, $p = 0.2$). Equally the interaction between species and environment was not significant for each of the diving behaviour variables. The thermal environment had a significant effect on the number of dives performed ($df = 5$, $F = 211.1$, $p = <0.001$), total submergence time ($df = 5$, $F = 247.5$, $p = <0.001$) and the time spent submerged per dive, $df = 5$, $F = 58.4$, $p = <0.001$).

The number of dives made increased with increasing surface temperature in both species (fig 7.3). While there was almost no activity at 30°C surface temperatures, a significant increase in activity was observed at 35°C and again at 40°C in both the stratified and uniform environments (Table 7.1). The same trend was observed in the geometric mean total submergence time (fig 7.4 and Table 7.2). There was no difference in the geometric mean time spent submerged per dive between trials at 30 and 35°C surface temperature (fig 7.5 and Table 7.3). Larvae dived away from the surface for a few seconds only and rarely reached the bottom of the tank. However there was a significant increase in dive duration when surface temperatures were increased to 40°C and larvae were frequently observed at rest on the bottom of the tank. Vertical temperature stratification only influenced larval diving behaviour when the surface temperature was 40°C. Larvae of both species performed fewer dives (table 7.1) but remained submerged for a longer period per dive (table 7.3) when the tank bottom was 35°C than when tank temperature was a uniform 40°C.

Table 7.1 Summary of significant independent t-test comparisons of geometric mean number of dives between environments. C.I. = confidence intervals.

Environment	Compared surface temperatures (°C), T1 v T2	Geometric mean no. dives at T1 (95%C.I)	Geometric mean no. dives at T2 (95%C.I)	df	t	p
An. gambiae s.s.						
Stratified	30 v 35	0.2 (0.1-0.3)	0.9 (0.7-1.2)	118	-6.5	<0.001
	30 v 40	0.2 (0.1-0.3)	3.1 (2.8-3.5)	118	-20.9	<0.001
	35 v 40	0.9 (0.7-1.2)	3.1 (2.8-3.5)	118	-10.6	<0.001
Uniform	30 v 35	0.2 (0.1-0.3)	1.1 (0.9-1.4)	118	-7.7	<0.001
	30 v 40	0.2 (0.1-0.3)	4.4 (4.1-4.9)	118	-25.3	<0.001
	35 v 40	1.1 (0.9-1.4)	4.4 (4.1-4.9)	118	-14.6	<0.001
Stratified v Uniform	40 v 40	3.1 (2.8-3.5)	4.4 (4.1-4.9)	118	4.9	<0.001
An. arabiensis						
Stratified	30 v 35	0.3 (0.2-0.4)	0.9 (0.7-1.1)	118	-5.4	<0.001
	30 v 40	0.3 (0.2-0.4)	3.0 (2.7-3.3)	118	-20.0	<0.001
	35 v 40	0.9 (0.7-1.1)	3.0 (2.7-3.3)	118	-12.4	<0.001
Uniform	30 v 35	0.2 (0.1-0.3)	1.0 (0.8-1.2)	118	-6.7	<0.001
	30 v 40	0.2 (0.1-0.3)	4.8 (4.4-5.2)	118	-27.8	<0.001
	35 v 40	1.0 (0.8-1.2)	4.8 (4.4-5.2)	118	-16.5	<0.001
Stratified v Uniform	40 v 40	3.0 (2.7-3.3)	4.8 (4.4-5.2)	118	7.7	<0.001

Table 7.2 Summary of significant independent t-test comparisons of geometric mean total submergence time between environments. C.I = confidence intervals.

Environment	Compared surface temperatures (°C), T1 v T2	Geometric mean total dive time at T1 (sec) (95%C.I.)	Geometric mean total dive time at T2 (sec) (95%C.I.)		df	t	p
An. gambiae s.s.							
Stratified	30 v 35	0.9 (0.4-.15)	11.1	7.3-16.7	118	-6.5	<0.001
	30 v 40	0.9 (0.4-.15)	100.1	91.8-109.3	118	-24.6	<0.001
	35 v 40	11.1 (7.3-16.7)	100.1	91.8-109.3	118	-10.6	<0.001
Uniform	30 v 35	1.1 (0.5-2.0)	13.5	9.3-19.4	118	-8.1	<0.001
	30 v 40	1.1 (0.5-2.0)	105.3	96.1-115.4	118	-22.9	<0.001
	35 v 40	13.5 (9.3-19.4)	105.3	96.1-115.4	118	-11.3	<0.001
An. arabiensis							
Stratified	30 v 35	1.6 (0.8-2.7)	9.8	6.4-14.8	118	-5.4	<0.001
	30 v 40	1.6 (0.8-2.7)	102.3	91.3-114.7	118	-19.3	<0.001
	35 v 40	9.8 (6.4-14.8)	102.3	91.3-114.7	118	-11.4	<0.001
Uniform	30 v 35	1.2 (0.6-2.2)	19.5	8-35.8	118	-6.5	<0.001
	30 v 40	1.2 (0.6-2.2)	112.0	78.8-130	118	-21.0	<0.001
	35 v 40	19.5 (8-35.8)	112.0	78.8-130	118	-10.9	<0.001

Table 7.3 Summary of significant independent t-test comparisons of geometric mean dive duration between experiment blocks. C.I. = confidence intervals.

Environment	Compared surface temperatures (°C), T1 v T2	Geometric mean at T1 (95% C.I.)	Geometric mean at T2 (95%C.I.)	df	t	p
An. gambiae s.s.						
Stratified	30 v 40	14.2 (12.0-16.7)	24.0 (23.8-25.8)	74	-6.6	<0.001
	35 v 40	16.9 (15.2-18.8)	24.0 (23.8-25.8)	108	-5.6	<0.001
Uniform	30 v 40	14.2 (12.0-16.7)	24.0 (23.8-25.8)	74	-6.6	<0.001
	35 v 40	16.9 (15.2-18.8)	24.0 (23.8-25.8)	108	-5.6	<0.001
Stratified v Uniform	40 v 40	37.9 (31.9-45.1)	24.0 (23.8-25.8)	118	4.9	<0.001
An. arabiensis						
Stratified	30 v 40	17.4 (14.4-20.9)	34.8 (32.2-37.6)	77	-8.2	<0.001
	35 v 40	18.1 (16.0-20.6)	34.8 (32.2-37.6)	103	-9.3	<0.001
Uniform	30 v 40	17.7 (14.4-21.7)	21.2 (20.0-22.6)	74	-2.4	0.02
	35 v 40	18.1 (16.1-20.4)	21.2 (20.0-22.6)	104	-2.6	0.01
Stratified v Uniform	40 v 40	34.8 (32.2-37.6)	21.2 (20.0-22.6)	118	10.1	<0.001

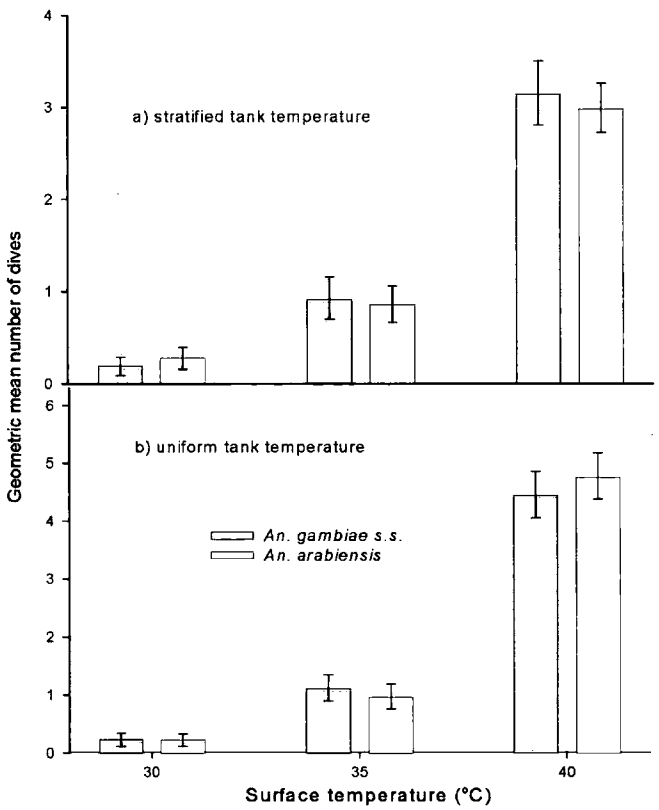

Fig 7.3 Geometric mean number of dives made in a 15 minute period by *Anopheles gambiae s.s* and *An. arabiensis*, at three surface temperatures within a) thermally stratified tanks, b) thermally uniform tanks. Bars represent 95%C.I.

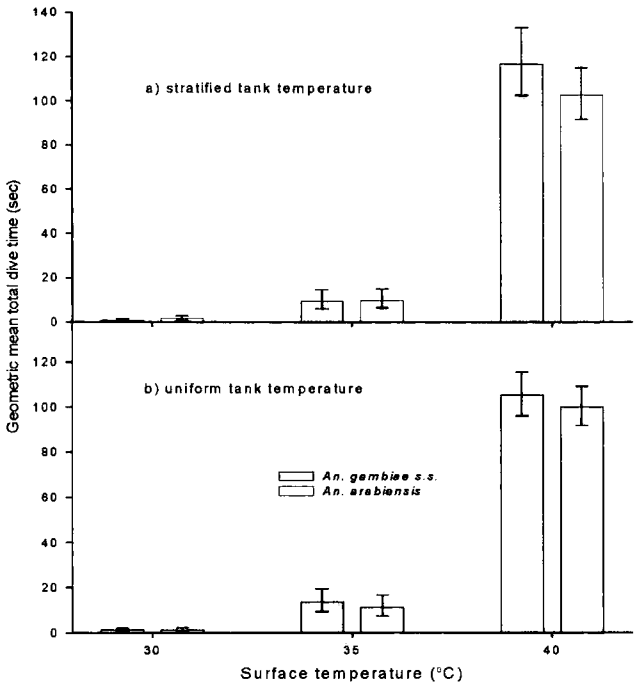

Fig 7.4 Geometric mean total dive time of *Anopheles gambiae s.s* and *An. arabiensis* at three surface temperatures, within a) thermally stratified tanks, b) thermally uniform tanks.

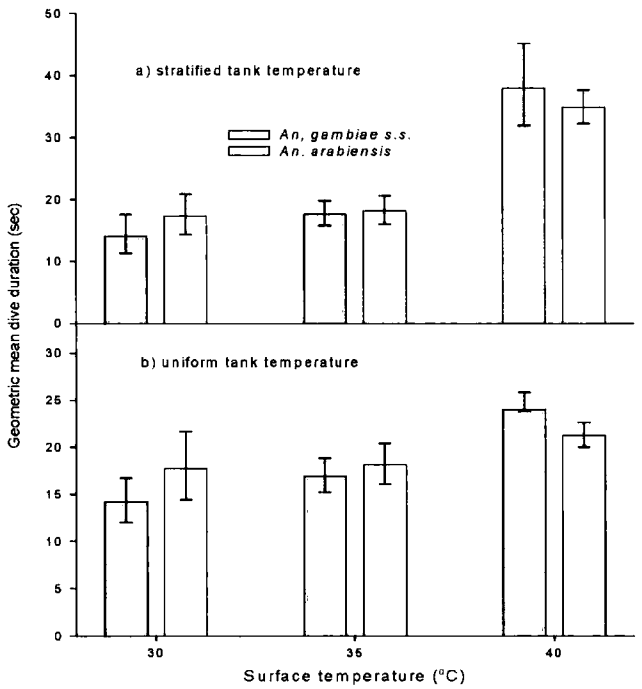

Fig 7.5 Geometric mean dive duration for *Anopheles gambiae s.s* and *An. arabiensis* at three surface temperatures, within a) thermally stratified tanks, b) thermally uniform tanks.

Survival of submerged larvae

Larvae of both species denied access to the water surface survived for 6 hours on average (*An. gambiae s.s.* mean survival and 95%C.I. = 351.0 (335.7-366.3) minutes; *An. arabiensis* = 365.0 (341.2-388.7) minutes). There were no differences in survival length between repeats or species (table 7.4).

Table 7.4 Univariate analysis of variance (type III) of the effect of repeat number and species on the survival length of submerged *An. gambiae s.s.* and *An. arabiensis* larvae.

Factor	Type III sum of squares	df	F	p
Species	2940	1	1.0	n.s.
Repeat no.	9030	2	1.6	n.s.
Species x repeat no.	2190	2	0.4	n.s.

Discussion

Water temperature governs the motility of mosquito larvae, and changes in water temperature can induce thermokinetic responses (Clements, 1999). Here it has been shown that the larvae of both *An. gambiae s.s.* and *An. arabiensis* respond to high surface temperatures by diving more and spending more time away from the water surface.

Larvae of both species were largely inactive at surface temperatures of 30°C, regardless of the presence of a thermal gradient. They dived very infrequently, and when they did, did so for short periods of 10-20 seconds. The only dives made were passive movements; larvae dropped from the surface film with little apparent muscular involvement. At 35°C surface temperatures the number of dives performed by both species increased but submergence time per dive was not longer than at 30°C. Active dives in which the body visibly contracted were more apparent at 35°C, but especially so at 40°C surface temperatures, conditions under which maximum diving activity was observed. The larvae are unlikely to be diving as a response to starvation (i.e. food seeking from the substrate), nor to the light or other stimuli. Larvae were well fed prior to any experiment and no changes were made to light exposure throughout any experiment. Negative phototaxism is less likely to be expressed in *An. gambiae s.l.* larvae, frequently exposed to direct sunlight in their preferred habitats, than in shade-

loving species. Furthermore it has been demonstrated that larvae habituate to frequently-changing light conditions (Clements, 1999). It therefore seems likely that larval diving was a direct response to water temperature.

It has previously been demonstrated that minimal larval activity occurs only near the lower threshold temperature (inactivation) and under near-optimum development conditions (Ivanova, 1940; Omardeen, 1957). Ivanova (1940) showed *An. messae* and *An. atroparvus* congregate and are virtually motionless in an area of 23-28°C when exposed to a horizontal surface temperature gradient of 15-42°C. *Aedes aegypti* are similarly motionless only at 23-32°C within a tank with a surface thermal gradient of 8-42°C (Omardeen, 1957). The optimal temperatures for larval development in *An. gambiae s.s.* range from 28-32°C (Bayoh and Lindsay, 2003). This suggests that *An. gambiae* larvae experiencing surface water temperatures of 30°C have no necessity to migrate to cooler waters unless motivated by starvation or the presence of predators. However, at constant 35°C larval development to adult is extremely low in many mosquito species (Shelton, 1973) including *An. gambiae s.s.* (Bayoh and Lindsay, 2003). Furthermore, at constant 40°C or above, mosquito larvae of most species do not survive more than a few hours. Fourth instar larvae of *An. minimus* are all killed by a five minute exposure to 41°C (Muirhead-Thompson, 1951), all first instar *Aedes aegypti* die within 24 hours at 40°C (Tun-Lin *et al.*, 2000) and *An. gambiae s.s.* larvae survive on average less than three days exposure to 40°C (Bayoh, 2001).

Despite the apparent risks of exposure to extreme temperatures, neither species spent a prolonged period of time away from the surface in any of the experimental situations. The longest total period spent below the surface in any experiment was just 35% of the total trial time, and occurred at conditions most encouraging for a long submergence i.e. 40°C surface temperature with 35°C bottom temperature. Neither species spent more than four minutes submerged for any one dive: this is comparable to periods of submersion in *Ae. aegypti* and *Culex pipiens* (Clements, 1999). However it has been demonstrated that both species are capable of surviving up to 6 hours submerged. The apparent reluctance to stay down for longer may be a result of the greater efficiency of breathing through the respiratory siphon, rather than by cutaneous respiration, as well as a response to increased risk of predation below the water surface as in this experiment there was no substrate to hide in. Forced diving to a depth of greater than 30cm has

been shown to cause significantly greater mortality in *An. gambiae s.s.* larvae than diving to a depth of less than 20cm (Tuno *et al.*, 2004). Age also influences submergence time; 4th instar larvae apparently have less capacity for cutaneous respiration than younger individuals and so must return to the surface more often (Clements, 1992).

Active dives and all resurfacing events involve fairly exaggerated movements likely to draw the attention of any nearby predators. Sih (1979) demonstrated that the capture of *Cx. quinquefasciatus* by *Notonecta hoffmanni* predators correlates positively with the amount of prey movement; larval activity drops significantly in the presence of predators. When attacked, larvae must actively escape to survive. This however will draw the attention of other predators. Mosquito larvae are particularly vulnerable when they move at the surface as they become visible to predators both above and below the water. This presents mosquito larvae with a trade-off situation. Movement away from high temperatures reduces the risk of thermal death but increases the risk of being preyed upon. For this reason diving in response to temperature is likely to be a last resort, only occurring when surface temperatures are critically high.

To understand how the larvae of *An. gambiae s.s.* and *An. arabiensis* survive exposure to high temperatures despite spending so much time at the surface, it must be appreciated that naturally-occurring water bodies reach these lethal temperatures for only short periods each day. The shallow ephemeral habitats populated by both species lose heat rapidly to the surrounding environment once the water temperature exceeds that of the air (Williams, 1987). These water bodies typically reach temperatures of 35-40°C for only 1-2 hours daily (Haddow, 1943; Young and Zimmerman, 1956; Bayoh, 2001). Some mosquito larvae are capable of resisting these short periods at extreme temperatures. Over 90% of *An. albimanus* survive 30 minutes at 41.5°C (Benedict *et al.*, 1991) and 92% of *Ae. aegypti* survive 60 minutes at 39°C (Christophers, 1960). With no opportunity for dispersal, mosquito larvae are restricted to the microclimate of the water environment in which they are laid as eggs. Because these habitats can quickly reach temperatures approaching upper lethal limits, larvae must have some rapidly inducible mechanism of heat resistance capable of protecting them, at least for the few hours when water temperature is at a maximum. In light of this is it interesting to note that 4th instar larvae may be more thermotolerant than younger stages. When placed in an

artificially-established temperature gradient, 4th instars of *Ae. taeniorhynchus* accumulate at mean temperatures 4°C higher than their 1st instar counterparts (Linley and Evans, 1971). This higher thermotolerance may relate to the production of Hsp70, inducible by the slightest of temperature elevations (Benedict *et al.*, 1991) and demonstrated to be up-regulated rapidly in several *Drosophila* species during and after exposure to potentially lethal heat stress (Krebs and Feder, 1997; Lansing *et al.*, 2000). Fully grown larvae, which show little division or enlargement of cells, gain more in survival terms from expression of Hsp70 than their 1st instar counterparts (Krebs and Bettencourt, 1999). This is because Hsp production can prove costly to growing life stages, inhibiting development by reducing cellular growth and division (Krebs and Bettencourt, 1999). However late instar larvae are not always the most thermotolerant stage. The four larval stages of *Ae. aegypti* have approximately the same order of resistance (Bar-Zeev, 1957), and 1st instar larvae of *An. minimus* are unaffected by an exposure which kills all 4th instar larvae (Muirhead-Thompson, 1951). In these situations it is likely that more than one method of thermotolerance is in operation.

Thermoprotection is a necessity for the larvae of tropical mosquitoes that inhabit exposed sites where daily and seasonal temperature fluctuations occur. It is probable that *An. gambiae s.s.* and *An. arabiensis* use several mechanisms to avoid or resist extreme water temperatures. Temperature avoidance through increased diving and submersion behaviour appears to be one such mechanism.

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CHAPTER VIII

The importance of temperature in the spatial distribution of *Anopheles gambiae sensu stricto* and *An. arabiensis* during the dry season in The Gambia

Abstract

The laboratory studies of this thesis have suggested an inherent difference between *Anopheles gambiae sensu stricto* and *An. arabiensis* in adult behaviour, and both adult and larval survival at high temperatures. Here I investigated whether the effects of high temperature in a natural environment paralleled those that occur in the laboratory. Studies were carried out in the dry season in The Gambia using field caught mosquitoes. Anopheline adults caught in exit traps were aspirated into a large cage covered in black cloth, from which they could escape into a smaller mesh-covered cage. When temperatures in the large cage increased on exposure to direct sunlight, mosquitoes appeared in the small cage and were removed and identified to species. There was no difference between the relative proportion of *An. arabiensis* exiting the covered cage at less than 40°C and that of *An. gambiae s.s.* (mean proportion of *An. arabiensis* in exit trap at <40°C = 0.24 (0.13-0.34), *An. gambiae s.s.* = 0.28 (0.20-0.35) although *An. gambiae s.s.* mosquitoes which exited the covered cage at less than 40°C were smaller than those that exited at greater than 40°C.

Collections of mosquitoes from house roofs and floors were made to assess whether the indoor distribution of *An. gambiae s.s.* and *An. arabiensis* was related to the temperature of the resting sites from which they were caught. No difference was observed between the relative proportion of *An. gambiae s.s.* caught from the roof at 07.00h and that of *An. arabiensis*, nor at 14.00h. When roof temperature was lower than 34°C, the roof catch represented close to 100% of the total catch. However 84% of mosquitoes of both species were driven from the roof to the floor when roof temperatures were higher than 37°C. Afternoon floor temperatures were on average 4.6°C (3.1.-6.1) cooler than roof temperatures ($df = 11$, $t = -6.8$, $p<0.001$).

Larvae were collected from diverse microhabitats within a rice-field breeding site. *An. gambiae s.s.* was the dominant species, and the ratio of *An. gambiae s.s.* larvae to *An.*

arabiensis did not differ between habitats with significant differences in maximum daily surface and bottom water temperatures. The finding that *An. gambiae s.s.* was the more prevalent species in both adult and larval dry season collections, contrary to predictions, is discussed in relation to experimental design and distribution of the chromosomal forms of this vector.

Introduction

Many Diptera have undergone metabolic adaptations to their environmental surroundings, so that the optimal conditions for survival, reproduction and development vary considerably between species of the same genus (Pritchard and Mutch, 1985). For instance, range boundaries in closely-related Australian *Drosophila* species are defined by an increase in the metabolic cost of living at marginal sites and a lack of genetic variance for traits that may enable species to overcome limiting temperatures (Hoffmann and Parsons, 1989). Adaptation to local microclimate can even lead to differences in the level of resistance to environmental stress expressed between populations of the same species. For example, highland populations of the fruitfly *D. buzzatii* in Argentina have adapted to a highly changeable thermal environment and exhibit much higher knock-down resistance at high temperatures than their lowland counterparts (Sorensen *et al.*, 2001).

The distribution of *Anopheles gambiae sensu stricto* and *An. arabiensis*, two closely-related mosquito species, can be broadly predicted using the environmental parameters of temperature and humidity (Lindsay *et al.*, 1998). In regions where these two species are sympatric, it is generally assumed that *An. arabiensis* predominates over *An. gambiae s.s.* in hotter, drier regions, and this is supported by several seasonal distribution surveys. In Kaduna, Nigeria, *An. arabiensis* represented 69% of 446 specimens caught indoors during 3 months of the dry season (Rishikesh *et al.*, 1985). Similarly in Chiga near Kisumu, Kenya, *An. arabiensis* represented over 99% of indoor resting mosquitoes caught at the end of an exceptional dry period lasting 5 months (White, 1972). The relative abundance of these two species in the rainy season is however very different, where nearly all mosquitoes collected are *An. gambiae s.s.*.

Despite the fact that *An. arabiensis*, *An. gambiae s.s.* and *An. melas* are the only members of the *An. gambiae* complex present in The Gambia (Bryan *et al.*, 1987), there are no comprehensive studies of the seasonal relative abundance and distribution of these species in that country. Quiñones *et al.* (1998) found *An. gambiae s.s.* represented 96% of human-landing catches; however these catches were only conducted in the rainy season. Lindsay *et al.* (1991) found that in the dry season in Wali Kunda, *An. arabiensis* adults comprised 69% of a limited survey size of mosquitoes collected from bednets.

A better tolerance of high temperatures in *An. arabiensis* may result in longer-term improvements in survival rates in hot and dry regions. The results of chapter III suggest that an inherent difference in survival at high temperature exists between adult *An. arabiensis* and *An. gambiae s.s.*. However it is possible that the temperature avoidance behaviour demonstrated in chapter IV, by driving mosquitoes to escape to cooler spots, may allow *An. gambiae s.s.* to persist in conditions better tolerated by *An. arabiensis*. The Gambia is a highly suitable location to study the effects of high temperature on the distribution and relative abundance of *An. gambiae s.s.* and *An. arabiensis*, as in the dry season mean maximum temperature reaches 38.8-40.2°C (Lindsay *et al.*, 1989), and indoor temperatures sometimes match those outdoors (Lindsay *et al.*, 1991).

An. gambiae s.s. and *An. arabiensis* larvae frequently occupy the same habitat; most typically sun-lit intermittent ephemeral puddles such as those that form in shallow depressions and marginal hoof-print pools, but also semi-permanent water bodies such as rice fields and marshy-edged ponds (White and Rosen, 1973; Minakawa *et al.*, 1999). When reared in a mixed-species population at intermediate temperatures, *An. gambiae s.s.* tends to out-survive and out-compete *An. arabiensis*, especially at high densities (Schneider *et al.*, 2000). The effects of high temperatures on this relationship are not well established, though the results of chapter VI suggest that survival and competitiveness in these two species is temperature dependent. In The Gambia larval habitats are restricted to the alluvial areas alongside the river (Bøgh *et al.*, 2003) and semi-permanent water bodies, especially during the dry season, because away from the river the substrate is dominated by porous sandstone soil, so that any rainwater puddles that form do not exist long enough to allow larval maturation (Thomson *et al.*, 1995). The Gambian rice fields that are irrigated during the dry season are therefore an ideal place to study the impact of water temperature on the distribution and interaction of *An.*

gambiae s.s. and *An. arabiensis* larvae, because it is highly likely that both species are restricted to breeding there. In the dry season the abundance of adult *An. gambiae s.l.* in The Gambia increases with increasing rice height (Lindsay *et al.*, 1991). Tall rice will shade habitats, creating lower temperature sites in which *An. gambiae s.s.* should out-compete *An. arabiensis*. Exposed sites will be much hotter and should support only *An. arabiensis*.

The study was designed to test three hypotheses. Firstly, that adult *An. gambiae s.s.* are driven earlier from sites experiencing high and increasing temperature than are *An. arabiensis*. Secondly, that the indoor distribution of *An. gambiae s.s.* and *An. arabiensis* is related to the temperature of the resting sites from which they are caught. Lastly, that the relative abundance of both species in dry season breeding sites is determined by water temperature.

Materials and methods

Study Site

The study took place at the villages of Wali Kunda, Saruja and Wellingara, and in the Jahally ricefields in the Central River Division of The Gambia, West Africa. These sites are situated on the south bank of the River Gambia, approximately 180km inland. The villages, supported by a rural economy, consist of discrete clusters of houses surrounded by flat open Sudan savanna. Free draining sandstone dominates the geology of the environment, with alluvial soils close to the river (Trolldalen, 1991). Rice is grown year round in paddies close to the villages and the river, supported by an irrigation system throughout the dry and wet seasons (Lindsay *et al.*, 1991). The study was carried out between May 9th–28th 2003, towards the end of the long dry season which lasts from November to June or July (Lindsay *et al.*, 1993).

Adult catch and temperature avoidance experiment

Initially 10 40cm^3 exit traps were fitted over bedroom windows in six compounds in Wellingara village. The traps consisted of a wire frame covered by mosquito netting, with the side facing indoors inverted to form an entrance tunnel tapering to a 3cm diameter opening. The traps were fixed in the middle of a piece of cloth secured over

the window so as to allow no point of exit from the window other than into the trap. Alternative positions for the exit traps were sought if mosquito numbers were low, and after the first week only five traps were used; these were hung in the most effective locations. The traps were set at 19.30h and any gaps around the traps were filled in with wads of cloth. Traps were left overnight and were collected at 06.00-06.30h the next morning. The entrances were plugged with cloth and the traps immediately transported back to the field station at Wali Kunda. Here they were covered with cloth soaked in water to maintain high humidity within the traps, and placed indoors away from the floor to avoid the risk of predation of the mosquitoes by ants.

It was only possible to use one trap per day for the temperature avoidance experiment because there was only one period of the day when outdoor temperature rose fairly constantly and from a cool enough initial temperature, and because it was possible to observe only one trap at any given time. As a result of the restricted opportunity for experimental replication, it was desirable to have a trap containing as many anopheline mosquitoes as possible. Most days individual trap catches were less than 50 adults, and so on these occasions the most successful trap was identified and mosquitoes from the other traps were carefully transferred into it using an aspirator.

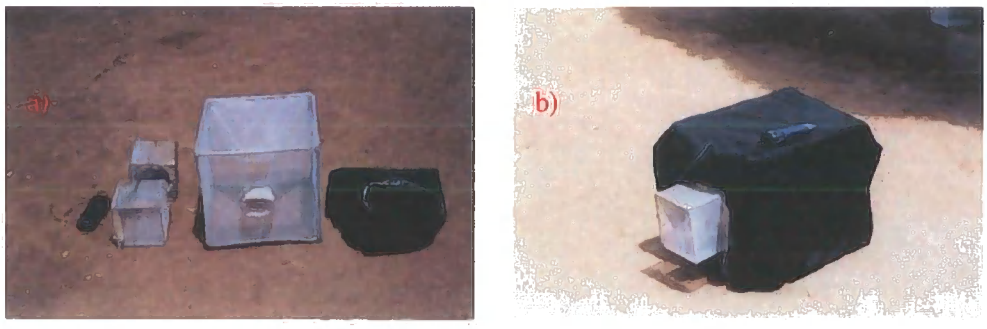

Fig 8.1 a) Equipment for thermotolerance experiment, showing, from left to right, digital thermometer, escape cages, holding cage and black cloth bag b) The equipment assembled.

When outdoor temperatures reached 30°C (usually 09.30-10.00 h), the exit trap was placed carefully into a black cloth drawstring bag and the mesh sleeve of the cage rolled back to create an aperture 12cm in diameter (fig 8.1a). An uncovered 15cm^3 mesh

'escape' cage with a similar sized opening was attached to the larger cage by means of three wire hooks, so that the apertures were connected. The black cage was then placed in full sunlight and out of any breeze (fig 8.1b). Two temperature probes (digital thermometers with probe type T, Digitron Sifam Instruments Limited, Torquay, UK) were inserted into the cage through small slits in the cloth, one in the top of the cage and one in the shaded side of the box. Mosquitoes were observed leaving the black cage and moving into the escape cage. The temperature at which the first mosquito entered the escape cage was recorded as the average temperature of the two probes. The escape cage was subsequently removed and immediately replaced with another once the probe in the shaded side of the black cage registered 40°C. This second escape cage was replaced with a third once the internal temperature exceeded 42°C. The experiment was stopped 30 minutes later. This procedure was repeated on five separate days.

Indoor resting site selection

Indoor collections were made between May 11th – 25th from five selected houses, two in Wellingara and three in Saruja. Houses were selected which had a low metal roof directly exposed to sunlight from which mosquitoes could be readily collected with an aspirator. One or two people slept under bednets in each room searched. At 07.00hrs the houses were searched for mosquitoes using a torch and an aspirator for 30 man minutes. The first 15 minutes were spent collecting mosquitoes directly from the metal surface of the roof (the 'roof catch'), approximately 240cm above the floor. The second 15-minute collection was restricted to mosquitoes found within approximately 60 cm of the floor (the 'floor catch'); usually from the floor itself, under the bed or around a water storage jar. Temperature readings were taken using a digital thermometer at the beginning and end of each collection. One reading was taken close to the roof surface and one from near the floor, both at points where mosquitoes were caught that day. Mosquitoes from each catch were transported to the field station in separate small mesh cages. The same house was revisited on the same day at 14.30hrs and two further collections made. Doors and windows were kept closed and rooms kept dark on collection days to minimise mosquito disturbance. Collections were made from each study house on three separate days. A whirling hygrometer (Brannan Thermometers, Cumberland, UK) was used to measure indoor relative humidity at 07.00 and 14.30hrs.

Larval distribution

The larval sampling area comprised 19 irrigated and planted plots within the Wellingara ricefield block, surrounded on all sides by large areas of dry unused plots. The plots were selected within 500m of Wellingara, as larval concentrations were highest closest to the village. Within the study area 11 sampling stations were selected where anopheline larvae had been found, and the edges of a cattle drinking pool chosen as a further station outside the ricefield (fig 8.2). Each station was selected to be representative of one of three habitat types (fig 8.3). The height of the rice and the depth of the water for each sampling station were determined using a scale marked onto the handle of a dipper. Water temperature was measured at 09.00, 11.00, 12.00, 15.00, 16.00 and 18.00hrs on three successive days for all sampling stations. Readings were taken from the top and bottom of the water column, always in the shade. The location of the sites allowed rapid coverage of the area so that all measurements were made within 10 minutes of the recorded time. For those sites where it was difficult to reach the water, the temperature probe was taped to the handle of the dipper.

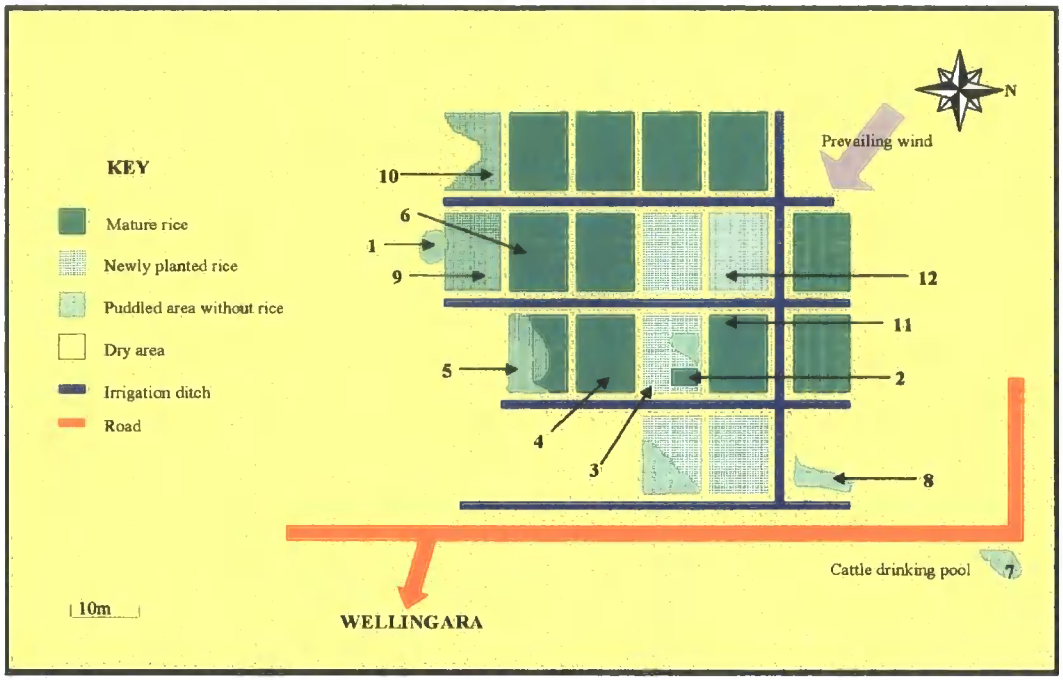

Fig 8.2 Map of the ricefield study area showing position of sampling stations

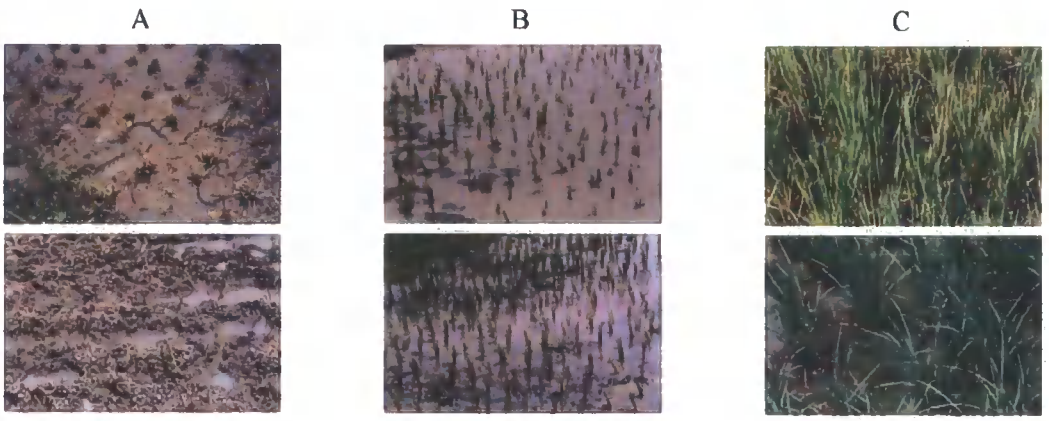

Fig 8.3 a) Habitat type 1: harvested plot or bare earth containing many water-filled footprints, hoofprints or depressions, fully exposed to sunlight b) type 2: Immature rice plants, well spaced out approximately 20cm apart, in open shallow water c) type 3: maturing or fully matured rice 40-50cm high, shading much of the water.

Larvae sampling

Larval density was estimated by calculating the number of larvae per dip at each sampling station. Ten dips were taken for each site on at least three separate occasions. Dips were taken with standard 350ml capacity larval dippers and poured into a white plastic tray (21x11cm) to facilitate identification of larvae. The presence of each larval stage was noted and the larvae pipetted into site-specific vials for transport back to the laboratory.

Sample processing

Adults

Cages were placed in full sunlight to kill adult mosquitoes by prolonged exposure to high temperature. The mosquitoes were sorted and all Anophelines stored individually in Eppendorf tubes containing desiccant. The right wing of all specimens was removed and measured, and mosquitoes identified by PCR (chapter II).

Larvae

Approximately 20 larvae from each sampling station (80 larvae for each habitat type) were individually stored in screw-capped vials containing 100% ethanol. Third and fourth instar larvae were preferentially stored for two reasons: as mature stages they best represented the species that survived and developed in the habitat type, and were less likely to be damaged during transport compared to the younger instars. Larvae were identified by the PCR method.

Analysis

A one-way analysis of variance (ANOVA) was used to assess differences between the mean number of mosquitoes in each escape cage. For the indoor collections, univariate analysis of variance was used to identify the relative importance of the fixed factors time, location and species as determinants of the number of mosquitoes, a paired samples t-test was used to compare morning with afternoon indoor temperatures, and a Wilcoxon signed ranks test was used to compare the indoor resting site distribution of the two species. For the larval data a Kruskal-Wallis non-parametric test was used to compare the relative proportion and density of the species between habitat types, and

multivariate analysis was used to identify the importance of habitat type, water depth and rice height to a range of temperature parameters.

Results

Adult catch

The adult collection comprised 673 mosquitoes, of which 88% (588) were female. 373 mosquitoes were caught in exit traps and 299 were caught during the indoor searches. *An. gambiae s.s.* represented 71% and *An. arabiensis* 29% of the 605 specimens identified by the PCR technique.

Temperature avoidance

A small number of mosquitoes did not exit the covered cage during the experiment, possibly due to injury during transportation. For this reason these mosquitoes were excluded from the analysis. Blood-fed and gravid females represented a large fraction of the total number of mosquitoes caught in exit traps and therefore it was necessary to include them in the data set, though the analysis made no distinction between mosquitoes at different stages of the gonotrophic cycle. Since it was difficult to regulate the temperature in the box with great precision, the numbers of mosquitoes that left at 40-42°C were combined with those that left at higher temperatures.

There was no difference between the relative proportion of *An. arabiensis* exiting the covered cage at less than 40°C and that of *An. gambiae s.s.* (mean proportion of *An. arabiensis* in exit trap at <40°C = 0.24 (0.13-0.34), *An. gambiae s.s.* = 0.28 (0.20-0.35); $\chi^2_{M\text{-}H} = 0.14$, df = 4, p = n.s.) *An. gambiae s.s.* mosquitoes which exited the covered cage at less than 40°C were smaller than those that exited at greater than 40°C (mean wing length of *An. gambiae s.s.* in exit trap at <40°C = 2.80 (2.69-2.95), at ≥40°C = 2.89 (2.85-2.96); t = -2.4, df = 146, p = 0.03, fig 8.4). There was no difference in size between *An. arabiensis* mosquitoes exiting at <40°C and ≥40°C. *An. arabiensis* was consistently larger than *An. gambiae s.s.* (mean wing length of *An. arabiensis* = 2.94 (2.89-3.00), *An. gambiae s.s.* = 2.87 (2.83-2.93); t = -2.5, df = 214, p = 0.02).

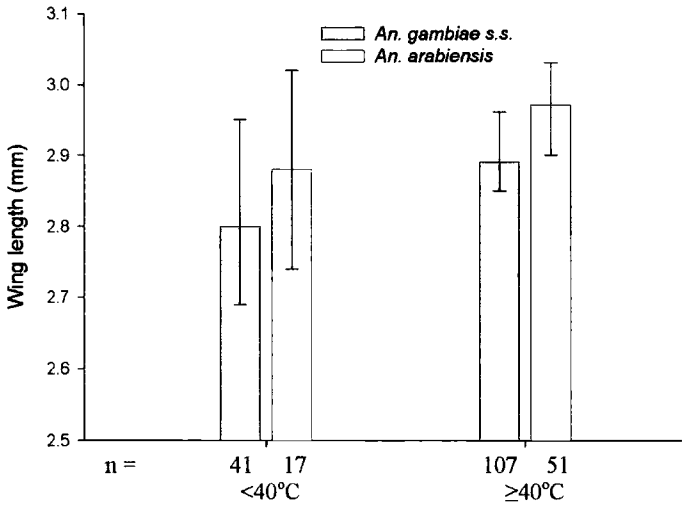

Fig 8.4 Wing length of mosquitoes exiting covered cage at <40°C and ≥40°C. Error bars represent 95%C.I.

Indoor resting site selection

An. gambiae s.s. represented 75% of the total number of mosquitoes caught and identified (table 8.1, fig 8.5), and as a result species was a significant factor in determining indoor catch size (table 8.2). However no difference was observed between the relative proportion of *An. gambiae s.s.* caught from the roof in the morning and that of *An. arabiensis* (mean proportion + 95%C.I. represented by roof catch at 07.00hrs for *An. gambiae s.s.* = 0.77 (0.56-0.98), for *An. arabiensis* = 0.77 (0.36-1.0); $\chi^2_{M\text{-}H} = 0.76$, df = 4, p = n.s.). Similarly there was no difference in the relative proportion of *An. gambiae s.s.* caught from the roof in the afternoon and that of *An. arabiensis* (mean proportion + 95%C.I. represented by roof catch at 14.00 for *An. gambiae s.s.* = 0.21 (0.03-0.39), for *An. arabiensis* = 0.20 (0.0-0.54); $\chi^2_{M\text{-}H} = 0.02$, df = 4, p = n.s.). Species data were therefore pooled to assess the affects of temperature on resting site selection.

Table 8.1 Indoor resting site catches and conditions

	Total number of mosquitoes caught, average temperature (T°C) and relative humidity (%RH) at 07.00hrs					Total number of mosquitoes caught, average temperature (T°C) and relative humidity (%RH) at 14.00hrs				
House	Roof		Floor		Room	Roof		Floor		Room
location	No.	T°C	No.	T°C	%RH	No.	T°C	No.	T°C	%RH
A, Wellingara	33	28.3	3	28.1	60	6	39.1	58	34.8	35
B, Wellingara	36	27.3	7	27.1	70	1	42.0	44	34.2	40
A, Saruja	27	29.1	0	29.0	50	11	37.8	20	35.2	40
B, Saruja	2	30.3	0	30.3	60	3	38.7	6	34.4	45
C, Saruja	26	31.4	3	31.6	50	5	39.1	8	35.5	30
Total	124		13			26		136		

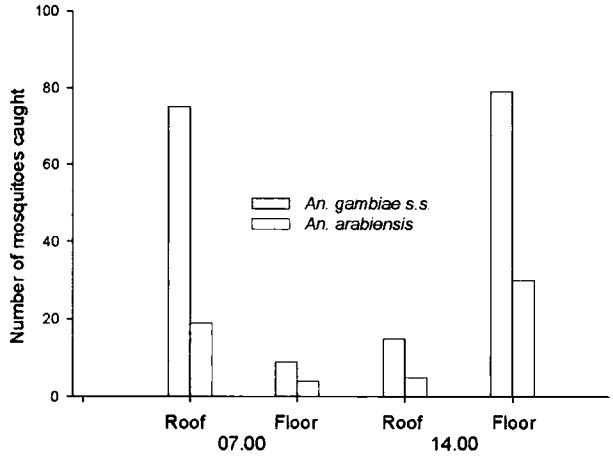

Fig 8.5 Total indoor resting site catches identified to species

Table 8.2 Significant univariate analysis of variance between-subjects effects

Factor	Type III sum of squares	df	F	p
Species	360.0	1	9.3	0.005
Time x Location	236.7	1	6.1	0.019
Time x Location x Species	202.5	1	5.2	0.029

The indoor catch size was not influenced by location or time independent of each other, but by a combination of the two factors (table 8.2). When accounting for indoor resting site selection it seems more appropriate to consider temperature rather than time. Indoor temperature was significantly higher in the afternoon than in the morning, both under the roof (mean roof temperature + 95% confidence intervals at 14.00hrs = 39.3°C (37.9-40.9), at 07.00hrs = 29.2°C (27.9-30.3); df = 11, t = 10.8, $p<0.001$) and near the floor (mean floor temperature at 14.00hrs = 34.8°C (34.2-35.4), at 07.00hrs = 29.1°C (27.8-30.4); df = 11, t = 12.3, $p<0.001$). When roof temperature was lower than 34°C, the roof catch represented close to 100% of the total catch (fig 8.6). Mosquitoes were driven from the roof to the floor when roof temperature increased to 36°C and higher. At 14.00hrs floor temperatures were on average 4.6°C (3.1.-6.1) cooler than roof temperatures (df = 11, t = -6.8, $p<0.001$).

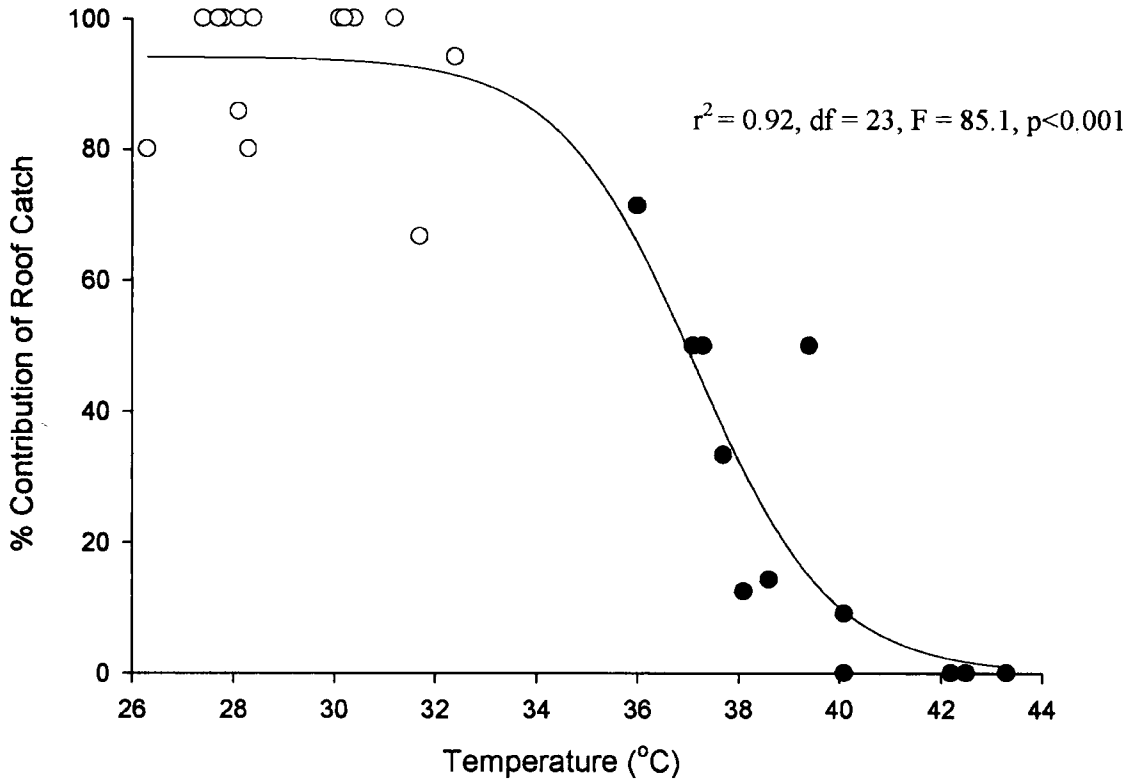

Fig 8.6 The effect of roof temperature on indoor mosquito distribution at 07.00hrs (○) and 14.00hrs (●). Line of best fit based on sigmoidal curve equation f = a/(1+exp(-(x-x0)/b)) where coefficients a = 94.0858, b = -1.3524 and x0 = 37.1327.

There was no intra-specific size difference between mosquitoes caught from the roof and those caught from the floor. This was consistent for both species and at both collection times (*An. gambiae s.s.* 07.00hrs df = 82, t = 0.9 p = n.s.; 14.00hrs df = 92, t = -0.5, p = n.s.: *An. arabiensis* 07.00hrs df = 21, t = -1.3, p = n.s., 14.00hrs df = 33, t = 0.5, p = n.s.). *An. arabiensis* was again consistently larger than *An. gambiae s.s.* (mean wing length of *An. arabiensis* = 3.03 (2.99-3.07), *An. gambiae s.s.* = 2.85 (2.83-2.87); df = 234, t = -9.1, p = <0.001).

Larval distribution

An. gambiae s.s. was the predominant species, representing 70% (155) of 220 successful identifications. *An. arabiensis* represented 30% (65/220). 11% of stored specimens could not be identified. Both species were present in 67% (8/12) of sites. The larval habitats selected presented a range of physical characteristics (table 8.3).

Table 8.3 Physical properties, larval density and larval species proportionality for all sites.

Habitat type (site)	Description	Temperature (°C)				Water depth (cm)	Rice height (cm)	Larval density (no. larvae/dip)	% *An. gambiae s.s.*
		Mean daily max.		Mean daily mean					
		Surface	Bottom	Surface	Bottom				
1(1)	Standing water seeped from adjacent plot through breach in mud wall	38.2	39.0	33.8	34.3	<10	Cut (<10)	2.63	76
1(5)	Water left in footprints as plot dries out	38.6	39.9	31.1	29.6	≤5	Cut (<10)	1.5	86
1(7)	Numerous hoof prints surrounding small cattle drinking pool	35.1	35.3	32.9	32.9	<10	No rice	1.73	100
1(8)	Shallow pools and cart track puddles containing some short grasses	35.5	35.9	30.3	30.6	5-10	No rice	0.93	100
2(3)	Young rice in shallow water	37.7	38.5	33.6	33.8	10	15	0.24	60
2(9)	Recently planted rice	38.0	38.4	31.1	30.4	10	15	0.18	100
2(10)	Young rice in shallow muddy water	37.9	37.8	30.3	30.3	8-10	10	0.15	67
2(12)	Young rice in shallow water	36.8	36.8	32.5	32.5	5	15	0.27	53
3(2)	Area of high emergent plant coverage and low water turbidity	34.5	33.7	33.2	32.8	15-17	50	0.15	100
3(4)	Fairly dense maturing rice and algal mats	34.2	33.6	33.2	33.0	<10	40	0.12	50
3(6)	Mature densely-packed rice	34.4	33.8	28.8	28.7	15	40	0.29	36
3(11)	Mature densely-packed rice	33.2	33.3	32.3	32.0	13-15	50	0.21	27

However the proportion of the stored specimens identified as *An. gambiae s.s.* did not differ between the three habitat types ($X^2 = 3.8$, df = 2, p = 0.2), despite the presence of

significant differences in maximum daily surface and bottom water temperatures (table 8.4) and also significant differences in larval density ($X^2 = 23.4$, df = 2, p = <0.001) between the habitat types. In fact no abiotic factors were found to affect the relative proportion of *An. gambiae s.s.* larvae present. Larval density increased with decreasing rice height ($r^2 = 0.36$, df = 11, F = 5.7, p = 0.04) but was not associated with any factor of site temperature. The height of the rice has a significant influence on mean daily maximum temperature at the water surface and at the bottom of the water column (table 8.4, fig 8.7), but water depth was found to be unrelated to any factor of site temperature.

Table 8.4 Significant multivariate analysis of variance between-subjects effects

Factor	Dependent Variable	Type III sum of squares	df	F	p
Habitat type	Mean maximum daily surface temperature	26.6	2	6.1	0.046
	Mean maximum daily bottom temperature	43.7	2	6.7	0.039
Rice height	Average mean daily surface temperature	18.8	1	10.9	0.021
	Average mean daily bottom temperature	14.7	1	8.3	0.034

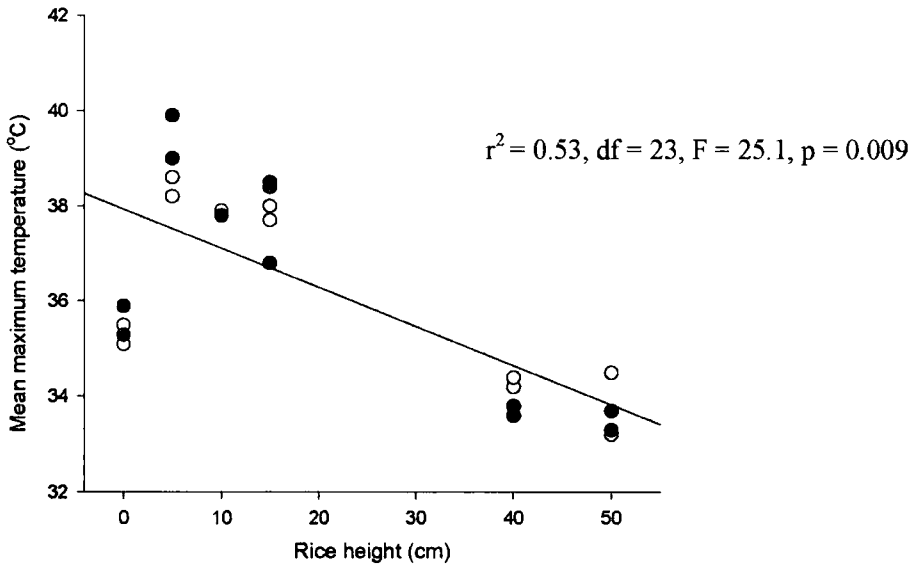

Fig 8.7 The effect of rice height on mean daily maximum site temperature at the top (○) and at the bottom (●) of the water column. Best fitting regression line based on the equation for a straight line y = b + mx where b = the y-axis intercept, when X = 0, and m = the slope of the line (DY/DX).

Discussion

Though *An. gambiae s.s.* dominates over *An. arabiensis* in the wet season in The Gambia (Bryan *et al.*, 1987; Lindsay *et al.*, 1993; Quiñones *et al.*, 1997), very few studies of the relative proportion of these species have been conducted during the dry season. Malaria transmission is limited outside the wet season (Greenwood *et al.*, 1989), suggesting breeding sites and vector numbers of both species are reduced at that time of year. However dry season irrigation can increase mosquito breeding (Mukiama and Mwangi, 1989; Ijumba and Lindsay, 2001), and limited evidence suggests a temporal change in species representation occurs as a result: *An. arabiensis* comprised 69% of 26 dry season identifications in 1987 and 1990 (Lindsay *et al.*, 1991). This study did not find *An. arabiensis* to be dominant over *An. gambiae s.s.* in the dry season, although the relative proportion of *An. arabiensis* adults (29%) is higher when compared with previous wet season collections where it represents less than 10% of catch numbers (Thomson *et al.*, 1995; Quiñones *et al.*, 1997) or is absent entirely (Lindsay *et al.*, 1991).

It was hypothesised that a greater tolerance of high temperatures would underlie the increase in the relative proportion of *An. arabiensis* in the dry season. However no inter-specific differences in temperature avoidance behaviour were observed. Dry season outdoor mean maximum temperatures reach 38.8-40.2°C in The Gambia (Lindsay *et al.*, 1989). When temperatures in the covered cage reached 40°C around 25% of mosquitoes of both species had already left the cage, presumably in search of cooler conditions. Those that remained were activated by temperatures only a few degrees higher. This suggests neither species are tolerant of exposure to dry season diurnal temperatures and probably spend much of the day indoors. The discovery that *An. arabiensis* is larger than *An. gambiae s.s.* is consistent with the situation in the laboratory, and is supported by data from other field studies (Hogg *et al.*, 1996; Petrarca *et al.*, 1998). The fact that the *An. gambiae s.s.* mosquitoes exiting the covered cage at less than 40°C were smaller than those exiting at greater than 40°C suggests adult size may have an influence on their tolerance of high temperatures, or at least on their avoidance of stressful conditions. Larger insects have a smaller surface area: volume ratio and may therefore be able to conserve water better at high temperatures. That this

pattern was not observed in *An. arabiensis* is probably a consequence of their under-representation in the sample. However it is surprising that no difference in avoidance behaviour was noted between the species despite this limitation. It is apparent that field specimens of both species are larger than laboratory specimens (Petrarca *et al.*, 1998). This holds true in this study, and may account for the increase in temperature required to activate the field-caught mosquitoes when compared with laboratory specimens (chapter IV). The design of the temperature avoidance experiment was necessarily altered from that of the laboratory study, and as a result it was not possible to exercise as much control over the temperature of the holding cage as was hoped for. In addition, it should be appreciated that the set-up induced a somewhat artificial behaviour, that is, the movement away from dark to light, which may also contribute to the increase in activation temperature. However the importance of this should not be overstated as it has been demonstrated that mosquitoes in a dark resting place experiencing sub-lethal rising temperature exhibit a reversal of the normal response to move towards shaded spots (Muirhead-Thompson, 1951).

The indoor resting site selection experiment reinforces the idea that mosquitoes are driven to seek cooler resting sites by high dry season temperatures. Indoor resting sites are generally favourable over outdoor sites because not only do they tend to be cooler and more thermally stable (Haddow, 1942; Muirhead-Thompson, 1951) but they also bring mosquitoes into closer contact with their human and animal hosts. However this indoor insulating effect depends on the construction materials used, as well as the thickness of both the walls and the thatch. Muirhead-Thompson (1951) hypothesised that in iron-roofed huts the temperature just below the roof may not be significantly cooler than outdoor screen temperatures. In the present study; roof temperatures in the afternoon reached 40°C on several occasions and previously in The Gambia it has been shown that during the hot dry season, indoor temperatures can match those outdoors, i.e. 40°C and above (Lindsay *et al.*, 1991). *An. gambiae s.s.* and *An. arabiensis* experience high knockdown and mortality after a few hours at such temperatures (Kirby and Lindsay, 2004) and they must move to seek out cooler spots. It was found that resting sites around water storage pots and under beds on the floor experienced temperatures 3-6°C cooler than under the roof. Similar indoor movement has been observed in *An. funestus* resting below the roof in metal-roofed houses in South Africa (De Meillon, 1934). Once roof temperature reached 40°C the mosquitoes were only found resting on

the floor. *An. gambiae s.s.*, as the less thermotolerant species, was expected to show a greater response to increasing roof temperatures and move down to the floor in larger numbers in the afternoon than *An. arabiensis*. That this was not observed suggests a more subtle difference between the species than is demonstratable in the laboratory. However it may also be the case that the transition from roof to floor takes place quite rapidly and that by 14.00hr temperatures were too high for either species to tolerate. This would explain why so few were left on the roof in the early afternoon. The relative proportion of *An. arabiensis* in the morning and afternoon collections (25%) was similar to that from the exit traps (32%). This suggests outdoor dry season temperatures in The Gambia force *An. arabiensis*, as well as *An. gambiae s.s.*, to spend most of the day seeking shelter indoors and that they do not enter houses late at night in significant numbers at this time of year.

Knowledge of anopheline larval ecology is limited. It is unclear what the regulatory factors are that control larval abundance and distribution (Minakawa *et al.*, 2002), yet a basic understanding of the mosquito larval stage would be of relevance to malaria control strategies. This study yielded three interesting findings perhaps relevant to malaria in The Gambia. Firstly, contrary to expectations, *An. gambiae s.s.* dominated the larval collection. This is not in accordance with dry season larval data from other areas of Africa including Cameroon (Robert *et al.*, 1992), Sudan (Dukeen and Omer, 1986) and coastal Senegal (Petrarca *et al.*, 1987), nor with the evidence that *An. arabiensis* generally predominates in irrigation schemes in arid areas (White, 1974; Ijumba *et al.*, 1990). Secondly, the relative proportion of the larval species was similar to the species composition of the adult collections. This is not always the case; for example in Kenya Minakawa *et al.* (1999) found *An. arabiensis* to be the most abundant larval species (representing 63% of the collection) but *An. gambiae s.s.* was more common in adult catches (57%). This may be a result of sampling technique and adult behaviour; *An. gambiae s.s.* tend to dominate collections reliant on indoor trapping methods because they are more endophilic than *An. arabiensis* (White, 1974). Given the low numbers of *An. arabiensis* larvae in the field and the potential bias in indoor trapping, a very low proportion of adults might have been expected. The fact that the ratio of *An. arabiensis* adults was similar to that of larvae in this study reinforces the idea that in the dry season in The Gambia *An. arabiensis* may spend more time indoors, and therefore feeding on humans, than anticipated. Finally the predominance of *An.*

gambiae s.s. in a rice irrigation area is unusual. In the rice fields of central and western Kenya, *An. arabiensis* is the more abundant species despite the presence of *An. gambiae s.s.* in surrounding rural habitats (Service, 1977; Highton *et al.*, 1979). The ricefield dominance of *An. gambiae s.s.* and the high percentage (67%) of sites shared by both species recorded in this study confirms the idea of extremely limited dry season breeding site availability in The Gambia, and particularly the ephemeral breeding sites usually more favoured by *An. gambiae s.s.*

This study hypothesised that *An. arabiensis* and *An. gambiae s.s.* larvae would dominate distinct microhabitats within the rice fields during the dry season according to site temperature. Spatial heterogeneity in the relative proportion of larval species has been demonstrated previously in a range of habitat types (Minakawa *et al.*, 1999), including rice fields (Russell and Rao, 1940), though temperature was not implicated. In fact the environmental variables that determined anopheline species occurrence were not identified. This study produced similar conclusions: though variation in the relative proportion of the two species seemed to occur between different sites no significant associations between environmental variables and the relative proportion of *An. gambiae s.s.* to *An. arabiensis* were detected, despite the heterogeneity in habitat mean maximum temperature and rice height. There are several possible explanations for this. Firstly, despite the intention to analyse the distribution of mainly third and fourth instars, most of the larval collection consisted of first and second instars. The power to determine the effect of habitat temperature on the progression of larval development and, most importantly, survival to late stage larvae and pupae, was therefore reduced. Temperature was not anticipated to influence first instar distribution as there is no evidence to suggest ovipositing females are influenced by the temperature of breeding sites, and *An. gambiae s.s.* and *An. arabiensis* often oviposit in the same site (White and Rosen, 1973). Ideally adult emergence traps would have been used in the field, but the time constraints of the study and the probable high mortality rates of larvae, which can reach 93% in ricefield populations (Service, 1977) would have resulted in a very small sample size. It may also be the case that many variables, each with only a small effect, influence species composition heterogeneity. In support of this idea, the abundance of *An. arabiensis* in permanent habitats has been shown to be affected by water temperature, depth, turbidity, elevated concentrations of HCO_3^-, low concentrations of NaCl and the presence of a range of flora (Robert *et al.*, 1998). Larval density, which

varied between sites in this study, has been shown to influence the interaction of the two species at 27°C (Schneider *et al.*, 2000) and may confound the effect of temperature on relative proportion of the species. Furthermore it may be true that the survival, development, competition and therefore relative proportion of *An. gambiae s.s.* and *An. arabiensis* are not as affected by short periods of extreme temperatures but that the average daily temperature is more important. In the present study the average mean daily temperature at the top and at the bottom of the water column did not differ between sites.

The absence of typical *An. gambiae s.s.* breeding sites and superior adaptation to restricted atypical breeding sites by *An. arabiensis* is given by Rishikesh *et al.* (1985) in explanation of the dry season dominance of *An. arabiensis* in Kaduna, Nigeria. The situation in The Gambia appears to differ from that in Kaduna insofar as the larval habitats in the dry season are similar to those available in the wet season; the main breeding sites are the flooded terraces bordering the river (Thomson *et al.*, 1995). As a result it seems plausible that the larvae of the *An. gambiae s.s.* population caught in the present study have adapted to the large permanent breeding sites perhaps more favoured by *An. arabiensis* elsewhere.

The analysis of the *An. gambiae s.s.* dry season population is made complicated by the fact that the species in The Gambia is polymorphic for three common independent inversions on the second chromosome (Bryan *et al.*, 1982). The distribution of these chromosomal forms seems at least partly dependent on climate (Bayoh *et al.*, 2001). The Bissau chromosomal form is common to coastal rice cultivated areas (Bryan *et al.*, 1982; Petrarca *et al.*, 1987), but it is very rare in Saruja (Bryan *et al.*, 1982), the site of the present study. Among the less common inversions the 2Rbc was the most prevalent in a 1979-81 sample, reaching frequencies of 2% in the east of the country (Bryan *et al.*, 1982). This inversion is characteristic of the Mopti chromosomal form, which is often associated with riverine and/or irrigated areas, and effectively exploits dry season larval habitats (Petrarca *et al.*, 1987). The Mopti form of *An. gambiae s.s.* dominates over *An. arabiensis* and makes up the majority of dry season *An. gambiae s.l.* collections in irrigated and rice cultivated areas of North and East Mali (Touré *et al.*, 1994; Dolo *et al.*, 2004) and Burkina Faso (Robert *et al.*, 1989). The site of the present study is of a similar environment and is typical of much of The Gambia, namely a riverine

environment surrounded by more arid areas of Sudan savanna. The dominance of *An. gambiae s.s.* in the present study might suggest a local increase in the Mopti form of this species, a form with a recent origin that is spreading from the inner delta of the Niger (Coluzzi *et al.*, 1985).

Though undeniably it is a reasonable assumption in the broadest sense that *An. arabiensis* dominates over *An. gambiae s.s.* in the hot dry season in Africa, the picture is more complicated at a local scale, most notably by interactions between physiological tolerances and resource availability, and the non-linear responses animals may show to abiotic factors in the presences of competition and predation (Chow and Clarke, 2000). Species will move, adapt or decline in response to climate; however marginal populations may survive through behavioural adaptation or acclimatisation by reducing high metabolic rates associated with stressful conditions (Hoffmann, 1995). It seems plausible that in the present study *An. gambiae s.s.* in the dry season is represented by acclimatised adults. Whether this is as a result of adaptation to high temperature larval rearing conditions or the spread of the thermotolerant Mopti form is unknown.

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CHAPTER IX

Water content and loss over time from *Anopheles gambiae sensu stricto* and *An. arabiensis* in a desiccating environment

Abstract

Although *Anopheles gambiae sensu stricto* and *An. arabiensis* occur sympatrically through much of sub-Saharan Africa, *An. arabiensis* is the more prevalent in areas presenting a high desiccation risk. This study examined the water content and loss over time of these two species using both infrared gas analysis (IRGA) and gravimetric methods. *An. gambiae s.s.* and *An. arabiensis* were found to have similar total water body content (~75%). However *An. gambiae s.s.* was found to lose a greater percentage of its weight after 60 minutes of exposure to 40°C and 30%RH than did *An. arabiensis*. This was reflected in the consistent significant inter-specific differences in the release of both CO_2 and water over time detected by IRGA. *An. gambiae s.s.* released CO_2 and water at a greater rate than *An. arabiensis*, after values were adjusted for differences in surface area between the species. *An. arabiensis* was also found to contain a disproportionably greater volume of haemolymph than *An. gambiae s.s.* These data would suggest that known inter-specific differences in knockdown and survival at 40°C and 30%RH may be due to better water preservation and replacement in *An. arabiensis* than in *An.gambiae s.s.*.

Introduction

Anopheles gambiae s.s. and *An. arabiensis* experience conditions of high heat and low relative humidity throughout much of their distribution. Where these species are sympatric it has been shown that *An. gambiae s.s.* predominates in saturated environments while *An. arabiensis* is more common in areas subject to desiccation. This chapter sets out to determine whether differences in water loss, tolerance of water loss and mechanisms of water loss resistance underlie this finding.

Water is essential for the maintenance of normal cellular structures such as membranes and proteins, it has a direct function in basic metabolic processes and is the solvent in which many biochemical reactions take place (Wigglesworth, 1972). Water also plays a part in heat distribution and temperature control because it has a high heat of

vapourisation and a high thermal capacity (Edney, 1977). Thus the removal of hydration layers is usually detrimental and often lethal to biological systems. Water loss and subsequent desiccation stress can occur at a particularly rapid rate in insects that occupy high temperature, low humidity environments. This is partly attributable to an increase in active ventilatory movements (Loveridge, 1968) but may also be explained by increased cuticular permeability resulting from changes in epicuticular structure and arrangement at high temperature (Toolson, 1982; Toolson and Kupersimbron, 1989; Gibbs *et al.*, 1998). The ability to withstand water loss is also affected by high temperatures; in *Drosophila* and other insects metabolic rates increase with increasing temperature (Hunter, 1964) and a high metabolism is known to limit the ability to tolerate other stress factors including desiccation (Hoffmann and Parsons, 1989).

Strategies for surviving periods of desiccation stress vary significantly between arthropod species. Several species are capable of adjusting the thickness and water-retentive quality of their cuticle in response to changing environmental conditions. For instance *Musca domestica* raised at 35°C produce more hydrocarbons by volume than those reared at 20°C (Noorman and Den Otter, 2002). Similarly, the lone star tick *Amblyomma americanum* exhibits a three-fold increase in cuticular wax deposition after feeding in preparation for off-host existence at lower relative humidities (Yoder *et al.*, 1997). Several insect species are capable of releasing CO_2 discontinuously (Williams and Bradley, 1998) and it has been suggested that discontinuous gas exchange is a mechanism for reducing respiratory water loss (Hadley, 1994). Ticks and fleas are capable of absorbing water from unsaturated air through the cuticle without a reduction in existing fluid levels (Kettle, 1995), and the capacity of the dipteran hind-gut to reabsorb water allows excretion to occur with minimum water loss (Coast, 2001). Desiccation resistance has also been associated with a reduction in locomotory activity (Williams *et al.*, 2004), an increase in heat shock protein transcription (Tammariello *et al.*, 1999) and an increase in haemolymph volume (Folk and Bradley, 2003).

In mosquitoes and other holometabolous insects, strategies for surviving periods of desiccation stress are also dependent on life stage. In *Aedes* species from Indonesia adult desiccation resistance was associated with conversion of glycogen to trehalose and accumulation of free fatty acids (Mogi *et al.*, 1996; Sawabe and Mogi, 1999).

Mosquito larvae including those of *An. gambiae s.s.* are highly water-permeable in air lose about 40% of initial body water in 4 minutes (Liu and Mazur, 2003). However in their natural environment the chitin-protein cuticle may reduce larval permeability and larvae replace lost fluids through drinking considerable volumes of water (Aly and Dadd, 1989). Though anopheline eggs are not adapted for long-term viability in the absence of water, being much more water permeable than those of *Drosophila* (del Pilar *et al.*, 1996) or *Aedes* (Sota and Mogi, 1992) species, the eggs of both *An. gambiae s.s.* and *An. arabiensis* can remain viable for up to 12 days in dry soil (Beier *et al.*, 1990; Minakawa *et al.*, 2001). The waxy permeability barrier deposited on the endochorion surface (Mazur *et al.*, 2001) may represent a significant short-term survival mechanism under desiccation stress.

Despite preventative measures, mean daily water loss rates can still be high and so tolerance of water loss is important and indeed common in xeric species. This tolerance can equate to a 10% loss of total body weight on a daily basis in Arizona tiger beetles (Hadley and Schultz, 1987) and up to 30% in the desert cockroach *Arenivaga investigata* (Edney, 1966). Body water content, expressed as a percentage of total weight, may also reflect adaptation to desiccation resistance (Edney, 1977; Mazer and Appel, 2001); a high water content perhaps representing a large water reserve that can be utilized at times of desiccation stress.

Insect water loss rates have been determined gravimetrically for over 70 years (Ramsay, 1935) for a range of orders including Hymenoptera (Johnson, 2000), Lepidoptera (Mazer and Appel, 2001) and Isoptera (Shelton and Grace, 2003). More recent studies have taken advantage of sensitive infrared water analyzers (Quinlan and Hadley, 1993; Williams *et al.*, 1998). Here both methods were employed to detect differences between *An. gambiae s.s.* and *An. arabiensis* in initial body water content, percentage water loss and differences in respiration and transpiration over time under environmentally stressful conditions.

Materials and Methods

An infrared gas analyzer system provided measurement of CO_2 and H_2O produced by groups of 60 female mosquitoes held at 40°C and 30%RH for 4 hours. CO_2 and H_2O levels were detected and recorded every minute of this period. CO_2 production was monitored as an indication of metabolic rate. The system utilised a standard and constant concentration of water vapour produced by a water vapour generator WG602 (ADC BioScientific, Hoddesdon, England). The generator unit was supplied with dry air at a constant pressure of 10 p.s.i. (approximately 0.6bar) from a compressed air gas cylinder (British Oxygen Company) fitted with a two-stage pressure regulator. The gas cylinder was connected to the vapour generator by a 1.5m long rubber tube of 5mm internal diameter. Water vapour was generated by crystalisation of ferrous sulphate, with vapour concentration dependent on salt temperature within the generator. The generator operated inside a programmable incubator (S.H. Scientific, Kent, U.K.) at 20°C, producing air with water partial pressure of 15±2mb. The generator was specifically run at a lower temperature than the experimental room ambient temperature of 30°C in order to restrict the amount of water in the air available to the mosquitoes and also to prevent 'dewing out' in the connecting tubes. The gas of known water partial pressure was routed from the generator into a leaf chamber analyser type LCA4 (ADC BioScientific, Hoddesdon, England) through a 170cm rubber tube of 3mm internal diameter. A T-type connector (Nalgene™, USA) was used to connect a 70cm plastic exhaust pipe of 3mm internal diameter to the rubber tube to draw off surplus air from the gas cylinder if the pressure changed. The LCA4 receives the air from the generator, passes it through a Portable Leaf Chamber type PLC4 and receives the air once more before the gas is vented away, in an 'open system' configuration. The mosquitoes were contained within the chamber. Measurements were carried out on the state of the incoming gas (the reference levels) and after passing through the chamber (the analysis levels). A trial run with no mosquitoes present in the chamber was used to obtain control levels of CO_2 and H_2O. These baseline values were subsequently subtracted from the experimental data values. The CO_2 and H_2O values were then adjusted to take into account differences in surface area and weight between the two species.

Surface area was calculated using the following equation:

s.a. (cm^2) = 0.0886 + 26.85(Wg) – 214.21(Wg^2)
where s.a. is surface area and Wg is weight in grams (Haagsma *et al.*, 1996).

When the generator crystal temperature equaled that of ambient temperature the generator supplied air at ~60%RH to the LCA4, therefore the LCA4 settings were used to pass air through iron II ferrous sulphate ($FeSO_4.7H_2O$) and indicating anhydrous calcium sulphate housed in the LCA4 itself, so that air at 30±5%RH was passed through the chamber. The PLC4 was placed in another programmable incubator at 40°C and this temperature was monitored by the LCA4.

Female mosquitoes were removed from 30cm^3 stock cages at age 3-6 days into 15cm^3 cages from which 60 were removed for each trial. A hand-held mechanical aspirator (Fulton, USA) was used to aspirate mosquitoes into a 50mm long plastic cylindrical holding tube of 26mm internal diameter. Two windows 25 x 20mm were cut in the tube and the holes covered with netting of 2mm mesh size. The two ends of the tube were also covered with netting. This allowed maximum airflow over the holding tube once introduced into the PLC4 chamber whilst preventing mosquito escape. Each batch of mosquitoes was anaesthetized by a 5 minute exposure to ethyl acetate and weighed as a group within the holding tube using a balance sensitive to 100μg. This was recorded as initial body weight. The holding tube was then placed back into a 15cm^3 cage held at high humidity, and the mosquitoes were allowed 15 minutes to fully recover. The holding tube was then sealed in the PLC4 chamber. A paper facemask was worn when placing the holding tube into the chamber so as to prevent any carbon dioxide and water vapour produced by the user from entering the chamber. The system was run for approximately 30 minutes before the mosquitoes were placed in the chamber in order to purge any residual air from the system. Once the mosquitoes were added 5 more minutes were allowed before recording commenced in order to purge the laboratory air which entered the system when the chamber was opened.

In total 24 trials were conducted. In 12 trials (6 for each species) recordings were made for the full 4 hours and in the other 12 the trial was stopped after 1 hour. At the end of all trials the mosquitoes were reweighed in the holding tube. The mosquitoes were then

removed and the tube reweighed. The holding tube was always weighed pre- and post-trial to demonstrate that trial conditions brought about no change in its weight. Post-trial groups of mosquitoes were placed in 9cm diameter Petri dishes containing 5g of anhydrous Ca_2SO_4 (Drierite), dried at 50°C for a minimum of 48 hours in a programmable incubator before being reweighed. Percentage total body water was calculated using the following equation:

% total body water = ((initial body weight – dry weight)/ initial body weight) x 100

Mann Whitney U tests were used to evaluate median values and percentages in all gravimetric comparisons. Independent samples t-tests were used to compare mean CO_2 and H_2O production at 30 minute intervals and a 1-way analysis of variance was used to compare CO_2 and H_2O production between time periods.

Haemolymph extraction

The centrifugation procedure for collecting haemolymph from adult female mosquitoes largely followed that described by Mack and Vanderberg (1978). The species were assessed 'blind'. Mosquitoes were anaesthetized in a refrigerator at 4°C, weighed as a group and then placed in test tubes chilled on ice before being transferred to the viewing platform of a dissecting microscope. An incision was made across the proboscis and into the abdominal wall of each mosquito using a fine-blade scalpel and a micro-needle. Forty female mosquitoes were loaded into each centrifugation tube. These consisted of a 1ml disposable pipette tip with 35mm removed from the wide end held inside a 1.5ml microfuge tube. A small glass wool plug in the pipette tip separated the mosquitoes from the collected haemolymph. Samples were centrifuged at 800g for 10 minutes after which the haemolymph was removed and the volume measured using a graduated 10µL Hamilton syringe. 1µL of coloured food dye was added to the sample to facilitate complete removal of the haemolymph from the tube and 1µL was subsequently subtracted from the recorded haemolymph volume. Mosquitoes were re-weighed after haemolymph extraction. An independent samples t-test was used to compare mean haemolymph volumes between species.

Results

The molar flow release of CO_2 mm^{-2} s.a. was consistently greater from *An. gambiae s.s.* than from *An. arabiensis* (table 9.1) during the first 150 minutes of the 240 minute period. Mean water loss mm^{-2} s.a. from *An. gambiae s.s.* was also significantly greater than that from *An. arabiensis* for all except the first 30 minutes of the 240 minute period (table 9.2). In both species, water loss was initially high but fell within the first 60 minutes and remained constant thereafter. There was no difference in mean water loss during the final 180 minutes of the trial from *An. gambiae s.s.* ($F = 1.3$, $df = 5$, p = n.s.) or *An. arabiensis* ($F = 1.7$, $df = 5$, p = n.s.).

Table 9.1 Mean CO_2 release over time from *An. gambiae s.s.* and *An. arabiensis* at 40°C and 30%RH

	CO_2 release (µmol mol^{-1} mm^{-2} s.a.)						
Time period	*An. gambiae s.s.*		*An. arabiensis*				
(min)	Mean	95%CI	Mean	95%CI	df	t	P
0-30	2.76	2.65-2.86	2.16	1.99-2.32	475	6.9	<0.001
30-60	1.58	1.52-1.65	1.07	0.97-1.17	475	8.1	<0.001
61-90	1.35	1.29-1.43	1.09	1.07-1.19	328	4.4	<0.001
91-120	1.28	1.22-1.34	1.06	0.97-1.15	328	3.8	<0.001
121-150	1.18	1.13-1.24	0.98	0.90-1.06	328	3.8	<0.001
151-180	1.06	1.00-1.12	1.04	0.96-1.11	328	0.4	n.s.
181-210	1.02	0.97-1.06	1.03	0.96-1.10	328	-0.3	n.s.
211-240	1.07	1.03-1.11	0.86	0.80-0.93	328	5.1	<0.001

Table 9.2 Mean H_2O release over time from *An. gambiae s.s.* and *An. arabiensis* at 40°C and 30%RH

	H_2O release (µbar mm^{-2} s.a.)						
Time period	*An. gambiae s.s.*		*An. arabiensis*				
(min)	Mean	95%CI	Mean	95%CI	df	T	p
0-30	175.9	165.0-186.8	159.2	141.6-176.8	475	1.6	n.s.
30-60	98.4	93.0-103.9	70.0	64.8-75.2	475	7.5	<0.001
61-90	76.6	71.9-81.3	46.6	43.0-50.2	328	10.1	<0.001
91-120	73.6	69.7-77.6	47.1	42.8-51.4	328	9.0	<0.001
121-150	77.7	73.9-81.4	49.0	43.8-54.2	328	8.8	<0.001
151-180	75.1	71.7-78.1	46.3	41.3-51.4	328	9.3	<0.001
181-210	72.7	68.7-76.8	58.7	54.8-62.6	328	4.9	<0.001
211-240	78.0	73.8-82.3	55.6	50.6-60.5	328	6.8	<0.001

Table 9.3 Progression of body weight loss over time in *An. gambiae s.s.* and *An. arabiensis* at 40°C and 30%RH

	An. gambiae s.s.		*An. arabiensis*				
	Median	IQR	Median	IQR	Z	df	p
Initial mosquito body weight (mg)	1.33	1.22-1.51	1.77	1.49-1.87	-2.8	23	0.006
% of initial weight after 60min exposure	78.1	74.2-80.1	82.8	79.7-84.8	-2.0	11	0.05
% of initial weight after 240min exposure	35.1	27.2-42.0	45.8	34.9-63.7	-1.4	11	n.s.
Dry weight (as % of initial weight)	25.8	22.2-28.2	23.9	19.9-28.6	-0.9	23	n.s.
% total body water	74.2	71.8-77.8	76.1	71.4-80.1	-0.9	23	n.s.

The initial body weight of *An. arabiensis* was significantly greater than that of *An. gambiae s.s* (table 9.3), and so subsequent comparisons of weight were calculated as percentages of initial body weight. There was no significant difference in the weight of the holding tube and lid before and after the experimental period (95%C.I. of the difference = -8.3 – 1.1mg, p = 0.12) so weight loss data could be treated with confidence. There was no inter-specific difference in the initial total body water content (table 9.3). There were significant differences in weight lost between the species after 60 minutes but not after 240 minutes; *An. gambiae s.s.* suffered a 21.9% reduction in body weight during the first 60 minutes, on average 4.7% greater than that of *An. arabiensis* at that stage. Initial body weight affected the percentage of weight loss that occurred after both 60 and 240 minutes (fig 9.2).

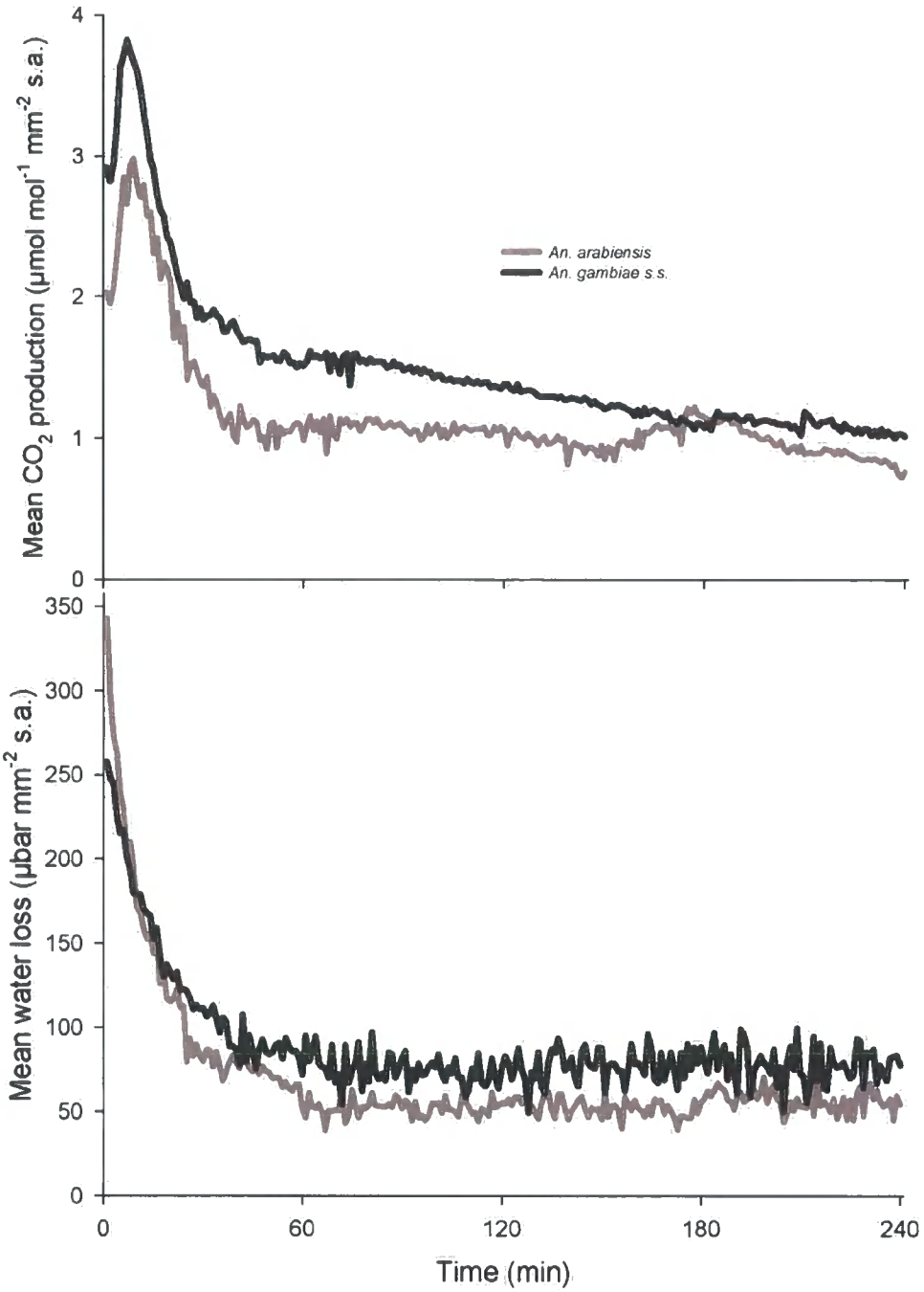

Fig 9.1 Mean CO_2 and H_2O release by *An. gambiae s.s.* and *An. arabiensis* recorded every minute during 4 hour exposure to 40°C and 30%RH. 95%C.I.s show a degree of overlap between the two species and are therefore not shown to maintain clarity of image.

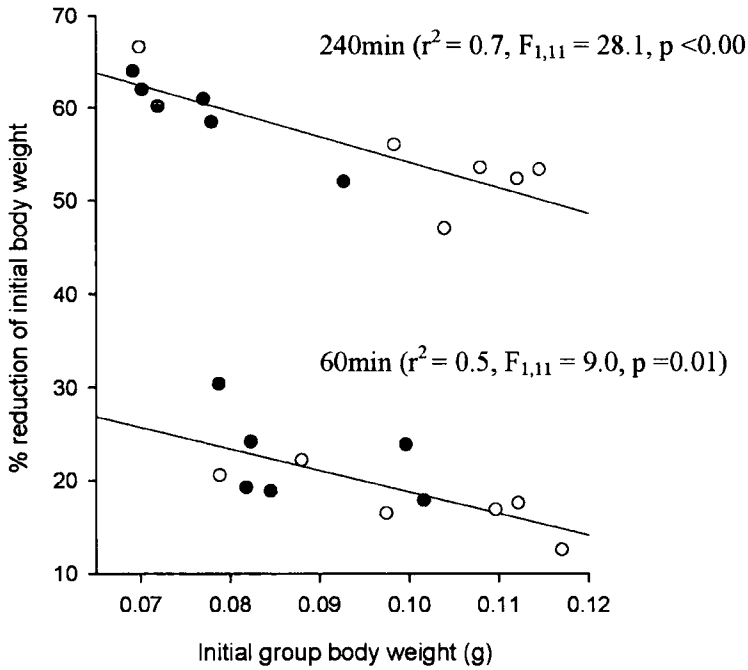

Fig 9.2 Negative relationship between initial mosquito body weight and percentage loss of body weight after 60 and 240 minutes at 40°C and 30%RH. Each circle represents a group of ~60 individual *An. gambiae s.s.* (●) or *An. arabiensis* (○) mosquitoes. Best fitting regression line based on the equation for a straight line y = b + mx where b = the y-axis intercept, when X = 0, and m = the slope of the line (DY/DX).

An. arabiensis contained 0.11 µL (0.06-0.17) mosquito^{-1} more haemolymph than *An. gambiae s.s.* (t = -4.5, df = 10, p = 0.001, table 9.4). Group weight was not recorded after haemolymph extraction as sample preparation often resulted in the removal of mosquito scales and legs which would have affected the measurement.

Table 9.4 Comparison of haemolymph volume in *An.gambiae s.s.* and *An. arabiensis*

Species	Group total body weight (mg)		Haemolymph volume (µL)		Haemolymph vol (µL) mosquito^{-1}	
	Mean	95%C.I.	Mean	95%C.I.	Mean	95%C.I.
An. gambiae s.s.	42.4	33.5-51.3	8.1	6.6-9.5	0.20	0.16-0.23
An. arabiensis	57.4	45.6-69.2	12.5	10.4-14.6	0.31	0.26-0.36

Discussion

An. gambiae s.s. and *An. arabiensis* necessarily have an excellent control of water balance because not only are they capable of surviving in desiccating environments (Lindsay *et al.*, 1998) they must also be capable of transporting and excreting large quantities of water during blood- and nectar-feeding; typically over 40% of the water contained in the ingested blood plasma is discharged within one hour of feeding (Clements, 1992). Water is also necessarily discharged by newly-emerging adults to reduce the haemolymph volume carried forward from the juvenile stages. *An. gambiae s.s.* adults exhibit an immediate post-eclosion burst of water excretion from the anus, lasting up to 12 minutes (Goma, 1964). In considering explanations for survival in desiccating conditions, three mechanisms are apparent that would identify a resistant species from a less resistant one. Firstly an increase in the amount of water in the body, secondly a reduction in the rate of water loss from the body and lastly a tolerance of a greater percentage of water loss.

Total body water content is variable both within and between insect species occupying a range of habitats (Edney, 1977). High percentage total body water content however does not always reflect adaptation to desiccating environments. This is because factors other than desiccation influence body water content. For instance adult beetles in which the integument contributes greatly to body weight have a lower water content than other species because the water content of arthropod cuticle is relatively low (Noble-Nesbitt, 1991). Desiccation resistance in cactophilic species of *Drosophila* does not correlate with total body water content (Gibbs and Matzkin, 2001) and that is also apparent in this study. In both cases this may reflect a trade-off between locomotor capacity and the storage of water for desiccation resistance (Lehmann and Dickinson, 2001). Insects carrying a large load show a reduced capacity for flight control rendering them susceptible to predation and preventing behavioural escape from stressful environments.

In both *An. gambiae s.s.* and *An. arabiensis* the pattern of water loss parallels the pattern of CO_2 loss, that is, high but falling during the first 60 minutes and then leveling out. This pattern of decreasing water loss rate with time during lengthy periods of desiccation stress has been observed in many insect species including locusts

(Loveridge, 1968), tenebrionid beetles (Edney, 1971), crickets (Hadley *et al.*, 1986) and cockroaches (Noble-Nesbitt and Al-Shukur, 1987). The most likely explanation for the decrease in both CO_2 and water release in at least the first 30 minutes is as a result of declining activity as the insects become familiar with new conditions. A first response to desiccation stressful environments would be to move away, especially in species with poor control over water retention. For example, the ectoparasitic mite *Varroa jacobsoni* has a high surface area : volume ratio and very little excess water to lose; it therefore responds to desiccation by seeking out a high relative humidity site or a new host to replenish lost water (Bruce *et al.*, 1997). It is known that humidity affects activity in insects: Bursell (1957) found tsetse flies were more active when desiccated and in dry air than humid air. The high initial activity increases metabolism and the demand for gas exchange results in increased spiracular opening and therefore respiratory water loss (Edney, 1977). Interestingly, Williams *et al* (2004) found that desiccation-selected flies subjected to the equivalent amount of desiccation exposure as non-selected flies (i.e. half of the time required to kill them at low RH%) do not exhibit this initial increase in activity in dry air. Unfortunately it was not possible to observe the activity of the mosquitoes within the incubator in the present study though the difference in CO_2 production within the first 60 minutes suggests a metabolic difference between the species that doesn't manifest itself in restricting water loss but may have some other function. Discontinuous gas exchange may play a part here but obviously this could not be detected because the CO_2 release was recorded from more than one mosquito.

Previous studies have found that desiccation resistant *Drosophila* populations exhibit a significantly lower mean rate of water loss than control populations (Gibbs *et al.*, 1997; Williams *et al.*, 1998). The consistent significant inter-specific differences detected by IRGA reveal that *An. arabiensis* loses water and releases CO_2 less rapidly over time than *An. gambiae s.s.* and suggests greater control over water loss in *An. arabiensis* confers better survival under these conditions. This is supported by the finding that *An. gambiae s.s.* loses a greater percentage of its weight during the first 60 minutes of exposure than *An. arabiensis*. The first 60 minutes of exposure seem to be the most critical; it has been shown that at 40°C and 30%RH *An. gambiae s.s.* suffers greater knock-down and mortality than *An. arabiensis* at this time (chapter III). Unfortunately in this experiment it was impossible to record the point of knock-down and thus relate water loss to knock-down directly because the incubator had to be kept closed to

maintain chamber temperature. Likewise water loss could not be directly related to mortality 24 hours later as the mosquitoes had to be weighed immediately after 60 or 240 minutes exposure which required killing those not already knocked-down. However by using the data of chapter III it is possible to infer that neither species is capable of surviving a loss in body weight of 50% or above as both species suffer ~100% mortality after 240 minutes exposure to 40°C and 30%RH.

That haemolymph is important to insect body water dynamics is not novel thinking. Mellanby (1939) proposed that insect haemolymph could act as a source of freely available water which could be distributed to tissues during periods of desiccation stress. It is essential to realise that haemolymph does not serve the purpose of oxygen transport in insects and so a constant blood volume is not necessary as long as there is sufficient circulation to provide for the transport of food materials, hormones and other substances. Thus haemolymph can indeed function as a water store and the water can be removed from the blood to maintain the water content of tissues and vice versa without any osmotic complications (Edney, 1977).
Evidence of this has been found in *Drosophila melanogaster* populations selected for desiccation resistance, which show a >6-fold increase in haemolymph volume relative to unselected control populations (Folk and Bradley, 2003). The role of haemolymph seems to relate to the capacity to adjust tissue inorganic solute content when total body water declines during desiccation. Folk and Bradley (2003) hypothesise that increasing tissue ion concentration may cause structural changes in cuticular proteins and therefore affect cuticular permeability. The disproportionably (according to body weight) greater volume of haemolymph extracted from *An. arabiensis* compared to *An. gambiae s.s.* suggests haemolymph in these mosquitoes may be as critical to desiccation resistance as it is in *Drosophila melanogaster*. Though there is no difference in the amount of total body water that they contain, perhaps *An. arabiensis* has more of this water freely available in the form of haemolymph than *An. gambiae s.s.*, the water body of which may be locked up and not usable in the same way as the haemolymph store.

It would be useful to establish whether water loss from both species is largely respiratory at 40°C and 30%RH. The parallel patterns of CO_2 release and water loss would suggest this was the case. It has been reasonably established that the greatest water loss experienced by most insects occurs as evaporation from the cuticle (Edney,

1977; Gibbs and Pomonis, 1995). However in some arid-zone beetles this has been reduced to such an extent that respiratory loss is the only significant loss of water; this occurs due to variation in the local humidity around the spiracles. The lower rate of water loss experienced by the tiger beetle *Cicindela obsoleta* when compared to closely related species has been attributed to differences in cuticular hydrocarbons: notably a greater production of branched alkanes and a reduction in the amount of unsaturated *n*-alkenes (Hadley and Schultz, 1987). Hydrocarbon composition is known to differ between *An. gambiae s.s.* and *An. arabiensis* (Milligan *et al.*, 1993). This suggests the role of hydrocarbons in these mosquitoes would be a valuable area to explore in future research.

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CHAPTER X

Induced thermotolerance and associated expression of the heat-shock protein Hsp70 family in adult *Anopheles gambiae sensu stricto* and *An. arabiensis*

Abstract

Heat shock proteins (Hsps) are synthesised as cellular thermoprotectants when temperatures are increased to levels substantially above normal. Here a preliminary investigation was conducted into the importance of the Hsp70 group of heat shock proteins to the thermotolerance of *Anopheles gambiae sensu stricto* and *An. arabiensis*. It was hypothesised that *An. arabiensis*, the more abundant of the two species in stressful high temperature, low humidity environments, would express higher levels of Hsp70 compared to *An. gambiae s.s.*. Groups of 50 adult mosquitoes of both species were exposed to 40°C for 60 minutes, with or without a period of prior heat hardening at 36°C for 60 minutes. Intraspecific heat-hardened and heat-shocked groups expressed a greater level of Hsp70 proteins than control groups in both species. Heat-shocked but not heat-hardened *An. arabiensis* exhibited a greater knockdown resistance than counterpart *An. gambiae s.s.* groups. However there were no apparent differences in the heat-shock responses of these two mosquito species. The expression of Hsp70 proteins cannot explain the difference in knockdown resistance between *An. arabiensis* and *An. gambiae s.s.* and therefore is probably of limited importance to their differential survival and geographical distribution.

Introduction

To survive and reproduce in a stressful environment, organisms must have effective mechanisms for tolerating or avoiding the most harmful conditions. The central phenomenon of one such mechanism of stress response is the rapid and specific induction of a set of proteins when organisms are abruptly exposed to temperatures well above their normal physiological growth conditions (Parsell and Lindquist, 1994). These proteins are known as heat shock proteins (Hsps); they are molecular chaperones found in the cell and are responsible for the correct folding, unfolding, assembly and secretion of multi-structured units, and the degradation or refolding of mis-folded or

aggregated proteins (Maresca and Lindquist, 1991). This is vital to the fitness of the individual cell and of the organism as a whole as these aggregates can cause cellular damage and death (Krebs and Feder, 1998). Evidence of the cellular protection function of Hsps has been gathered from an extensive and diverse set of organisms including yeast (Craig et al., 1991), *Drosophila* (Krebs and Bettencourt, 1999; Lansing *et al.*, 2000) , fish (Iwama et al., 1999) and humans (Wu et al., 1985). In fact it seems that Hsps are expressed by almost all organisms that have a high conservation of primary structure after exposure to high temperatures (Krebs and Feder, 1997).

Induced thermotolerance, also referred to as heat hardening, is the result of exposure to a sub-lethal heat stress that confers a survival advantage when the same individuals are subsequently exposed to temperatures in the lethal range for that species. Heat pre-treatments elicit resistance not just to high temperatures but to a range of other stresses as well (Parsell and Lindquist, 1994). Often a relatively short period of exposure is sufficient, and both the temperature of the hardening period and the length of recovery time prior to heat shock are significant in predicting the survival of the study specimens. In insects this phenomenon has been observed in adults and larvae of *Drosophila* (Feder *et al.*, 1996; Dahlgaard *et al.*, 1998) and in the larvae of the mosquito *Anopheles albimanus* (Benedict *et al.*, 1991). Such tolerance-inducing treatments generally also induce hsp synthesis (Feder and Hofmann, 1999), and it is striking that hsps are induced at very different temperatures in different organisms yet in each case, at a temperature that constitutes a stress for that particular organism (Parsell & Lindquist, 1994).

The first members of the 70-kD heat shock protein family known as hsp70 were identified in the 1970s, and they is now considered one of the evolutionarily most highly conserved protein families (McKay et al., 1994). Heat shock proteins can all be categorised as either induced by stress, constitutive, or both constitutive and inducible by stress (Morimoto *et al.*, 1994). It is apparent that not all of the hsp70 family members are functionally interchangeable (Hightower et al., 1994). Some hsp70s are constitutively expressed such as Hsc70 and Hsc73, the transcript abundance of which is not increased after heat shock in *Drosophila* (Craig *et al.*, 1983). Others hsp70s are induced by heat or cold, and in multicellular organisms there are tissue-specific versions (Lindquist and Craig, 1988). Hsp70 is the primary inducible heat-shock protein produced by both larval and adult *Drosophila*, and there is some evidence that its

production confers resistance to high temperatures (Parsell and Lindquist, 1994; Dahlgaard *et al.*, 1998; Krebs and Feder, 1998). Up-regulation of hsp70 has also been found to correlate with induced thermotolerance in locusts (Barclay and Robertson, 2000) and larvae of the medfly *Ceratitis capitata* (Stephanou *et al.*, 1983).

Despite the frequent induction of hsps by heat stress in laboratory studies, the importance of hsps in stress tolerance is still subject to some dispute. Until recently very few studies have been carried out on natural field populations or even using ecologically relevant stress intensities in the laboratory. This work is crucial to determining how common hsp expression actually is in nature. It is emerging that there is considerable variation in the ability to withstand stress and the level of hsps produced between species, and even within species, between seasons and with geographical distribution (Feder *et al.*, 1996; Krebs and Feder, 1997; Sorensen *et al.*, 2001; Zatsepina *et al.*, 2001). Hsps appear to have an affect on development, fecundity and life span as well as stress resistance (Rutherford and Lindquist, 1998; Sorensen and Loeschcke, 2001; Minois and Vaynberg, 2002), and so the relationship between hsp expression and thermotolerance may be further clouded.

To understand how hsps result in stress tolerance at an organismal level it is useful to characterise the stress response along gradients that occur in nature (Feder and Hofmann, 1999). Here I address the question of whether organisms from environments with much stress have a different or increased stress response compared to those from environments with less stress. The mosquito species *An. gambiae s.s.* and *An. arabiensis* provide a useful model. *An. arabiensis* is found in regions of Africa and at times of year where *An. gambiae s.s.* is not (Mekuria *et al.*, 1982; Rishikesh *et al.*, 1985; Petrarca *et al.*, 2000). These areas are characterised by more stressful conditions of higher temperatures, lower humidities and therefore greater desiccation risk than areas in which *An. gambiae s.s.* is the predominant species. It is hypothesised that in order to survive in high stress environments, especially in light of its more exophilic nature (Githeko and Service, 1996), *An. arabiensis* will express higher levels of hsp70 compared to *An. gambiae s.s.* The relationship between induced thermotolerance and the expression of inducible heat shock proteins has apparently not been studied in mosquitoes up to now.

Materials and methods

Bioassay

To describe between-species variation in adult Hsp70 concentration after heat shock, with and without prior heat hardening, 150 unfed 3-day old female mosquitoes of both *An. gambiae s.s.* and *An. arabiensis* were reared in the standard manner (chapter II). Fifty adults were exposed to air temperatures of 36°C for 1 hour then allowed a 2 hour recovery period at 27°C and 50±5%RH. This period was used because levels of Hsp70 have previously been shown to be highest 2 hours after heat hardening (Dahlgaard et al., 1998). These adults, referred to as the heat-hardened group, were then exposed to the potentially lethal stress conditions of 40°C and 30±5%RH for 1 hour. A separate cohort of 50 adults, referred to as the heat-shocked group, did not receive the heat hardening treatment and was also assayed simultaneously under the same stress conditions for 1 hour. The proportion of thermotolerant flies was scored at the end of the period; mosquitoes unable to right themselves after probing with the tip of a pipette were scored as knocked down and therefore not thermotolerant. The heat hardened and heat-shocked groups of both species were then frozen at -70°C along with a control group of 50 adults maintained at 27°C with no period at 36 or 40°C. The control and heat shocked groups were counted and handled in the same way as the heat-hardened populations i.e. removed and placed in an incubator at 27°C. 50 flies per treatment were used in order to reduce random variation in body size between trials, and to give a large enough working sample mass.

Protein preparation

Protein was extracted from all three groups by homogenisation in an antiprotease cocktail. Samples were homogenised without prior thawing, using a hand-held homogeniser, in 1ml of extraction buffer consisting of ice-cold phosphate buffered saline (PBS) containing 1μg ml^{-1} pepstatin A, 50μg ml^{-1} leupeptin and 2μg ml^{-1} antipain (Sigma-Aldrich Inc.). 10mM phenyl methyl sulphonyl fluoride (PMSF) in 100% ethanol was added to the homogenate to give a final concentration of 100μg/ml, and this was centrifuged at 13,000g at 4°C for 30 minutes. The supernatant was pipetted into new microfuge tubes, 100μl was retained to measure the protein concentration and the remainder was stored at -70°C until needed. The protein concentrations of all samples were determined in triplicate using a Lowry assay (Lowry *et al.*, 1951) and compared to

a standard curve created using bovine serum albumin (BSA) using a Jenway (Geneva) colorimeter at optical density OD750.

Protein concentrations were low and so samples were concentrated by the chloroform/methanol procedure. 400µL of methanol were added to 30µL of the protein sample and 70µL ddH_2O. This was vortexed then pulsed in a bench centrifuge up to 15,000g. 100µL of chloroform were added and the mixture was again vortexed and pulsed up to 15,000g. 300µL of ddH_2O were added and vortexed, then centrifuged for 1 minute at 15,000g. As much of the top layer as possible was removed without disturbing the intermediate phase. 100µL of methanol were added and again vortexed then centrifuged for 4 minutes at 15,000g. The supernatant was removed to leave the pellet, which was allowed to dry for 30 minutes before being re-suspended in 5µL sample loading buffer, and 8µLH_2O, and stored at -20°C. When all the samples had been prepared, they were thawed, 2µL of 1M dithiothreitol (DTT) were added and then heated in a dry heating block at 95°C for 5 minutes, cooled on ice and centrifuged at 15,000g at 4°C for 2 minutes. Samples were loaded onto a 9% SDS polyacrylamide gel (SDS-PAGE) and separated at 170V for 60-80 minutes. The gel was stained with Coomassie blue for 2-3 hours and de-stained for 1-2 hours at 30°C with gentle agitation on a flat-bed shaker.

Western blot technique

The electrophoretically separated proteins were transferred from the SDS-PAGE to a nitrocellulose membrane (Hybond™ – ECL™), by electrophoresis at 10mA for 45 minutes then 15mA for 1hr, following standard procedure (Sambrook *et al.*, 1989). Binding sites on the nitrocellulose were blocked for 1 hour at 20°C using a powdered milk blocking solution (5% dried milk, 0.2% Tween20 in Tris-buffered saline (TBS)). The nitrocellulose was then incubated with a monoclonal antibody hsp70 clone 3A3 (Affinity Bioreagents™, Colorado) raised in mouse and used at 1:5000 dilution at 4°C overnight. The membrane was washed in TBS before incubation with the secondary immunological reagent, an HRP-conjugated anti-mouse IgG (Sigma-Aldrich Inc.) at 1:2000 dilution for 1 hour, then washed again. Luminol was oxidised by HRP in the presence of hydrogen peroxide and the chemiluminescence emitted was detected by exposing the membrane to X-ray film.

Quantification of the film image bands was performed using a personal densitometer (Molecular Dynamics Inc.) and ImageQuant™ software. The membrane was washed and probed again using monoclonal anti β-actin primary antibody produced in mouse (Sigma-Aldrich Inc.) and the same secondary antibody to confirm that protein concentrations were consistent between samples.

Statistical analysis

The Chi-squared test was used to compare the knockdown resistance of the heat-hardened and heat-shocked groups of both species to each other and to previous results of knockdown under the same conditions (chapter III).

Results

The heat-shocked *An. arabiensis* group exhibited a 20% greater level of knockdown resistance than the equivalent *An. gambiae s.s* group after exposure to 40°C and 30%RH (*An. arabiensis* 64% resistance, *An. gambiae s.s.* 44%, df = 1, $X^2 = 4.03$, p = 0.04). However there was no significant interspecific difference in the level of knockdown resistance experienced by the heat-hardened groups (*An. arabiensis* 66%, *An. gambiae s.s.* 52%, df = 1, $X^2 = 2.03$, p = n.s.). 100% knockdown resistance was recorded from the control groups of both species, and so knockdown was considered to be entirely a result of the stressful exposure conditions. The levels of knockdown resistance in the heat-shocked and heat-hardened groups of both species was not significantly different to that experienced by groups exposed to the same conditions in an earlier experiment (table 10.1).

Table 10.1 Chi-squared comparison of knockdown resistance between studies

	Proportion of knockdown resistant mosquitoes (%)					
Species	Heat-shocked chapter III	Heat-shocked group	Heat-hardened group	df	X^2	p
An. gambiae s.s.	38/100 (38)	22/50 (44)		1	0.5	n.s.
An. gambiae s.s.	38/100 (38)		26/50 (52)	1	2.7	n.s.
An. arabiensis	75/100 (75)	32/50 (64)		1	2.0	n.s.
An. arabiensis	75/100 (75)		33/50 (66)	1	1.3	n.s.

The coomassie blue staining of the SDS-PAGE gel revealed no obvious differences in protein expression between species or groups (fig 10.1).

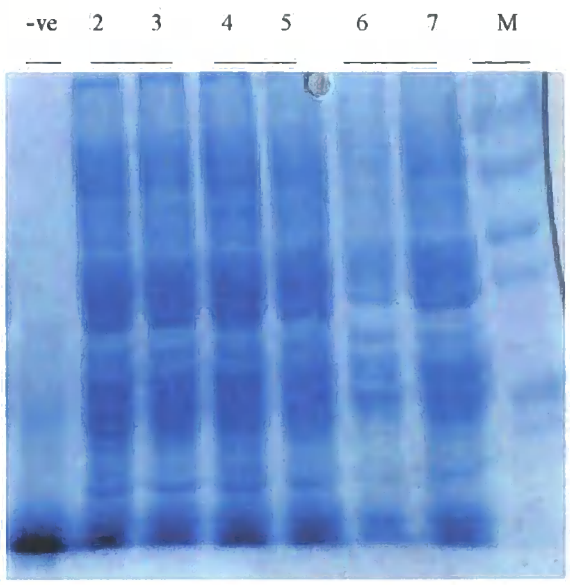

Fig 10.1 Coomassie blue stained SDS-PAGE gel of total extracted protein from control (lanes 2&3) heat-shocked (4&5) or heat-hardened (6&7) populations of *An. gambiae s.s.* (LH lanes) and *An. arabiensis* (RH lanes). Lane 1 = negative control. Lane 8 = Molecular markers

Proteins in the size range of the Hsp70 family, 70kDa to 78kDa, were detected by the monoclonal antibody (fig 10.2). A base level of expression that occurred in control populations seems likely to represent constitutive members of the family. Hsp expression was approximately two times greater in both heat-shocked and heat-hardened groups than in the control groups in both *An. gambiae s.s.* and *An. arabiensis* though there were no obvious differences between the groups exposed to the heat stress. There are no interspecific differences in hsp production between any of the groups (table 10.2).

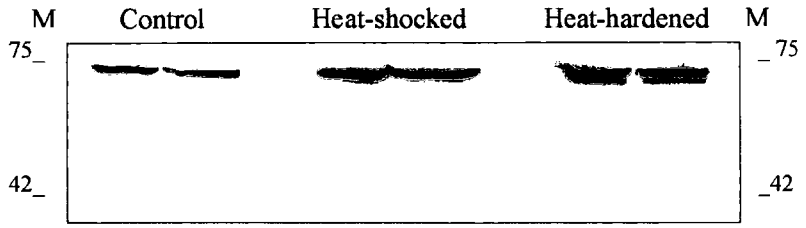

Fig 10.2 Immunological detection of heat shock protein members of the Hsp70 family expressed by control, heat-shocked, or heat-hardened populations of *An. gambiae s.s.* (LH lanes) and *An. arabiensis* (RH lanes). Marker: Sigma prestained 'blue' marker.

Table 10.2 Quantification of expressed heat shock protein

Group	Species	Volume (OD units)	% relative density
Background level	-	106	0.50
Control	*An. gambiae s.s.*	1868	8.72
	An. arabiensis	1871	8.74
Heat-shocked	*An. gambiae s.s.*	3359	15.78
	An. arabiensis	4865	22.71
Heat-hardened	*An. gambiae s.s.*	4787	22.34
	An. arabiensis	4568	21.32

Discussion

Induction of heat shock proteins in many organisms has been seen to only occur at temperatures that constitute a stress for that particular organism (Parsell and Lindquist, 1994). Hsp expression was greater in both heat-shocked and heat-hardened groups than in the control groups suggesting some purely inducible members of the hsp70 family were induced by exposure of whole adult mosquitoes to high temperature. So it can be said that 40°C and 30%RH is a stressful environment for both *An. gambiae s.s.* and *An. arabiensis*.

Hsp expression and heat stress resistance are complex traits that can evolve together (Dahlgaard *et al.*, 1998) and independently of one another (Bettencourt *et al.*, 1999; Lansing *et al.*, 2000).There is a wealth of evidence suggesting heat-shock proteins improve the thermal resistance of insects exposed to stressful conditions. Expression of Hsp70 correlates positively with thermotolerance in *Drosophila melanogaster* embryos

(Welte *et al.*, 1993), and in both laboratory and naturally occurring larval populations (Krebs and Feder, 1997; Krebs and Feder, 1998). In adult populations of *D. melanogaster* and *D. buzzatii* hsps act as short term agents of thermotolerance (Loeschcke *et al.*, 1994; Dahlgaard *et al.*, 1998). Up-regulation of hsp70 in adult locusts correlates with induced thermotolerance and importantly it stabilises neuromuscular signalling underlying thermoprotection of muscle contraction force and thus altering the thermosensitivity of an escape behaviour critical for survival (Barclay and Robertson, 2000). At a genetic level, by generating a strain bearing extra copies of the *Hsp70* gene and comparing stress resistance to that exhibited by an 'excision' strain lacking the extra copies, it has been shown that this single gene affects Hsp70 concentration in whole larvae and pupae, which in turn affects their tolerance of natural thermal stress (Feder *et al.*, 1996; Feder and Krebs, 1998). Similarly, molecular variation in the *hsp68* gene has been shown to contribute to natural heritable variation for knockdown heat resistance in *D. melanogaster* (McColl *et al.*, 1996). In the present study however, greater knockdown resistance exhibited by *An. arabiensis* when compared to *An. gambiae s.s.* did not correlate with a greater expression in the level of heat shock proteins and so it seems unlikely that hsps make a contribution to the higher levels of thermotolerance witnessed in *An. arabiensis*. In other cases the relationship between hsp levels and thermotolerance is not apparent. Sørensen *et al.* (2001) found that a population of *D. buzzatii* originating from a high temperature environment had a greater degree of knockdown resistance, but a lower level of hsp70 expression, than a population from a low temperature environment. The level of Hsp70 production by adult male *D. melanogaster* has been shown to greatly exceed that by females, yet female survival after heat shock generally exceeds that of males (Dahlgaard *et al.*, 1998).

In several cases a period of pre-shock heat-hardening has resulted in increased thermotolerance, though not always with a coincident increase in heat shock protein expression. Pre-shock treatment at 37°C before heat shock treatment at 41.5°C resulted in a six-fold increase in *An. albimanus* larval survival compared to a control group pre-shock treated at 25°C, though no relationship with Hsps was established (Benedict *et al.*, 1991). Heat-hardened larvae of the medfly *Ceratitis capitata* produce greater levels of heat-shock proteins and are more thermotolerant than larvae that are not hardened (Stephanou *et al.*, 1983). Heat hardened larval honey bees *Apis mellifera* were found to

express inducible Hsp72 whereas larvae incubated at lower temperatures synthesized only the constitutive Hsc73 protein (Chacon-Almeida *et al.*, 2000). The period of heat hardening in the present study apparently did not promote up-regulation of Hsp production in either species when compared to levels in the heat shocked groups, though there is a suggestion of some effect in *An. gambiae s.s.* There could be several explanations for this. Some Hsps are rapidly induced and just as rapidly inactivated. The inactivation of hsp70 can occur with extraordinary speed - in early embryos it occurs within just a few minutes after return to normal temperatures and thermotolerance is lost at a similar rate (Parsell and Lindquist, 1994). This inactivation could have occurred during the 2 hour recovery period. Alternatively it may be the case that pre-exposure provides benefit by extending the period of thermotolerance by affecting the rate of inactivation and therefore duration, but not the level, of expression. The lack of a difference in knockdown resistance between the heat-shocked and heat-hardened groups might reflect the similarities in hsp expression, but it is also possible that recovery from the hardening period had not been completed, and that the stress from hardening treatment and subsequent heat shock is therefore cumulative. This absence of improved resistance has been observed in *D. melanogaster* during the first 2 hours after heat-hardening despite Hsp70 levels reaching a maximum in this period (Dahlgaard *et al.*, 1998).

Hsp expression is not a constant but changes during development and in response to physiological conditions in a manner that does not always correlate with changes in thermotolerance (Parsell and Lindquist, 1994). At temperatures warm enough to induce Hsp expression but insufficient to cause rapid death, hsps may have few benefits and a variety of costly consequences (Sørensen *et al.*, 2001). *D. melanogaster* adults regularly exposed to a conditioning treatment, consisting of non-lethal stress that induces hsp production, produce significantly fewer offspring than females kept at a constant temperature (Krebs and Loeschcke, 1994). Larva-to-adult survival at 25°C is negatively correlated with Hsp70 level in the same species (Feder *et al.*, 1992). Retarded cell development has also been observed in *Drosophila* cells expressing hsp70 at normal temperatures (Feder *et al.*, 1992). Evolution at high temperatures with long-term induction of hsps may favour decreased expression over time of inducible hsps because of these deleterious effects. It may however favour increased reliance on constitutive hsps.

This chapter represents only a preliminary investigation into the importance of the Hsp70 family to mosquito thermotolerance. The monoclonal antibody hsp70 clone 3A3 used detects the strongly inducible Hsp70, and also Hsp72, which is induced exclusively under stress conditions (Kang *et al.*, 1995). Unfortunately, for the purposes of this experiment, it also detects the cognate protein Hsc70 and the mitochondrial protein p75. It was hoped that these proteins might be separable by electrophoresis in a high % SDS-PAGE gel but this appears not to have happened. There are two ways to resolve the heat-induced forms from their constitutively expressed counterparts. One method would be to use a primary antibody specific for the 70-kDa inducible member of the Hsp70 family. The monoclonal antibody 7.FB is highly specific to inducible Hsp70 in *Drosophila* (Velazquez *et al.*, 1983) but it is not commercially available, and many difficulties can arise when applying such specific probes to non-standard organisms (Hightower, 1995; Feder and Hofmann, 1999). The alternative would be to run the samples through two-dimensional gel electrophoresis. Obviously the set of experiments presented here need to be rigorously reproduced. Rearing150 females from the same generation, in order to reduce sources of natural variation between experimental groups, proved to be a major difficulty preventing experimental repeats.

Inducible stress tolerance is increasingly understood to result from numerous molecular mechanisms of which Hsps are only one (Feder and Hofmann, 1999). Other mechanisms include change in cell membrane organisation (Revathi *et al.*, 1994; Swan and Watson, 1999) and the accumulation of osmotic stress protectants such as sorbitol and trehalose (Salvucci *et al.*, 2000). As a result the unequivocal attribution of heat shock tolerance to specific Hsps demands more than correlative evidence. The thermal phenotype of multicellular organisms comprises hundreds of traits – a background that can confound the assessment of the role of a particular Hsp (Feder *et al.*, 1996). The mosquito species investigated here also possess mechanisms other than biochemical means of avoiding damage from environmental stress. Owing to the evolution of behavioural and physiological mechanisms, the relative importance of inducible heat-shock proteins in *An. gambiae s.s.* and *An. arabiensis* could have become subordinate to alternative methods of protection against stressful conditions.

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CHAPTER XI

Conclusions

The spatial differentiation in distribution between *Anopheles gambiae sensu stricto* and *An. arabiensis* can be described at three scales – continental, regional and local.
In large areas of tropical sub-Saharan Africa the species are sympatric (Coetzee et al., 2000). *An. arabiensis* dominates in areas characterized by low rainfall, in the northern most areas of the range in Sudan (Petrarca et al., 2000) and Ethiopia (Mekuria et al., 1982), and also at the southern fringes of the range in Mozambique (Mendis et al., 2000) and South Africa (Braack et al., 1994). More specifically *An. arabiensis* is the predominant species where average annual precipitation is less than 500mm (Rishikesh *et al.*, 1985; Petrarca *et al.*, 2000). It therefore appears better adapted to drier conditions than *An. gambiae s.s.* At a finer spatial scale other climatic effects such as maximum daily temperatures and humidity become important. In the hot dry season of The Gambia, when maximum temperatures exceed 40°C and relative humidity (RH) drops as low as 20%, *An. arabiensis* predominates over *An. gambiae s.s.* (Lindsay *et al.*, 1991). Similarly in Tanzania, *An. arabiensis* is the more abundant species when day-time humidity is below 60%RH and during the months of highest maximum temperatures (White *et al.*, 1972). *An. arabiensis* is also the more dominant species in lowlands and is less common than *An. gambiae s.s.* at high altitudes in equatorial regions (Lindsay and Martens, 1998), but not in the Ethiopian Highlands. At a finer scale still these two species can be separated by the relative availability of host species; *An. arabiensis* can be found in game reserves where *An. gambiae s.s.* is not (White, 1974). In addition to spatial segregation there is temporal variation in the relative distribution of these two species. For example in the Kilombero Valley, Tanzania, *An. gambiae s.s.* and *An. arabiensis* occur sympatrically during the rainy season but separate into different habitats in the dry season (Charlwood et al., 2000). The main hypothesis of this thesis anticipated major differences between the behaviour and physiology of *Anopheles gambiae s.s.* and *An. arabiensis* that reflected these spatial and temporal differences.

Adult thermotolerance: behaviour and physiology

I have shown that adult survival of *An. arabiensis* is greater than that of *An. gambiae s.s.* at high temperatures and low humidity, thus providing one explanation for their distribution at a regional scale. At 40°C and 30%RH it took nearly twice as long to kill 50% of *An. arabiensis* than it did *An. gambiae s.s.*. By contrast at 30°C there was no difference in survival between the species. In a separate series of experiments adults of both species were found to exhibit behavioural avoidance of increasingly high temperature, flying away to a cooler location. Again there was a distinction in the response of the species; *An. gambiae s.s.* actively avoided exposure to temperatures lower than those that induced a response in *An. arabiensis*. Although adult temperature avoidance may allow *An. gambiae s.s.* to persist at the fringes of the *An. arabiensis* distribution, a better tolerance of high resting temperature in *An. arabiensis* may result in longer-term improvements in survival rates under such conditions. This behavioural response of both species to high temperature was also apparent in metal-roofed houses in The Gambia, although no difference between the behaviour of the species was observed. Mosquitoes were driven from the roof to the cooler floor when roof temperature increased to 36°C and higher. Both *An. gambiae s.s.* and *An. arabiensis* are largely inactive during the day and so this daytime movement was considered an escape response, manifest as a short burst of activity in the presence of near-lethal conditions.

Longer periods of activity, measured by an acoustic actograph, in *An. gambiae s.s.* and *An. arabiensis* were clearly temperature-related and could not be sustained at 40 or 35°C in either species. This implies that it is not the ability of adult *An. arabiensis* to disperse, seek hosts and lay eggs at higher temperatures than *An. gambiae s.s.* that separates the species, but more simply their ability to survive such conditions and then carry out these processes when conditions are more favourable. It could be suggested that the higher wing-beat frequency of *An. gambiae s.s.* may generate more heat during flight than *An. arabiensis*, to the extent that sustained flight at high temperature is more costly to *An. gambiae s.s.* Activity in both species was highest at 30°C; mosquitoes flew for longer at 30 than at 20°C, although there was no increase in the number of flights made. It was anticipated that *An. gambiae s.s.* might be more active than *An. arabiensis* at the lower temperatures, but this was not apparent. This is evidence that *An. arabiensis* appears better adapted to existence under a wide range of conditions and supports the

idea that *An. arabiensis* is more tolerant of microclimatic instability than *An. gambiae s.s.* (Petrarca et al., 1991).

No differences were seen in the expression of Hsp70 between the adults of *An. gambiae s.s.* and *An. arabiensis*. Expression in both species was higher in populations that were both heat shocked and heat hardened when compared to control populations, but this did not relate to improved knock-down resistance. It seems the importance of heat shock proteins to the survival of these species in stressful environments is limited and they cannot explain the differences in the distribution of these two species, at least from the evidence of this thesis.

Adult water loss resistance

The results of gravimetric and infrared gas analysis studies conducted for this thesis suggest that a key mechanism underlying the predominance of *An. arabiensis* in desiccating conditions is their greater ability to resist water loss and tolerate desiccation than *An. gambiae s.s.* Though both species had a similar total water body content (~75%), *An. gambiae s.s.* lost a greater percentage of its weight (22%) after 60 minutes of exposure to high temperature and low humidity than did *An. arabiensis* (18%). The physiological explanation for this is that *An. gambiae s.s.* released CO_2 and water at a greater rate than *An. arabiensis* under these conditions. Furthermore, *An. arabiensis* contained a disproportionably greater volume of haemolymph than *An. gambiae s.s.* Haemolymph in insects can function as a water store and the water can be removed from the blood to maintain the water content of tissues (Edney, 1977).

Larval thermotolerance: behaviour and physiology

Although *An. gambiae s.s.* and *An. arabiensis* commonly share larval habitats (Laird, 1988) *An. gambiae s.s.* is thought to out-compete *An. arabiensis* in these situations (Schneider et al., 2000). However competition between the two species had not, up to now, been investigated at high temperatures. Because survival to adult and development rate probably have the most influence on the recruitment of new individuals to insect populations, it was interesting to find a greater survival to adulthood of *An. arabiensis* larvae at 35°C and *An. gambiae s.s.* at lower water temperatures. Greater larval survival at high temperature may contribute to the dominance of adult *An. arabiensis* in dry season collections and at sites experiencing high water temperatures all year round.

Differences in larval survival may also help explain situations where *An. arabiensis* is the first species to take advantage of the rains in the early rainy season when water temperatures are likely to still be high. At 25°C *An. gambiae s.s.* reached pupation faster than *An. arabiensis* which may confer a competitive advantage for this species in ephemeral habitats common to the wet season but less apparent in the dry season (Lyimo et al., 1992). By contrast at 35°C there was no difference in development rate, suggesting this advantage is lost at high temperatures. Larval rearing temperature affected emerging adult size of both species in a similar way. Adult size decreased with increasing temperature, and *An. gambiae s.s.* remained consistently smaller than *An. arabiensis.* Insufficient resource acquisition might eventually limit growth rates at increased temperatures. For example, in aquatic environments oxygen availability decreases with increasing water temperature (Davis, 1975). Consequently growth, although initially faster at high temperature, slows down sooner as the rate of resource uptake becomes limiting. In ectotherms this may select for an ability to reduce size at maturity adaptively in response to high rearing temperature (Atkinson and Sibly, 1997). However in *An. gambiae s.s.* and *An. arabiensis* it is adaptive for adults to be large. Larger mosquitoes survive for longer and produce more offspring than smaller individuals (Lyimo and Takken, 1993; Ameneshewa and Service, 1996). Adult *An. arabiensis* emerging at 35°C were still larger than *An. gambiae s.s.* adults emerging at 25°C, but it has not been determined whether size constraints act on these two species in the same way. However it has been shown that *An. arabiensis* at 35°C are larger than *An. gambiae s.s.*, despite developing at the same rate. If an organism is to maintain the initial faster growth rate seen at high temperatures it needs a greater capacity for resource acquisition than it exhibits at lower temperatures (Atkinson and Sibly, 1997). It may be that *An. arabiensis* larvae are better adapted than *An. gambiae s.s.* to acquire resources at high temperatures. At lower temperatures this ability is superfluous but may incur a maintenance cost. In the case of *An. gambiae s.s.* this cost may have been removed by selection at low temperature over many generations, permitting faster development than seen in *An. arabiensis.*

This thesis hypothesized that the relative proportion of *An. gambiae s.s.* to *An. arabiensis* larvae in any breeding site in The Gambia would be influenced by the water temperature of that site, given the differential survival of the two species is temperature-dependent. However no factor of site temperature was found to affect the relative

proportion of *An. gambiae s.s.* present at that site. Most of the larvae collected from breeding sites were first or second instar and it may be that the effect of temperature on larval survival is only apparent in the latter stages of development. An important aspect of these sites is the diel variability in temperature to which the larvae are exposed. *An. gambiae s.s.* larvae were abundant in all sites studied where the mean surface temperature was lower than 35°C. This implies that larvae are capable of surviving short periods of exposure to temperatures at and above 35°C, suggesting that the distinction in larval survival may be less obvious in the field than the laboratory, and therefore a less important contribution to an explanation of the distribution of the species. In the laboratory it has been shown that both species avoid high surface water temperatures by diving to cooler water. This behavioural mechanism could allow avoidance of damaging temperatures, but it may be of limited use in nature, especially for *An. gambiae s.s.* which thrives in shallow ephemeral pools (Laird, 1988). In more permanent sites such as rice fields where a thermal gradient in the water column might exist, this diving behaviour may be more beneficial to survival rates.

Biological implications and the importance of adult size

The description of multi-dimensional ecological niches by mathematical or statistical means has been, and continues to be, a practice common to the study of an enormous range of organisms (for examples see Pisek *et al.*, 1973; Begon *et al.*, 1986). However most of these studies do not take the necessary next step and determine the mechanisms used by species to occupy particular climate envelopes. This thesis demonstrates how simple physiological and behavioural changes can allow one species to out-compete another in a particular habitat. As a result of these findings it is tempting to propose that *An. arabiensis* is an arid-adapted successor of *An. gambiae s.s.* similar to the aridity-tolerant Mopti chromosomal form of *An. gambiae s.s.* However when predicting the effect of abiotic factors on insect abundance and distribution, it is important to consider historical, as well as ecological, factors. To assume present distributions are limited by the present climate ignores the importance of past environmental conditions and speciation events. In the case of *An. gambiae s.s.* and, to a lesser extent *An. arabiensis*, their range expansion may have mirrored the growth of human and domestic animal populations more so than prevailing climate as a result of their dependence on humans for feeding and breeding sites (Donnelly *et al.*, 2001). It could therefore equally be argued that *An. gambiae s.s.*, is an anthropophilic successor of *An. arabiensis* that has

lost the ability to tolerate high temperatures as a result of its closer association with human behaviour. The apparent mixing of genomes between these species (Besansky *et al.*, 2003), despite their unquestioned species status, demands elucidation of the genome proportions that resist introgression. Presumably those proportions include the genes directly involved in speciation; these genes could underlie the tolerance of hot and dry conditions that have allowed range expansion of *An. arabiensis* into environments inhospitable to *An. gambiae s.s.* (Besansky *et al.*, 2003).

It is noticeable that the adults of *An. arabiensis* were consistently bigger than those of *An. gambiae s.s.* in all the experiments described in this thesis. Many species of mosquito show considerable variation in adult size, which may reflect the conditions of larval development (Lyimo *et al.*, 1992; Yuval *et al.*, 1993; Mpho *et al.*, 2002). Therefore it was of particular interest to note that the difference between the species is so consistent considering their close genetic relationship and their identical laboratory rearing conditions. Given the universal phenomenon of temperature change, from short term diel fluctuations to longer-term climate changes, and the importance of body size in mosquito ecology, there is a fundamental need to understand the effect of temperature on body size in ecotherm populations. This is an issue of wide-scale biological importance and one this thesis has only touched upon. In interpreting Bergmann's rule it can be said that selection at high temperature favours individuals with a large surface area to body mass that should facilitate heat loss (Atkinson and Sibly, 1997). Lyimo (1993) found monthly mean wing lengths, and therefore body size, of female *An. gambiae s.l.* to be negatively correlated with air temperature in accordance with the rule. In my experiments it is apparent that mosquito larvae give rise to smaller adults under hotter conditions, and yet paradoxically it seems as though it may be adaptive for *An. gambiae s.s.*, and especially *An. arabiensis*, to be larger in desiccating environments. In the case of *An. arabiensis* it appears that the ability to conserve water is more important than the ability to lose heat. As well as greater body mass, *An. arabiensis* have bigger wings than *An. gambiae s.s.* which may improve radiative heat loss and compensate for its smaller body surface area to mass ratio. *An. arabiensis* is also found where it is cold and dry (Ameneshewa and Service, 1996), and a large body size would therefore appear to permit survival in a greater range of environments than a small body size. *An. quadriannulatus* survives at high temperature in Zanzibar but also at high altitude in Ethiopia and Zimbabwe (White, 1974). A

population of *An. quadriannulatus* originating from South Africa are of a similar size to the *An. arabiensis* strain used in this thesis and are significantly larger than *An. gambiae s.s.* (Takken *et al.*, 2002).

It is important to realize however that optimal size is affected by factors other than growth limitation by temperature, in particular mortality rates and time constraints on life cycle. To be considered adaptive, a particular response must impart greater fitness than alternative responses (Atkinson and Sibly, 1997). Delayed maturity resulting in larger adults may be more costly in fitness terms than the compensation of increased fecundity conferred by a larger body size. This is because these are opportunistic mosquito species exhibiting a high reproductive output strategy and utilizing ephemeral habitats.

The difference in size between *An. gambiae s.s.* and *An. arabiensis* may also reflect seasonal variation in larval habitat availability. In the rainy season there is greater variation in breeding sites, leading to greater variation in larval densities and food availability, two factors that also affect emerging adult size. Lyimo (1993) found average female body size of *An. gambiae s.l.* was smaller in the rainy season than the dry season. Only permanent water bodies such as rice fields and ponds are sustained through most dry seasons, not necessarily because ephemeral puddles dry out too quickly due to the high temperatures but that they are not sustained by rainfall. In the dry season permanent water bodies produce larger adults as they offer more food and more predation resulting in more food per larva.

Public health implications

The public health importance of this research is of an indirect nature only. The transmission of vector-borne parasites varies in intensity through space and time much more so than directly transmitted pathogens (Rogers et al., 2002), largely because highly variable vector-related factors such as abundance, biting rate and mortality rate are crucial to predicting the basic case reproductive number, R_0. It should be possible to calculate malaria risk purely on the basis of current and previous vector population dynamics (Rogers et al., 2002). The competence of the mosquitoes *An. gambiae s.s.* and *An. arabiensis*, as vectors of malaria, is determined by intrinsic and extrinsic factors such as physiology and anti-parasite defence systems, feeding behaviour and

microclimate. This thesis has demonstrated differences in the physiology and survival of these species across a range of environmental conditions, data that could be of use to models predicting future malaria outbreaks. Although both species are highly efficient malaria vectors, where they are sympatric the less anthropophilic nature of *An. arabiensis* could lead to an decrease in malaria transmission should climate change result in lower rainfall and higher temperatures and therefore promote its survival over *An. gambiae s.s.*. Contrary to this hypothesis, an increase in the incidence of malaria epidemics has been linked to increasing environmental temperature in Zimbabwe (Freeman and Bradley, 1996), Kenya (Malakooti et al., 1998) and also Ethiopia, where such an increase is additionally associated with decreasing rainfall and relative humidity (Tulu, 1996). Although no direct link between these changing conditions and mosquito abundance was demonstrated, they should promote the survival of *An. arabiensis* over *An. gambiae s.s.* However the latter species is absent in much of Zimbabwe and Ethiopia, and the increase in malaria transmission may be a result of an increase in the range of *An. arabiensis*, or more likely of accelerated parasite development rather than a change in vector composition. Expansion of the *An. arabiensis* range seems to be occurring in Nigeria, though largely into regions already occupied by *An. gambiae s.s.* (Onyabe and Conn, 2001) and with no evidence yet of a change in malaria transmission. Clearly the relationship between the relative abundance of mosquito species and the risk of malaria is complex, and so for control purposes both *An. gambiae s.s.* and *An. arabiensis* have been regarded as being of equal importance up to now. Improving the knowledge of how small changes in climatic factors influence the range boundaries of these vector species could be of great importance to public health and disease control organizations operating at regional and international levels and this research has provided some insights. For instance, it is argued that the inadvertent transport of live mosquitoes aboard aircraft arriving into disease-free countries from areas with endemic vector-borne diseases could become a serious problem, and indeed during the last 30 years several European countries have reported cases of 'airport malaria' (Gratz *et al.*, 2000). However 95% of all airport malaria in European countries occurs during the summer (Guillet *et al.*, 1998). This clearly indicates the importance of suitable conditions for vectors leaving the aircraft. Given the extremely low level of flight activity observed in both *An. gambiae s.s.* and *An. arabiensis* at 10°C I would speculate that these mosquitoes would not be physically capable of leaving aircraft at external temperatures lower than this.

At present the only practical methods of identifying *An. gambiae s.s.* and *An. arabiensis* are expensive and impractical for use in the field with large mosquito samples. Had a large and consistent difference in behaviour or survival been observed between the two species it might have formed the basis of a field-based identification technique. Unfortunately no inter-specific differences demonstrated in this thesis could be reliably used for this purpose.

There is a need to expand the basic data presented in this thesis in two directions. Firstly it would be useful to mimic more closely the biotic and abiotic factors of the natural environment of these two species. The niche of a species in any given dimension cannot always be conceived of simply in terms of maximum and minimum values but should allow for daily and seasonal fluctuations (Begon *et al.*, 1986). For instance, ephemeral breeding sites exhibit a predictable daily rise and fall in water temperature that could be reasonably well simulated in the laboratory using more sophisticated incubators. Secondly, and possibly more informative, it is necessary to look for a genetic basis for the behavioural and physiological differences between the species if the current distribution of these two species reflects subtle evolutionary changes over time.

The data presented in this thesis show that through behavioural and physiological mechanisms, predominantly in the adult stage, *An. arabiensis* is better adapted to survive hotter and drier conditions than *An. gambiae s.s.*.

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