Influence of environmental characteristics on the habitat of and behavioural interactions between *Anopheles* species in South Africa

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, South Africa in fulfilment of the requirements for the degree of Master of Science.

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DECLARATION

I declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science (MSc Med) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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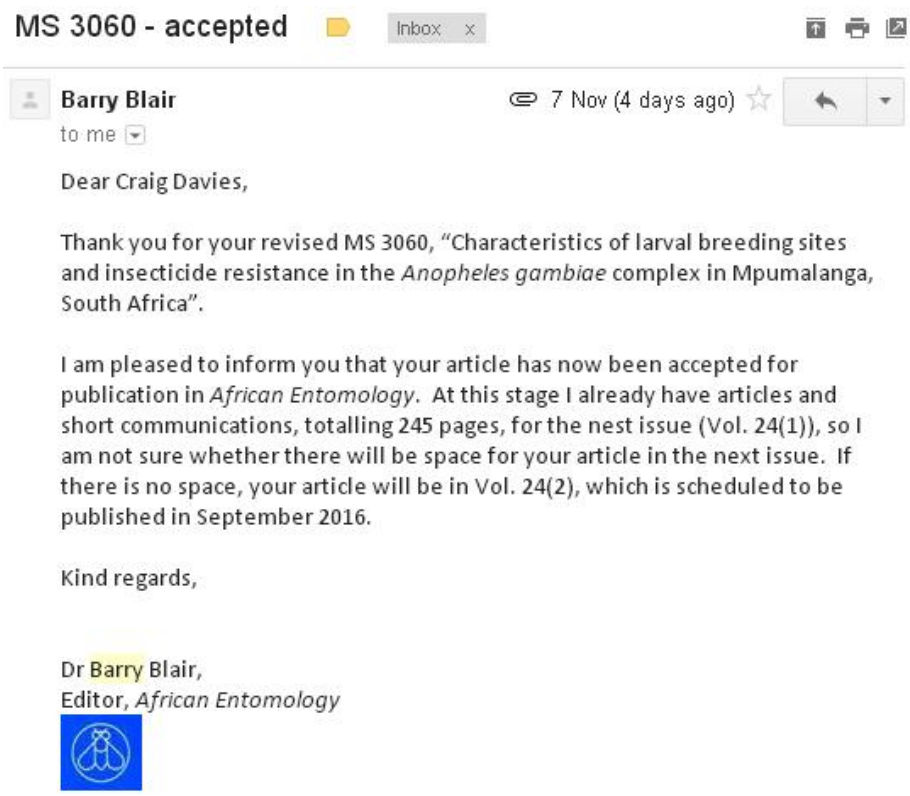
(Signature of candidate)

16th day of March 2016

Publications and presentations/ conferences arising from this study

Publications

Davies, C., Coetzee, M. and Lyons, C.L. (2016). Characteristics of larval breeding sites and insecticide resistance in the *Anopheles gambiae* complex in Mpumalanga, South Africa. *African Entomology*, 24(2) (In Press).



MS 3060 - accepted Inbox x

Barry Blair 7 Nov (4 days ago)
to me

Dear Craig Davies,

Thank you for your revised MS 3060, "Characteristics of larval breeding sites and insecticide resistance in the *Anopheles gambiae* complex in Mpumalanga, South Africa".

I am pleased to inform you that your article has now been accepted for publication in *African Entomology*. At this stage I already have articles and short communications, totalling 245 pages, for the nest issue (Vol. 24(1)), so I am not sure whether there will be space for your article in the next issue. If there is no space, your article will be in Vol. 24(2), which is scheduled to be published in September 2016.

Kind regards,

Dr Barry Blair,
Editor, *African Entomology*

Davies, C., Coetzee, M. and Lyons, C.L. (2016). Competitive behaviour and response of *Anopheles arabiensis* and *An. quadriannulatus* under different temperature treatments. *Parasites and Vectors,* (In Review).

Poster presentations/ Conferences attended

Davies, C., Coetzee, M. and Lyons, C.L. 2014. Insecticide resistance in the *Anopheles gambiae* complex from the Lowveld region of the eastern Mpumalanga Province. University of the Witwatersrand, Faculty of Health Sciences Research Day, Johannesburg, South Africa.

Davies, C., Coetzee, M. and Lyons, C.L. 2015. Insecticide resistance in the *Anopheles gambiae* complex from the Lowveld region of the eastern Mpumalanga Province. 2nd Annual Pan-African Mosquito Control Association Conference, Dar es Salaam, Tanzania.

ABSTRACT

This project explored the ecological conditions of aquatic breeding sites of *Anopheles gambiae sensu lato* immatures in the Lowveld region of eastern Mpumalanga Province, South Africa. The aim was to determine the environmental conditions influencing anopheline abundance as well as abiotic parameters which associated with vector productivity. In addition to this, the levels of insecticide resistance to the three dominant compounds used in vector control in the region were assessed. Taking into account the sympatric occurrence of the major malaria vector in South Africa (*An. arabiensis* Patton) and its sibling, non-vector species (*An. quadriannulatus* Theobald), a laboratory study was devised which investigated the outcome of intra- and inter-specific competition under constant and fluctuating temperature regimes.

There was a heterogenous distribution of anophelines across aquatic habitats in Mpumalanga with small-scale variation in salinity and Total Dissolved Solids (TDS) influencing species composition and *Anopheles arabiensis* was found in all sites surveyed with low numbers occurring where salinity levels were elevated. *Anopheles merus* associated with high salinity and TDS (Pearson's Product Moment, $r = 0.922$, $p < 0.05$) whilst *An. quadriannulatus* dominated in breeding sites within 50m of a building or road. *Anopheles gambiae* complex members were susceptible to the insecticides tested with possible resistance (97%) to DDT in *An. merus*.

Under laboratory conditions, temperature and competitive scenarios affected the life-history traits of both species studied here. The treatment 18 - 35°C generally reduced survivorship except for *An. arabiensis* in mixed, larval species treatments where it was similar to values reported for 25°C. Survivorship of both species at 20 - 30°C was not significantly impacted and the adult production was high across species treatments. The development rates at 25°C

and 20 - 30°C were significantly different between species when reared alone and in mixed species treatments from larvae and from eggs**.** The effect of temperature was more pronounced at 18 - 35°C with *An. arabiensis* developing faster under both competitive scenarios and *An. quadriannulatus* slower, notably when in the presence of its competitor ($p < 0.05$).

In the field component of this study, *Anopheles arabiensis* exploited all the habitats surveyed. It is therefore recommended that larval control operations should include all available breeding sites, focusing efforts during the dry season when these sites are limited and discreet within the landscape. In the laboratory component, it was possible to test whether or not community composition of anophelines at the adult stage was regulated by different temperature and competitive conditions at the larval stage to better understand the ecological conditions that determine anopheline composition and relative abundance. Taken together, the results of each component emphasize the need for local scale studies, especially under conditions of changing temperatures and rainfall patterns. The results of responses to temperatures and biotic interactions are necessary data for use in models predicting the impact of climate change on malaria vector mosquitoes.

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The field work undertaken in this project would not have been possible without the dedicated and passionate team at the Mpumalanga Malaria Control Programme headed by Mr. F. Mbokazi who tirelessly assisted with larval collections and navigated us around the study area. In addition, my appreciation goes out to Dr. Candice L. Lyons, Dr. Power Tshikae, Nancy Stephen and Godira Segoea at the Vector Control Reference Laboratory for their help and friendship in the field and beyond.

Special thanks to all the students and staff at the Vector Control Reference Laboratory who assisted me on a number of levels to achieve the lab work. Dr. Riaan Christian and Dr. Yael Dahan were always there to assist throughout training and when I needed advice and reagents. The colonies used in the lab study are maintained through a combined effort of the technicians and colony sitters and I thank them for making eggs available for me to work with.

Thank you to my family for their moral support, encouragement and love throughout.

ETHICS APPROVAL

Human Research Ethics Committee (Medical)

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Ref: W-CJ-150911-1 11/09/2015

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof Maureen Coetzee.**

Project title: **Research on mosquitos.**

Reason: This waiver covers all research on mosquitos and mosquito parasites by Professor Coetzee, her staff and students as long as no humans or human tissues are involved. The waiver lasts 5 years and may be renewed.

UNIVERSITY OF THE WITWATERSRAND
2015 -09- 11
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat : Zanele Ndlovu.

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ABBREVIATIONS

CDC – Centre for Disease Control

DDT – Dichlorodiphenyltrichloroethane

DNA – Deoxyribonucleic Acid

DVS – Dominant Vector Species

EIR – Entomological Inoculation Rate

GPS – Global Positioning Software

IRS – Indoor Residual Spraying

IVM – Integrated Vector Management

LSM – Larval Source Management

LTM – Long Term Mean

ORNL DAAC – Oak Ridge National Laboratory Distributed Active Archive Centre

PCR – Polymerase Chain Reaction

SASRI – South African Sugarcane Research Institute

SAWS – South African Weather Service

TDS – Total Dissolved Solids

USGS – United States Geological Survey

WHO – World Health Organization

CHAPTER ONE - INTRODUCTION

1.1. Introduction

1.1.1. Malaria and vector ecology

Global estimates for reported cases of clinical malaria infections have declined by 30% since 2000, whilst related deaths have shown a 47% decline over the same period (WHO 2014). Although trends point to an overall improvement, the disease still poses a significant threat to the health of many people worldwide. This is especially true in sub-Saharan Africa where approximately 85% of global cases are reported, mostly affecting high risk groups such as children under five and pregnant women (WHO 2014).

Human malaria is the transmission of protozoan parasites of the genus *Plasmodium* (*P. falciparum*, *vivax*, *ovale*, *malariae*) (Haemosporida: Plasmodiidae) by a competent vector of the genus *Anopheles* (Diptera: Culicidae). In Africa, the dominant vector species (DVS) of the parasites are also the most efficient and effective malaria vectors worldwide (Gillies and De Meillon 1968; Snow *et al.* 2005). As a result, the entomological inoculation rates – a measure of the number of infective bites per person per year - in some parts of the continent are amongst the highest globally, increasing rates of human morbidity and mortality (Snow *et al.* 2005). On the continent, the DVS that are most commonly implicated in transmission are three species of the *Anopheles gambiae* Giles complex (*An. gambiae s.s.* Giles*, An. coluzzii* Coetzee & Wilkerson and *An. arabiensis* Patton); one of the *An. funestus* Giles group (*An. funestus s.s.* Giles); *An. nili* Theobald and *An. moucheti* Evans (Sinka *et al.* 2012; Coetzee *et*

al. 2013a). Additional secondary vectors, such as *An. merus* Dönitz, are also present and may become more important under certain conditions, such as when high densities are achieved (Cuamba and Mendis 2009; Kipyab *et al.* 2013).

Where malaria is not hyper-endemic and immunity in the indigenous population is low to absent, a significant health threat exists and devastating epidemics can occur when the normal transmission scenario changes temporarily, such as during prolonged periods of raised temperature and humidity, or permanently, usually resulting from environmental change that shifts the ecological setting to one which favours higher endemicity (Nájera *et al.* 1998; WHO 2000). South Africa falls under the malaria endemic countries, albeit in a state of low risk, where transmission in the north-eastern region is largely restricted to the summer months when temperatures are suitable for vector survival and parasite development (Maharaj *et al.* 2013). Neighbouring countries, specifically Zimbabwe and Mozambique, where the transmission of malaria is still high and programmes are in the control phase or are limited, will continue to pose a risk to local elimination of the disease (Moonasar *et al.* 2013). South Africa has met the WHO's pre-elimination criteria and aims to completely eliminate the disease by 2018 by means of coordinated efforts and expanding areas of control such as larval source management (Ngomane and de Jager 2012; Moonasar *et al*. 2013).

Field evidence for the range of ecological conditions which affect the spatio-temporal dynamics of anopheline vector communities is central to malaria control interventions. That climate plays a large role in the distribution of insects is undeniable (Andrewartha and Birch 1954; Sutherst *et al.* 1995) and like many insect species, mosquito vectors are strongly reliant on environmental conditions for survival, directly or indirectly (Lindsay *et al.* 1998). In particular, temperature plays a pivotal role in insect development, and reduced growth rates and lower survivorship have been observed under low and high temperatures (Huffaker 1944; Rueda *et al.* 1990; Lyimo *et al.* 1992; Bayoh 2001; Bayoh and Lindsay 2003; Bayoh and

Lindsay 2004; Lyons *et al.* 2013). In addition, moisture availability has also been shown to influence the composition of the dominant vector species (Lindsay *et al.* 1998; Craig *et al.* 1999; Minakawa *et al.* 1999; Bayoh and Lindsay 2003). The availability of breeding habitats is dependent on rainfall and Govere *et al.* (2000) report increasing vector numbers when the rains in the Lowveld of Mpumalanga begin, showing a peak during mid-rainy season in January when most adults are caught by human landing catch method of trapping. Moderate rainfall favours vector productivity as heavy rains tend to flush the breeding sites out (Paaijmans *et al.* 2007).

The effect of temperature on larval stages may help explain the spatial and temporal distribution of *An. gambiae s.s.* and *An. arabiensis*, where lower water temperatures typically favour the former, and higher temperatures the latter (Kirby and Lindsay 2009). On a regional scale, Lindsay *et al.* (1998) report that the ranges of these two dominant vector species (DVS) could be defined by climatic conditions over a gradient from East to West Africa. Their distribution follows the known patterns of peak occurrence of each where they both occur sympatrically. *Anopheles arabiensis* is seen to predominate where air temperatures are highest and conditions are drier, whilst at lower air temperatures under moist conditions *An. gambiae s.s* predominates (White 1972; Lindsay *et al.* 1998; Kirby and Lindsay 2004).

At the local scale, clear differences exist in the way that species exploit different habitat types resulting in differences in, for instance, vector and non-vector abundance and composition (Nájera *et al.* 1992; Bøgh *et al.* 2003; Shililu *et al.* 2003). Certain ecological conditions may favour the proliferation of one species over another which has implications for vector-borne disease transmission (Juliano and Lounibos 2005; Bevins 2007; Beck-Johnson *et al.* 2013; Takken *et al.* 2013). Describing the relationships between ecological parameters and larval species composition and abundance is required for larval management strategies (Killeen *et al.* 2002a; Fillinger *et al.* 2004; WHO 2013a). Geomorphology has a determinant effect on

local hydrology as well as on water quality through its effect on soil chemistry and the surrounding vegetation, thus influencing the structure of the ecosystem (Rioux *et al.* 1968; Savage *et al.* 1990; Rejmankova *et al.* 1993). Alterations in the physical and chemical structure of the breeding site during the course of the mosquito's aquatic life stages, for instance from an influx of fresh water, will also change the suitability of the habitat and presence and interactions of the different species. As the breeding site persists, the growth of semi-aquatic vegetation and an increase in predator abundance may reduce mosquito larvae numbers (Gillies and De Meillon 1968; Service 1977; Munga *et al*. 2006).

Female anopheline breeding site choice is variable, and uncommon larval habitats have been recorded when preferred breeding sites were unavailable due to rarity or excessive density of larvae leading to overcrowding (Patton 1905; De Meillon 1938; Munga *et al.* 2006). Oviposition of eggs in clear, temporary to semi-permanent water bodies that may be natural or man-made, fresh or partially saline, has been observed in members of the *An. gambiae* complex. They are rarely found in permanent bodies of water such as wetlands (e.g. De Meillon 1947; Muirhead-Thomson 1951), as opposed to *An. funestus s.s.* which breeds mainly in more permanent, stable sites (De Meillon 1951). These differences in larval site choice have important consequences for malaria epidemiology. The unstable nature of breeding sites of *An. gambiae* means that epidemics are commonly associated with proliferation of this species as the number of temporary sites increases during the rainy season, whereas *An. funestus* associates with a higher degree of malaria endemicity (De Meillon 1951; White 1974; Nájera *et al.* 1992; Alonso *et al.* 2011).

Mosquito populations are strongly influenced by the biotic and abiotic conditions within their environment and the need to understand the physical nature of larval habitats is essential for vector control interventions such as larviciding. As the cost and logistical complexity associated with larval source management in larger ecosystems is high (Sharp and le Sueur

1996; Coetzee *et al.* 2013b), identification of the most prolific breeding sites would allow for prioritisation, reducing the costs associated with interventions (Gu and Novak 2005).

1.1.2. *Anopheles gambiae* complex and malaria in South Africa

There are numerous *Anopheles* species present in the malarious regions of South Africa, the majority of which are non-vectors (Gilies and De Meillon 1968; Govere *et al.* 2000; Ngomane *et al.* 2007). The dominant vector species here is *An. arabiensis*, a member of the *An. gambiae* complex (Gillies and Coetzee 1987; Coetzee *et al.* 1993; Govere *et al.* 2001; Gericke *et al.* 2002). This complex comprises eight, morphologically indistinguishable, named species that vary in their ecology and vectorial capacity (Coetzee *et al*. 2013a). Along with *An. arabiensis*, *An. merus* and *An. quadriannulatus* Theobald from the *An.* gambiae complex can be found in the Mpumalanga region (Coetzee *et al.* 1993; Sinka *et al.* 2012).

The ecological requirements of each species in South Africa are not well reported. However, the general biology of *An. arabiensis* and *An. quadriannulatus* larvae suggests that a wide range of ecological conditions are exploited. Over the distribution of the *An. gambiae* complex, *An. arabiensis* typically favours drier conditions, and savannah habitats are well-suited to its presence (Coetzee *et al.* 2000; Sinka *et al.* 2012); however, it may also be found in forested sites where land disturbance has occurred (Coetzee *et al.* 2000). The larvae of both *An. arabiensis* and *An. quadriannulatus* are commonly found in natural, clear, sunlit, small and temporary freshwater pools (le Sueur and Sharp 1988; Gimnig *et al.* 2001; Edillo *et al.* 2002) although larvae have been reported in permanent bodies as well as in artificial sites (Fillinger *et al.* 2009). Larval densities appear to be negatively affected by the presence of

vegetation as a result of increased shading (Chandler and Highton 1976) which may reduce algal levels in the water – a source of nutrition for mosquito larvae (Gimnig *et al.* 2002).

Although many of the members of the *An. gambiae* complex display typical breeding site preference, resting and feeding behaviours, there is also a great degree of plasticity present within this group. In several African regions, *An. arabiensis*, although traditionally thought of as an exophilic and zoophilic species (Duchemin *et al.* 2001; Mahande *et al.* 2007), displays endophilic and anthropophilic behaviour (Tirados *et al.* 2006) and is often associated with greater incidences of malaria than the more commonly assumed anthropophilic and endophilic species: *An. gambiae s.s.; An. coluzzii;* and *An. funestus s.s.* (Kent *et al.* 2007; Coetzee *et al.* 2013a; Lwetoijera *et al.* 2014). These changes are largely dependent on the local environmental conditions, the insecticide used in IRS (White 1972; Sharp and le Seuer 1991) and the genotypes present at a site (Coluzzi *et al.* 1979).

The sympatric Southern African non-vector of malaria, *An. quadriannulatus*, occurs particularly where livestock are common. It is predominantly zoophilic in its feeding behaviour (Hunt and Mahon 1986; Takken and Knols 1999), with reports of anthropophagy in very few localities (e.g. Pates *et al.* 2001). Its sibling species, *An. merus*, is traditionally thought of as a secondary vector and is not thought to be responsible for malaria epidemics within South Africa, however, it has been implicated in transmission in Madagascar (Pock-Tsy *et al.* 2003), Kenya (Kipyab *et al.* 2013), Tanzania (Temu *et al*. 1998) and in neighbouring Mozambique (Cuamba and Mendis 2009). Early research on the feeding behaviour of *An. merus* suggests this species is zoophilic and mainly exophilic (Mutero *et al.* 1984), with a peak in its biting cycle in South Africa occurring after 24h00 when temperatures are around 23°C, and low activity reported during the two hours preceding dawn and following dusk (Sharp 1983). Behavioural plasticity as seen in anophelines highlights the importance of localised studies.

The vector, *An. arabiensis*, is common in the low-lying inland areas of the malarious region of South Africa and the larvae can be found where salinity is low (Munhenga *et al.* 2014), although its presence under brackish conditions alongside a river in rural Gambia suggests tolerance of more saline conditions in the field (Bøgh *et al.* 2003). The ability of *An. arabiensis* to occupy sites that are larger, flowing, turbid and partially shaded is in contrast to that of *An. gambiae s.s.* (Shililu *et al.* 2003; Shililu *et al.* 2007). In the eastern Mpumalanga province of South Africa, *An. arabiensis* and *An. quadriannulatus* commonly share breeding sites (e.g. Munhenga *et al.* 2014). Their sibling species, *An. merus*, is known to favour breeding in saline waters and is found along the eastern coast of Africa extending inland to a significant degree where it is often associated with saltpans (Paterson 1964; Mosha and Mutero 1982; Coetzee and Cross 1983; Gillies and Coetzee 1987). Although *An. merus* favours saline conditions, laboratory studies suggest that this species does well in freshwater and low numbers found in freshwater field sites (e.g. Munhenga *et al.* 2014) may be attributed to the presence of predators (Mosha and Mutero 1982; Coetzee and le Sueur 1988).

In South Africa, malaria transmission by *An. arabiensis* has declined substantially over the last decade and many parts of the malarious regions report infection rates of less than five cases per 1000 population, with some areas at less than one case per 1000 (Moonasar *et al.* 2013; WHO 2014). Approximately 10% of the population of South Africa live in the malaria endemic provinces of Limpopo, Mpumalanga and Kwazulu-Natal and are prone to malaria transmission (Statistics South Africa 2012). According to the WHO malaria incidence levels (WHO 2014), the status of South Africa has moved from the control phase to elimination with the exception of Vhembe in Limpopo Province which falls under the pre-elimination threshold but above that for the elimination threshold (Maharaj *et al*. 2013).

Vector control in South Africa has at its core Indoor Residual Spraying (IRS) to reduce malaria incidence (Mabaso *et al.* 2004). Moving to the elimination phase in South Africa will

require increases in financial as well as human resources and, in view of vector control strategies, more targeted approaches, such as management of the breeding habitats of anophelines, are necessary (Moonasar *et al.* 2013). Behavioural plasticity in the dominant vector in South Africa, *An. arabiensis*, in response to the application of intra-domiciliary adulticides (Sharp and le Seuer 1991) further warrants the need to develop the component of control programmes targeting the immature stages.

1.1.3. Vector control and insecticide resistance

Malaria vector control programmes have mainly relied on adult vector control, the efficacy of which is reported on by numerous authors (WHO 2000; Killeen *et al.* 2002b). Chemical control as well as larval source management has proven successful in reducing malaria vector abundance elsewhere (see for example: Molineaux and Gramiccia 1980; Killeen *et al.* 2002b; WHO 2010). These interventions can facilitate a reduction in vector populations to sufficient numbers such that clinical control and chemoprophylaxis interventions can contribute to eliminating parasite transmission in indigenous populations (Mouchet 1997).

Recent years have seen an increased interest in vector control tools moving away from insecticides targeting adult *Anopheles* spp., to more integrated, sustainable methods to interrupt or reduce malaria transmission (WHO 2004; Chanda *et al.* 2008). Due to the problem posed by insecticide resistance (Ranson *et al*. 2009), expanding environmental management and larviciding operations have been incorporated as key objectives of Integrated Vector Management (IVM) aimed at influencing vector biology and morbidity, running in synergy with chemical control and public health interventions (WHO 2004; Maharaj *et al.* 2013; WHO 2013a). Over the last several years, larval source management has

experienced a resurgence in interest as an integral component to control programmes (Utzinger *et al.* 2001; Killeen *et al.* 2002a; Fillinger *et al.* 2003; Shililu *et al.* 2007; Maheu-Giroux and Castro 2013; Tusting *et al.* 2013) and support for the efficacy of larval-control methods is illustrated in the complete eradication of accidentally introduced *An. gambiae sensu stricto* into upper northeast Brazil during the 1930s (Soper and Wilson 1943) as well as from the Upper Nile Valley in Egypt (Shousha 1948). More recently, Fillinger and Lindsay (2006) showed a 95% decrease in anopheline larval densities in sites in Kenya which had been treated with microbial larvicides. Similar results have been reported in neighbouring Eritrea (Shililu *et al.* 2007).

Although great strides have been made in the chemical control of malaria vectors, insecticide resistance is a global problem and this is especially true of those compounds used in both agriculture and public health (WHO 2010). During a malaria epidemic in South Africa at the end of the 1990s, Hargreaves *et al*. (2000) reported resistance in one of the major South African malaria vectors of that time, *An. funestus s.s.* which, having been eradicated from South Africa in the 1950s, saw a resurgence in numbers and associated malaria cases (Hargreaves *et al.* 2000; Brooke *et al.* 2001; Govere *et al.* 2002). Resistance to newly introduced compounds was detected using standard WHO bioassays (WHO 2013b) while at the same time bioassays revealed continued susceptibility to DDT (Hargreaves *et al*. 2000; Brooke *et al.* 2001).

The combined use of pyrethroids in agriculture and in public health campaigns focusing on malaria control, likely conferred resistance in southern African populations of *An. funestus,* whilst vectors belonging to the *An. gambiae* complex showed continued susceptibility to the compounds used in control (Hargreaves *et al.* 2003). Resistance to pyrethroids contributed to the failure of local malaria programmes, in contrast to sustained reduction in malaria transmission in neighbouring Swaziland where DDT was still in use (Govere *et al.* 2002). In

2000, reintroduction of DDT into malaria control programmes in the malarious regions of South Africa once again led to the disappearance of the highly endophilic vector *An. funestus s.s.* and an associated reduction in malaria cases (Coetzee *et al.* 2013a).

The supplementary role that adult chemical control by means of indoor residual spraying plays in IVM requires the continued monitoring of insecticide resistance in local vector populations. There are a limited number of available insecticides which have been approved as low risk for human health and ecological systems and resistance to these in populations of *An. gambiae s.l.*, as well as in other vector species, has been reported across the African continent (Ranson *et al.* 2009). The pyrethroid class of compounds is important here as this is the only class authorised for use in the treatment of bed nets (Zaim *et al.* 2000; WHO 2006). However, their use in agriculture has led to selection for resistance in anopheline vectors (Akogbeto *et al.* 2005; Ranson *et al.* 2009). Issues such as this can undermine control efforts and it is vital for programmes to adopt measures to mitigate the development of insecticide resistance. The WHO protocol (2006) provided recommendations and procedures for testing resistance to adulticides in mosquitoes and routine monitoring of susceptibility to the compounds used in control is an important requirement for malaria control programmes.

1.1.4. The larval environment

Conditions in the larval environment have a significant effect on the development time of mosquito progeny, thus influencing the number surviving to the pupal stage and adulthood (Lyimo *et al.* 1992; Lyons *et al.* 2013). These conditions may lead to a change in the transmission scenario which has important implications for mosquito-vector borne disease epidemiology (Juliano and Lounibos 2005; Bevins 2007; Beck-Johnson *et al.* 2013; Takken *et*

al. 2013). A number of different abiotic and biotic factors can affect the development rate, emergence time, survivorship, adult size and the susceptibility to infection by *Plasmodium* parasites in anopheline mosquitoes (Rueda *et al.* 1990; Lyimo *et al.* 1992; Bayoh and Lindsay 2003; Bayoh and Lindsay 2004; Costanzo *et al.* 2005; Juliano and Lounibos 2005; Lyons *et al.* 2013). The range of ecological conditions influencing the breeding sites means that adult productivity is not homogenous and landscape level changes in ecological conditions affects species composition (Juliano 2009). Bøgh *et al.* (2013) investigated the community composition of vectors along the Gambia River and found that anopheline productivity varied, with different species of the *An. gambiae* complex exploiting different habitats.

Associated with the rainy season are highly seasonal trends in peak densities of malaria vectors in the dry savannah regions in southern Africa (Govere *et al*. 2000). Elsewhere in Africa investigators have shown that open, sun-lit water bodies of a temporary nature are mostly exploited (Taylor *et al.* 1993) whereas in the dry season the residual pools left behind by receding rivers and other smaller habitats, such as those that are man-made, maintain the larvae of vectors of the *An. gambiae* complex (White and Rosen 1973).

When multiple species exist together and interact, the effects of competition can be observed through altered survival rates (Koenraadt and Takken 2003) and the possible displacement of one or more species (Barrera 1996; Lounibos *et al.* 2001). In a landscape that changes in time and space, competitive advantage may shift depending on the local ecological conditions. The impacts of these changes can facilitate the landscape level coexistence of competitors (Juliano 2009). Owing to the sympatric distribution of vector and non-vector species, larvae will come into frequent contact and ultimately compete for space and resources (Schneider *et al.* 2000; Kirby and Lindsay 2009; Paaijmans *et al.* 2009b).

The outcome of competition among mosquito larvae is context dependent and within a spatially heterogeneous environment, the conditions at the breeding site contribute to

determining the competitive advantage of one species over another. In addition, the presence of antagonist species has an influence on the composition of other species. For instance, *Enallagma* damselfly assemblages are markedly different among permanent larval sites, a finding McPeek (1990) attributes to the presence of predators. In mosquito populations, where strict predators are present in larger, more permanent sites, the larvae of *Aedes* species are eliminated (Banerjee *et al.* 2010). Among members of the *An. gambiae* complex, negative effects of cannibalism, predation and inter-specific competition on larval survival have been reported even in the absence of food deprivation, and are likely density dependent (Koenraadt and Takken 2003).

Under certain scenarios, it may therefore be possible that a dominant vector species could outcompete a non-vector species leading to significant changes in the vector-borne disease status of a region. A significant variable influencing the development of mosquito larvae, and one which has been well studied in *An. gambiae s.l.*, is temperature. In other insect species, low temperatures during the immature stages result in a prolonged development time, a finding also reported in anopheline larvae (Bayoh and Lindsay 2003; Lyons *et al.* 2013). Other life history traits, such as survival to the adult stage, are likewise influenced by temperature in the larval habitat and these differ among members of the *An. gambiae* complex (Kirby and Lindsay 2009; Paaijmans *et al.* 2009b; Beck-Johnson *et al.* 2013).

These examples illustrate some of the abiotic and biotic factors that influence mosquito populations. The effects and outcomes of these interactions at immature stages influence the species densities and composition of adult mosquitoes and contribute to determining local mosquito-borne disease epidemiology. Understanding the habitat conditions and surveying the contribution of malaria vectors to the total species composition in an area enables targeted larval control operations to be undertaken. Mosquito larvae also interact with other species, and the presence of two or more anopheline species, vector or non-vector, and their

interactions with each other and the environment, could have significant implications for the malaria incidence in a particular region.

1.2. Rationale

Continuous research into the various methods of vector control can contribute to improving current IVM control programmes. Larval source management (LSM) has been used as a supplementary tool in IVM for reducing malaria vector numbers (Walker 2002; Tusting *et al.* 2013; WHO 2013a). However, an assessment of the ecological and epidemiological conditions is required to ensure that LSM strategies are suitable for a given area. An understanding of the local vector ecology, especially at the larval stage, is imperative for the establishment of these interventions (Killeen *et al.* 2002a; Fillinger *et al.* 2004; Ferguson *et al.* 2010; WHO 2013a). Studies on local larval bionomics in Mpumalanga Province are few and the current study provides vital information for malaria control programmes on the main malaria vector in this region. In addition to understanding the ecological conditions that influence larval abundance and composition, monitoring insecticide resistance in local populations is vital for predicting the continued success of the chemical control of adult vector mosquitoes (Molineaux and Gramiccia 1980; Haji *et al*. 2013; WHO 2013b). The continued appearance of insecticide resistance across Africa warrants continued monitoring of the resistance status in local populations. This not only aids in understanding resistance in this region, but also contributes good resistance records to control programmes in the area.

In the larval environment, changes in the ecological conditions may favour one species of mosquito over another. This will ultimately influence the community of vectors that have survived into adulthood which has direct implications for malaria epidemiology. One variable

of significant importance is temperature and this has been linked to development in all invertebrates including mosquitoes (e.g. Rueda *et al.* 1990; Lyimo *et al.* 1992; Alto and Juliano 2001; Lyons *et al*. 2013). Under different temperatures, the development and survival of one species can shift which has implications for population dynamics at the adult (vectorial) stage of malaria transmitting *Anopheles*. One of the focuses of research in vector control is to better understand the ecological conditions that determine localised anopheline composition and relative abundance, and determine the influence that these have on the vector community. In this case, the effect of temperature and competition on the survivorship and development time of the immature life stages of two species of the *An. gambiae* complex was conducted. This allowed for insights into how adult population structure is regulated by competition under different temperatures at the immature stage. To do so, fluctuating temperatures as well as constant temperatures were investigated. The former was done in an attempt to mimic field settings and to deduce what the resultant outcome of such interactions might be under field conditions. Few studies have investigated the importance of fluctuating temperatures in determining basic biology and life history traits in anophelines, despite the importance of such information in the context of a changing climate (but see Huffaker 1944; Parham *et al.* 2012; Lyons *et al.* 2013).

1.3. Objectives and hypotheses

The aims of this study included:

- characterising the larval breeding sites of *Anopheles gambiae s.l.* in the Lowveld region around Hectorspruit in Mpumalanga Province;

- determining levels of insecticide resistance in local mosquito populations in Mpumalanga province; and
- determining the outcome of intra- and inter-specific competition between two members of the *Anopheles gambiae* complex under constant and fluctuating temperatures in controlled settings.

The main hypotheses include:

1. larvae of anopheline vectors and non-vectors in Mpumalanga associate with certain conditions in the breeding site which can be determined;
2. there is no insecticide resistance present in populations of members of *Anopheles gambiae* complex in Mpumalanga; and
3. under different temperature treatments, there is no difference in the outcome of intra- and inter-specific competition in the larvae of *An. arabiensis* and *An. quadriannulatus*.

CHAPTER TWO - CHARACTERISTICS OF LARVAL BREEDING SITES AND INSECTICIDE RESISTANCE IN THE *ANOPHELES GAMBIAE* COMPLEX IN MPUMALANGA, SOUTH AFRICA.

2.1. Introduction

The province of Mpumalanga, in north-east South Africa, contains a diversity of *Anopheles* species (Diptera: Culicidae), and includes the notable vector *Anopheles arabiensis* which is responsible for the continued seasonal transmission of malaria in the region (White 1974; Sharp *et al.* 1990; Govere *et al.* 2000). The saltwater breeder and minor vector *An. merus*, implicated in malaria transmission elsewhere (Temu *et al.* 1998; Pock-Tsy *et al.* 2003; Cuamba and Mendis 2009), is also present. The Malaria Control Programme in Mpumalanga, employing DDT for indoor residual spraying (IRS), which has been the main vector control method since 1946 (Gear *et al.* 1981), has eliminated the largely anthropophilic, indoor resting vector *An. funestus*, often associated with epidemics in the region (Govere *et al.* 2001).

The emergence of resistance to drugs in *Plasmodium falciparum* and multiple insecticides in African anophelines has over the years required malaria programmes to increasingly concentrate on vector control through management of the environment as well as biological control (Keiser *et al.* 2005; Elkhalifa *et al.* 2008; WHO 2013a). Although not new to vector control, these two approaches have been documented as effective tools in diverse places such as India (Rajagopalan *et al.* 1987) and Brazil (Soper and Wilson 1948; Killeen *et al.* 2002b). Larval breeding sites in the malarious areas of South Africa have been targeted for

modification and larval control from as early as 1905 but did not contribute significantly to the reduction in malaria cases (Coetzee *et al*. 2013a). The Mpumalanga Malaria Control Programme's efforts in larval source management rely largely on larviciding with insecticides of biological or chemical origin applied to the aquatic breeding habitats (Ngomane and de Jager 2012). Knowing the local seasonal pattern of transmission allows larviciding operations to be scaled-up to coincide with periods in which malaria cases peak (Silal *et al.* 2013). Continued low level larviciding during winter months coinciding with the dry season in the malarious regions of southern Africa also offers benefits in the reduction of source populations prior to the transmission season (Shililu *et al.* 2003; Fillinger *et al.* 2004; Shililu *et al.* 2007; WHO 2013a).

Although ecological management of larval breeding sites is a valuable tool in malaria control programmes, this approach requires knowledge of local larval ecology (Fillinger *et al.* 2004; Ferguson *et al.* 2010; WHO 2013a). Important in this regard is an understanding of the environmental characteristics that favour vectors of malaria and which may influence their relative abundance in an area (Rejmankova *et al.* 1991; Kudom *et al.* 2011). Identification of the most productive breeding sites is a key component of larval source management (WHO 2013a) which, in comparison to IRS, allows for higher coverage due to the inability of larvae to migrate substantial distances from the assaulting compound (Killeen *et al.* 2002a). Implementation of larval source management has led to an effective decline in larval numbers and adult densities in many African countries such as Eritrea (Shililu *et al.* 2007), Ethiopia (Elkhalifa *et al.* 2008) and Tanzania (Geissbühler *et al.* 2009).

As ecosystems where anopheline larvae occur can show great variation, operations targeting larvae of vector mosquitoes require the identification and characterisation of conditions of possible aquatic breeding sites in an area (Killeen *et al.* 2002b; WHO 2013a). Several authors describe the ecological conditions which support larvae of the *An. gambiae* complex

in southern Africa (see for example, De Meillon 1947; le Sueur and Sharp 1988). However, studies on local larval bionomics in Mpumalanga Province are few. Good rainfall is vital for the proliferation of larval habitats but excessive rainfall may have a negative effect on breeding sites and thus anopheline numbers (Paaijmans *et al.* 2007).

Results from studies suggest that breeding site productivity is largely under the influence of the type of habitat (Edillo *et al.* 2002; Minakawa *et al.* 2004; Shililu *et al.* 2007; Majambere *et al.* 2008) with those arising from anthropogenic activity producing significantly greater numbers of adult vectors compared to natural water bodies (Matthys *et al.* 2006; Mutuku *et al.* 2006; Shililu *et al.* 2007; Kudom *et al.* 2011; Mereta *et al.* 2013). Elsewhere, ecological parameters such as: water turbidity (Sattler *et al.* 2005), habitat size (Minakawa *et al.* 2004; Minakawa *et al.* 2005), lower temperatures (Minakawa *et al.* 2006; Mereta *et al.* 2013), nitrate concentrations (Gimnig *et al.* 2002*;* Ndenga *et al.* 2012) and distance to the nearest house (Minakawa *et al*. 1999) have been shown to influence larval numbers of vectors and non-vectors of the *Anopheles gambiae* complex and the *Anopheles funestus* group. In addition, biological conditions such as: larval density (Gimnig *et al.* 2002) and predator/ competitor presence (Mereta *et al.* 2013) influence malaria vector numbers. Identifying the key habitat characteristics and relating this to vector productivity is important for incorporating larval source management into integrated vector management programmes (WHO 2013a).

In South Africa, the morphologically identical non-vector *An. quadriannulatus* and the minor vector *An. merus*, can be found in sympatry with the dominant vector, *An. arabiensis* (Coetzee *et al.* 2000). All of these species share larval breeding sites and adults have been reported resting indoors throughout the region (Odetoyinbo and Davidson 1968; Hunt and Mahon 1986). The behaviour and role of *An. merus* in malaria transmission in Mpumalanga, South Africa is unknown. In Mozambique, a decrease of 8.4% of *An. merus* has been reported following IRS intervention, in comparison to a 93% and 84% decline in *An.*

arabiensis and *An. funestus* numbers, respectively (Sharp *et al.* 2013) suggesting exophilic behaviour in this species where there are human hosts. Its role as a minor vector has been reported along the Kenyan coast (Kipyab *et al.* 2013), the Tanzanian coast (Shiff *et al.* 1995; Temu *et al.* 1998), in Madagascar (Pock-Tsy *et al.* 2003) and southern Mozambique (Cuamba and Mendis 2009) especially when this vector predominates and human hosts are readily available (Temu *et al.* 1998; Kipyab *et al.* 2013). Assessing the relative proportions and local ecological conditions of *An. gambiae* complex members in eastern Mpumalanga contributes to the wider malaria control programme by monitoring vector numbers and proportions during routine surveillance. It further aids in forecasting the need for adult control as well as in measuring the impact of previous interventions (WHO 2013a).

Larval sites selected by gravid females are variable and uncommon larval habitats have been recorded when preferred breeding sites are unavailable due to rarity or excessive density of larvae leading to overcrowding (Patton 1905; De Meillon 1938). *Anopheles gambiae s.l.* members (which includes *An. arabiensis*) typically favour sites characterised by small size, short longevity and higher light intensity, whereas *An. funestus s.s.* prefers shaded sites of relative permanence (De Meillon 1947; Sinka *et al.* 2012). As a freshwater breeding species, *An. arabiensis* is common in the low-lying inland areas of Mpumalanga except where salinity is high as reported by Munhenga *et al.* (2014) in the neighbouring Limpopo Province. Under brackish conditions there is a tendency for *An. merus* to dominate (Kipyab *et al.* 2013; Munhenga *et al.* 2014), however, larval survival of this species is reportedly good in freshwater and at up to 60% salinity under laboratory conditions (Mosha and Mutero 1982; White *et al.* 2013). The non-vector species *An. quadriannulatus* commonly co-occurs with *An. arabiensis* (Gillies and Coetzee 1987; le Sueur and Sharp 1988). Munhenga *et al.* (2014) report the presence of *An. quadriannulatus* under conditions most favourable to *An. merus* in Mafayeni, northern Kruger National Park. However, under laboratory conditions its ability to

survive to maturity is diminished significantly in increasingly saline waters (Coetzee and le Sueur 1988).

In this chapter, the results from a field study on the breeding site requirements of *Anopheles* spp. in eastern Mpumalanga are presented and an examination of the relative proportions of *An. gambiae s.l.* and their insecticide resistance status is given. Information gathered can aid malaria control personnel in identifying larval breeding sites based on environmental parameters where vector species are common. As South Africa moves toward a scenario where no local cases of malaria are acquired, information gathered in this study can be used in conjunction with hotspot mapping of high transmission sites and appropriate, targeted interventions such as larviciding can take place in sites with known vector densities. In addition, data may contribute to identifying and profiling the demographics of the major vector and non-vector species in the sites based on the prevailing environmental characteristics (Maharaj *et al*. 2013; Moonasar *et al*. 2013).

2.2. Aims and objectives

The aims of this study were firstly to characterise the larval breeding sites of anopheline species in the Lowveld region around Hectorspruit in Mpumalanga Province. Using abiotic parameter data collected in the field I aimed to determine the environmental conditions that correlated with larval community composition. An investigation into the insecticide resistance profiles of *Anopheles* populations in the region was also undertaken. Specific questions pertaining to the field study included:

- What conditions characterise the breeding sites of anopheline species in the area? In this regard, do the physical characteristics of the breeding habitat influence the composition and abundance of *Anopheles* larvae present?
- Given the reported suitable breeding site requirements of anopheline species such as *An. merus*, can we expect to find a significant difference in the proportion of individuals found in more saline sites?
- Are larvae of some *Anopheles* spp. more likely to be found in certain classes of habitat such as riverbeds or ephemeral streams?
- Are similar breeding habitats composed of similar species assemblages?
- What is the composition of malaria vectors at each site and how does this compare across the sites?
- Does the distance to nearby man-made objects (houses and roads) influence the composition and abundance of anophelines?
- What are the current profiles of insecticide resistance among *Anopheles* species from the area?

2.3. Materials and methods

2.3.1. Study site

This study took place in the Mpumalanga Province of South Africa, 30 km west of the border of Mozambique, around the town of Hectorspruit (25°31’59’’S, 31°41’59’’E) in the Nkomazi Municipality. Annual temperatures in the area range from an average minimum of 8°C in the dry season to an average maximum of 26°C in the hot and rainy season, although

temperatures can peak above 35°C during the hottest times of the year according to the South African Weather Service (2014). The annual rainfall of 650 mm falls predominantly in the summer months, with peaks in the first quarter of the year. Total annual rainfall from October 2013 to September 2014 in the Malelane district of Nkomazi Municipality was 1155 mm, 147 % of the long term mean (LTM) of 786 mm and in the month prior to sampling [March] rainfall exceeded this value, peaking at almost three times the annual long term mean for March (data from the South African Sugarcane Research Institute 2014).

Field work was conducted during April 2014, nearing the end of the rainy season, which normally follows the 2 – 3 month lag period of high rainfall associated with peak transmission (Govere *et al.* 2002). Members of staff from Mpumalanga's Malaria Control Programme assisted with locating known larval breeding sites in the surrounding areas. At each site, larvae were sampled from breeding habitats and characteristic environmental features were assessed.

2.3.2. Mosquito collections

2.3.2.1. Field collections

Mosquito larvae were collected using larval dippers or scoops. Sampling was conducted from 10:00 and continued until 15:00 with two to three sites visited per day. On average, approximately an hour and forty minutes was spent in active searching for larvae at each site.

Water samples were collected from each site and stored in plastic bottles. Upon collection, mosquito larvae were kept in containers with the same water from which they were collected.

At a later stage, these were transferred to 50% distilled water and 50% site water mixture. Anything that was not readily identified as an anopheline larva was removed from the samples before transportation and after transfer into larval bowls so as to minimize predation by organisms such as Odonata nymphs.

A temporary outdoor insectary was set up in the field following collection. Mosquito larvae kept in water from their specific breeding site were fed optimally on a mixture of ground dog biscuits and yeast extract. Emergent adults were maintained on a 10% sugar solution. Adults that emerged during this time were tested for insecticide resistance. Those that were not tested, or remained alive following exposures, were killed using ethyl alcohol and stored on silica gel for later identification. Transport of the remaining mosquito larvae to the Botha De Meillon Insectary at the Vector Control Reference Laboratory of the National Institute of Communicable Diseases in Johannesburg was done in plastic bottles containing water specific to each site, diluted to 50% with distilled water. Conditions in the insectary have a constant temperature of around 25°C and a relative humidity of 80%.

2.3.2.2. Habitat characteristics

To assess larval community structure in response to different habitat conditions, descriptive data of the breeding habitats was collected. The substrate type, consisting of muddy or sandy/coarse substrate, was also noted. Site classes were recorded into the following categories: floodplain, riverbed, stream, river bank, wetland and other (Table 2.1).

Table 2.1 Description of categories of habitat used in site classification.

Habitat category	Description
Floodplain	Area above river bank, prone to flooding
Riverbed	Bed of channel in which a river flows, large (>2m wide)
Stream	Channel in which a stream flows, small (<2m wide)
River bank	Land alongside the river bed, generally on an incline
Wetland	More permanent water body characterised by wetland vegetation
Other	Roadside puddles, artificial containers

Separate samples of water from each site were collected and stored at 1–4°C for a maximum of three days following guidelines from the Waters and Rivers Commission by Hosking Chemical Services (Australian Department of Water 2009). Measurements of salinity, pH and total dissolved solids (TDS) were tested in the laboratory in Johannesburg using the Consort C5020 (Consort, Turnhout, Belgium). Instrument results were reported as g/ L or % seawater converted from the Consort C5020 unit of measure: ppt (35 g/l NaCl = 100% seawater) (USGS 2014). In order to ensure consistent measurements, containers were removed from cold storage as and when recordings were made and the temperature was allowed to increase to 8°C for all samples to ensure standardisation. Three measurements for each abiotic condition were carried out and the average taken.

In addition, Global Positioning Software (GPS) coordinates were recorded using a handheld GPS (eTrex©, Garmin International Inc., Olathe, USA). Estimates of the distances to houses

and roads were made by line of sight and corroborating this in Google Earth (Mountain View, CA, U.S.A.) software as these factors can influence larval habitat and localised species composition (Minakawa *et al.* 2004). Land surface temperature data for the period March 30th to May 25th 2014 was extracted from the Oak Ridge National Laboratory Distributed Active Archive Centre website (ORNL DAAC, Tennessee, U.S.A.). The dates included here were selected as they encompassed the study period and occurred at least one month prior to field work in which anopheline females would have been active in securing reproduction and breeding sites. The subset of data was obtained from the product MOD11A2 (MODIS Satellite Global Subset, ORNL DAAC), centred on the GPS coordinates recorded in field at each site where the size of the subset grid was approximated at 3 km wide and 3 km high.

2.3.2.3. Insecticide resistance profiling

All anopheline larvae sampled were reared to adult and tested for insecticide susceptibility using WHO test-kits and insecticide impregnated papers following standard test procedures (WHO 2013b). Diagnostic dosages of the insecticides Deltamethrin (0.05%), Bendiocarb (0.1%) and DDT (4%) were tested. Mosquitoes were exposed for one hour in standard WHO exposure tubes. In most cases, one hundred individuals per species, per insecticide were tested, as recommended by the WHO. Susceptible controls were sourced from the SENN-BASE colony housed at the Botha de Meillon insectary (Johannesburg) and colonised from Sennar, Sudan in 1980 and is susceptible to Deltamethrin, Malathion, Bendiocarb and Dieldrin. Post exposure, adults were allowed to rest in a tube containing insecticide free paper and given a 10% sugar solution in cotton wool which was placed on top of the gauze of the tube.

Following a 24-hour holding period, the number of dead adults was scored. Possible resistance is measured as mortality less than 98%, 24-hours post-exposure, while confirmed resistance is mortality less than 90% (WHO 2013b). Adults that remained alive were killed using ethyl acetate and stored on silica gel for later identification. Separation of *An. gambiae* complex member from non complex anophelines was difficult and all anophelines were exposed together. As such, some tubes contained very low numbers of *An. gambiae* complex members and a high number of repetitions were conducted to ensure that the numbers of *An. gambiae s.l.* required for WHO protocol were achieved. As a result, reporting of standard error was not possible. Results of susceptibility testing were submitted initially to the Mpumalanga Malaria Control Programme and then to the WHO aggregated global database for the Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM) (WHO 2012).

2.3.2.4. Identification of anopheline adults

Initial identification of anopheline adults based on morphological characteristics described in Gillies and Coetzee (1987) was carried out using the identification key and a stereo-microscope. Specimens that were identified as *An. gambiae* complex were then further processed for species identification up to species level using the polymerase chain reaction (PCR) technique outlined by Scott *et al.* (1993). An estimate of proportions of each anopheline species relative to the others, as well as to the total anopheline number, was determined for each site.

The PCR technique (Scott *et al.* 1993) uses nucleotide sequences in the intergenic spaces of the ribosomal DNA, which are specific to each species. Briefly, PCR uses a small portion of

intact DNA, from a leg or wing, in a pre-prepared master mix containing the enzyme Taq-polymerase. The mixture goes through a process of heating and cooling in order to amplify the DNA for later use in gel electrophoresis.

Individual specimens of anophelines were stored on silica gel in separate eppendorf tubes to prevent contamination of DNA of one individual with another. The source of DNA throughout the PCR assays was either of wing or leg origin and conditions were kept sterile using 70% alcohol. A PCR master mix containing the following reagents was prepared according to the number of samples requiring identification each time the assay was run: 1.25 µL 10 x dNTP; 0.5 µL $MgCl_2$; 1.25 µL 10 x buffer (consisiting of 1 mM KCl, 100 mM Tris-HCl at a pH of 8.3); 4.9 µL deionised H_2O; 0.5 µL *An. quadriannulatus* primer; 1.0 µL each of *An. arabiensis*, *An. gambiae*, *An. merus*, Universal primer; and 0.1 µL Taq-polymerase. Primers consist of 20-base oligonucleotides (Table 2.2).

All reagents, excluding the Taq, were centrifuged and vortexed prior to making the master mix. Intact DNA samples were added to 0.2 mL tubes into which 12.5 µL of the master mix was introduced. In addition, positive and negative controls were run with each PCR assay. The positive controls were obtained from known colonies in the Botha de Meillon Insectary at the Vector Control Reference Laboratory, whereas the negative control consisted of master mix without DNA.

Sample tubes were placed into a PCR machine (Bio-Rad C1000 Touch™ Therma-Cycler) and subjected to cyclic processes of heating and cooling. The protocol requires that the cycle begins with an initial denaturation temperature of 95°C for two minutes, followed by 30 denaturation cycles at 94°C lasting 30 seconds each, annealing of primers at 50°C for a 30 second period, a 30 second extension at 72°C and a five minute final extension at 72°C.

Table 2.2 Primer sequences used in diagnostic PCR identification of the members of the *Anopheles gambiae* complex (Scott *et al*. 1993).

Primer (anneals to species)	rDNA primer Sequence (5' to 3')	Melting Temperature (° C)	Product Size
UN (Anneals to rDNA of all 5 species) (universal)	5' GTG TGC CCC TTC CTC GAT GT 3'	58.3	
GA (*An. gambiae*)	5' CTG GTT TGG TCG GCA CGT TT 3'	59.3	390 bp
AR (*An. arabiensis*)	5' AAG TGT CCT TCT CCA TCC TA 3'	47.4	315 bp
ME (*An. merus/melas*)	5' TGA CCA ACC CAC TCC CTT GA 3'	57.2	464 bp
QD (*An. quadriannulatus)*	5' CAG ACC AAG ATG GTT AGT AT 3'	42.7	153 bp

Following amplification, the samples were removed and 2.5 µL of loading dye was added to the product. An electrophoresis gel was prepared beforehand by adding 10 grams of agarose gel (SeaKem® LE Agarose, Lonza, South Africa) and 400 mL of 1 x TAE Buffer and microwaving until the solution is clear. Ethidium bromide solution (12 µL) (Sigma-Aldrich, St. Louis, USA) was added to the 2.5 % TAE solution after it was allowed to cool. The solution was then poured into a casting tray and allowed to set. A molecular weight ladder which is used as a reference point during gel analysis was loaded into the first and last wells on the gel. The PCR products containing DNA with loading dye were loaded into the wells in the gel and electrophoresis was set at 400 mA/ 100 V for approximately one hour and twenty minutes. The gels were removed and placed into a GeneSnap Vacutec G-Box (SynOptics Ltd., Cambridge, England) for gel documentation and chemiluminescence imaging. Images

were saved electronically and the diagnostic PCR bands in the gels were used to identify species of the *An. gambiae* complex.

2.3.3. Data analysis

Data analysis was performed in SPSS version 20 (IBM Inc., Chicago, USA). To test if there was a significant difference in the mean number of individuals of each species of the *An. gambiae* complex, per site, a non-parametric Kruskal-Wallis test for independent samples was used as the assumptions for a one-way ANOVA were violated (normality and homogeneity of variance) (Chan *et al.* 2003). Temperature data were obtained from the MODIS satellite and were used to test if there was a correlation between species occurrence and abundance.

As the data were not from a normal distribution, the assumptions for a linear regression were not met. In order to explore the correlation between the dependent variable, anopheline species composition, and the predictor abiotic variables (e.g. salinity and pH), a Pearson's Product Moment correlation was run to test if there is a significant association between the two variables.

2.4. Results

2.4.1. Species identification

During the sampling period, a total of 3074 anopheline larvae were collected and reared to adult for later identification. Of these, 2027 were morphologically identified as *Anopheles gambiae s. l.* with the remaining 1047 comprising other anopheline mosquitoes. Fig. 2.1 shows an agarose gel stained with ethidium bromide (2.5 %) for PCR identification of the members of the *An. gambiae* complex.

The relative abundance of *An. gambiae* complex and non-*An. gambiae* mosquitoes according to site is shown in Fig. 2.2. Mgobodi (MG) and Jeppe's Rust (JR) were dominated by non-*An. gambiae* complex anophelines comprising 88.4% and 94.4% of the total, respectively. This number decreased in Magudu (MD) as the number of *An. gambiae* complex anophelines increased to 39.6%. The remaining sites: Block A 1a (BA1a); Block A 1b (BA1b); Mzinti (MN); Vlakbult (VB); and Masibekele (MB) were dominated by *An. gambiae* complex mosquitoes with relative proportions of 77.4%; 100%; 71.7%; 59.2%; and 97.9%, respectively (Table 2.3).

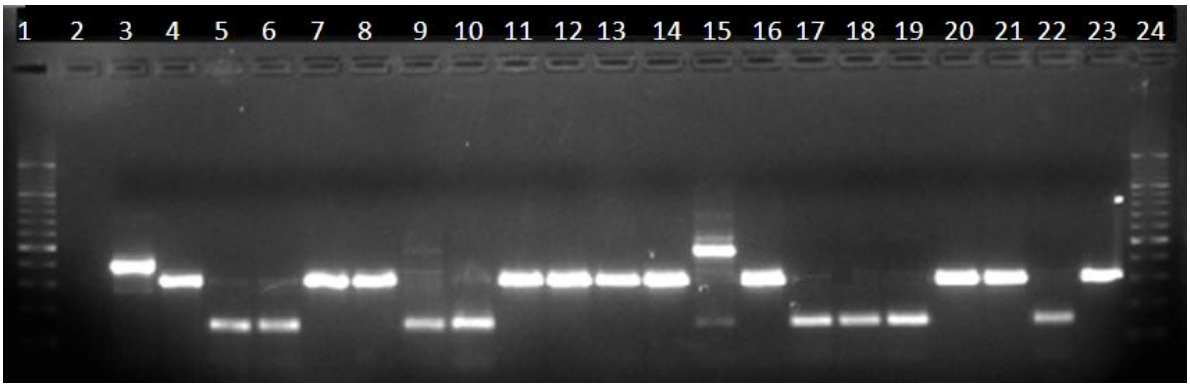

Figure 2.1 An agarose gel stained with ethidium bromide (2.5 %) for PCR identification of members of the *An. gambiae* complex. Lane numbers 1 – 24 indicate well position where numbers 1 and 24 are the molecular weight markers; lane 2: negative control; lane 3: *An. gambiae* positive control; lane 4: *An. arabiensis* positive control; lane 5: *An. quadriannulatus* positive control; lanes 6, 9, 10, 17, 18, 19, 22 positive for *An. quadriannulatus*; lanes 7, 8, 11, 12, 13, 14, 16, 20, 21, 23 positive for *An. arabiensis*; and lane 15 positive for *An. merus*.

The distribution and relative abundance of each species of the *Anopheles gambiae* complex found in the Nkomazi Municipality in South Africa is presented in Table 2.3 with a graphical representation in Fig. 2.3. Changes in the relative abundances and the presence/ absence of *An. gambiae s. l.* species were found within geographically separated sites in the region (Table 2.3). The primary malaria vector in the region, *An. arabiensis*, was recorded in all sites and contributed the greatest relative proportion of *Anopheles* spp. in the Magudu site.

The sibling species *An. quadriannulatus*, was also recorded in all sites and contributed the greatest proportion in Vlak Bult and Mzinti, whereas *An. merus* dominated in sites in Block A and Masibekele. There were no *An. merus* recorded in sites surveyed in the south-west of the area, while in the east this species contributed the highest proportion to species composition (Fig. 2.3).

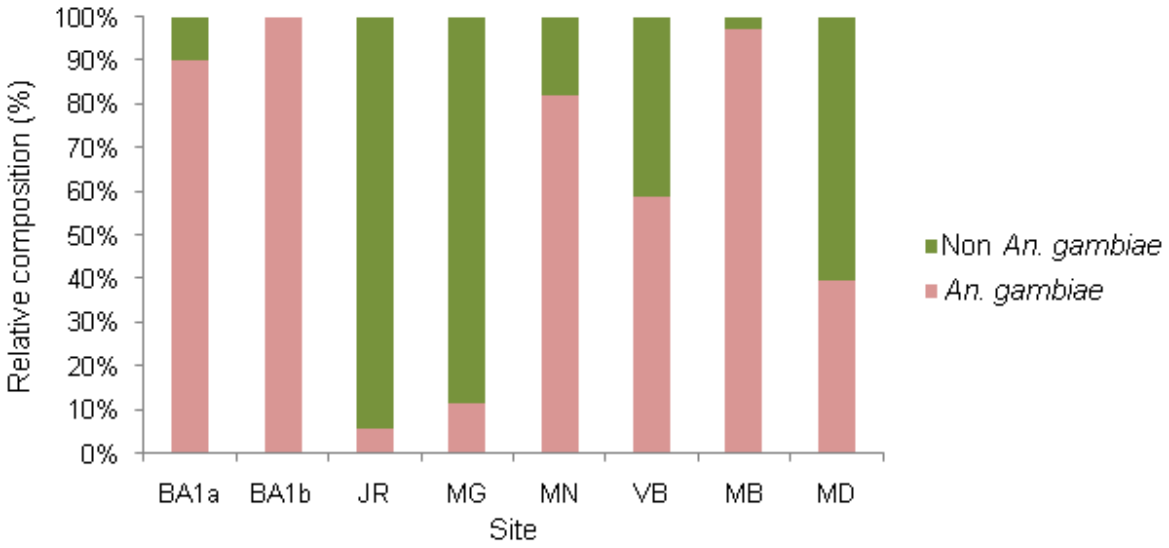

Figure 2.2 Relative composition (%) of *Anopheles gambiae* (pink) and non-*An. gambiae* (green) complex mosquitoes across sites in Nkomazi Municipality in eastern Mpumalanga. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

The non-*An. gambiae s.l.* species, *An. pretoriensis* was the dominant anopheline in Jeppe's Rust and Mgobodi. Other species of significance were *An. marshalli*, *An. rufipes* and *An. maculipalpis*. Additional species which were recorded but were in low numbers (< 10) were: *An. coustani*, *An. theileri*, *An. squamosus*, *An. tenebrosus* and *An. demeilloni* (Table 2.3).

There was a significant difference in the mean abundance of all species of anophelines from each of the geographically separate sites (Kruskal-Wallis ANOVA, $p < 0.05$, df = 6, H_2 = 17.09). There was no significant difference in the overall mean abundance of *An. gambiae s.l.* members: *An. arabiensis* (n = 437), *An. merus* (n = 422) and *An. quadriannulatus* (n = 373).

Table 2.3 Summary of the collections of anopheline mosquitoes in the Nkomazi Municipality in eastern Mpumalanga, South Africa. Percentages indicate the contribution of each species to the total number of *An. gambiae s.l.* members per site, where n is the actual number of specimens collected. Highlighted cells show the species which contributed the greatest proportion to the total at each site. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

	Characteristics	**Site**							
	Species composition	**BA1a**	**BA1b**	**JR**	**MG**	**MN**	**VB**	**MB**	**MD**
An. gambiae s.l.	*An. arabiensis* % (n)	8% (15)	1% (1)	86% (6)	95% (40)	39% (147)	31% (42)	41% (119)	81% (67)
	An. quadriannulatus % (n)	30% (56)	3% (4)	14% (1)	5% (2)	55% (205)	57% (76)	5% (14)	18% (15)
	An. merus % (n)	62% (122)	95% (111)	0	0	6% (21)	11% (14)	54% (154)	0
	% *An. gambiae* of total anophelines	77.4 %	100 %	5.6 %	11.6 %	71.7 %	59.2 %	97.9 %	39.6 %
Other *Anopheles* spp.	*An. coustani* (n)	2	0	2	8	4	2	0	0
	An. pretoriensis (n)	2	0	70	244	137	55	12	117
	An. rufipes (n)	8	0	46	42	3	5	0	0
	An. marshalli (n)	37	0	0	0	1	0	0	0
	An. maculipalpis (n)	0	0	0	24	2	24	0	10
	An. tenebrosus (n)	3	0	0	0	0	4	0	0
	An. squamosus(n)	0	0	0	1	0	0	0	0
	An. theileri (n)	0	0	0	1	0	0	0	0
	An. demeilloni (n)	1	0	0	0	0	0	0	0

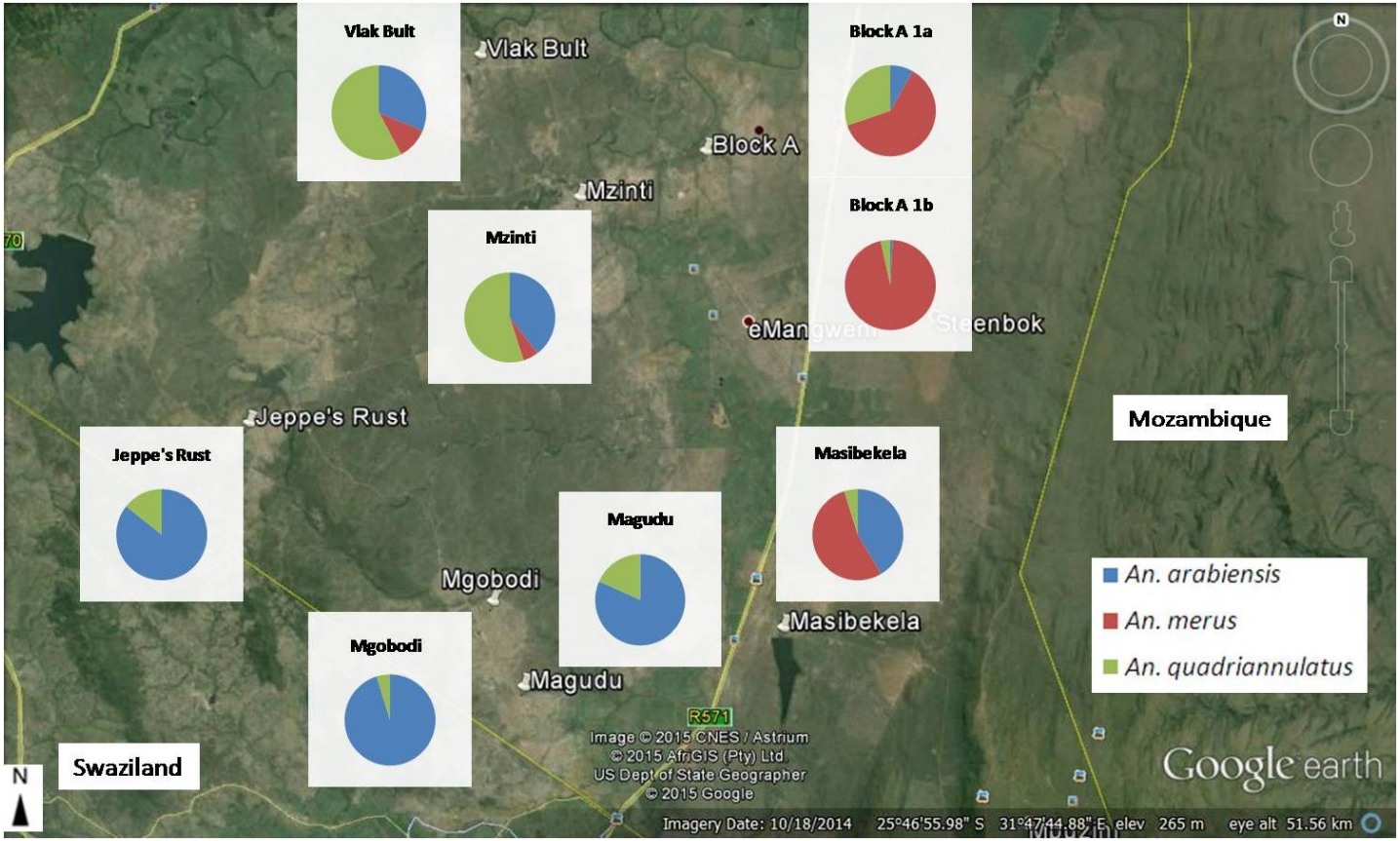

Figure 2.3 Distribution map and relative abundances for the members of the *Anopheles gambiae* complex according to the various sites surveyed in the eastern Mpumalanga region. Pie charts are representative of the proportion of *An. gambiae* complex members to one another. Map data licenced to AfriGIS and Google Imagery (2014).

2.4.2. Habitat characteristics

Habitat characteristics recorded at each site are given in Table 2.4. All sites were sunlit either fully or for a portion of the day and in the latter case, the side walls of the incised streambed in Jeppe's Rust caused shading during the early morning and late afternoon. Where semi-aquatic vegetation, such as sedges, was present, such as in sites Vlak Bult and Mzinti, it is likely that there is a small degree of shading, however the breeding site was still characterised as being open/ sunlit. In the majority of breeding sites surveyed culicine larvae, water beetles (Hemiptera) and dragonfly (Odonata) nymphs were observed. In the river bank site in Block A, no mosquito predators were observed; however, culicine larvae were recorded in high abundance.

Mean day and night temperatures were uniform for the period selected across the sites apart from in Mzinti where day temperatures were significantly lower (One-way ANOVA, $p < 0.05$, $df = 7$, $F = 9.97$) (data extracted from: ORNL DAAC, Tennessee, U.S.A.). Numbers of individuals of the non-vector species *An. quadriannulatus* were negatively correlated with day time temperatures (Pearson's Product Moment, $r = -0.870$, $p < 0.05$) and positively correlated with night time temperatures (Pearson's Product Moment, $r = 0.889$, $p < 0.05$).

When sites were ordered by increasing salinity (Fig. 2.4), *An. merus*, a species known to favour more saline waters, was absent in three sites (Magudu, Jeppe's Rust and Mgobodi) where salinity was low (< 1 g/ L salinity). However, in two sites with slightly higher salinity, Vlak Bult and Mzinti, *An. merus* was present in small numbers, contributing 11 % and 6 %, respectively, to the total number of *An. gambiae s. l.* mosquitoes collected. At slightly higher saline concentrations (> 3 g/ L salinity), such as that found at sites BA1a, BA1b and MB, the relative proportion of *An. merus* increased significantly contributing 62 %, 95 % and 54 %, to the total number of mosquitoes collected, respectively.

Anopheles merus showed a significant positive correlation with salinity (Pearson's Product Moment, $r = 0.971$, $p < 0.05$), however, testing for a significant relationship between the two variables was not possible due to heterogeneity of variances invalidating the assumptions for a regression. Both *An. arabiensis* and *An. quadriannulatus* showed no significant correlation with the predictor variable salinity. In sites which had high readings of salinity, *An. arabiensis* contributed a relatively greater proportion to the numbers (41%) in site MB than it did in other sites with similar salt concentrations (Table 2.3, Fig. 2.4).

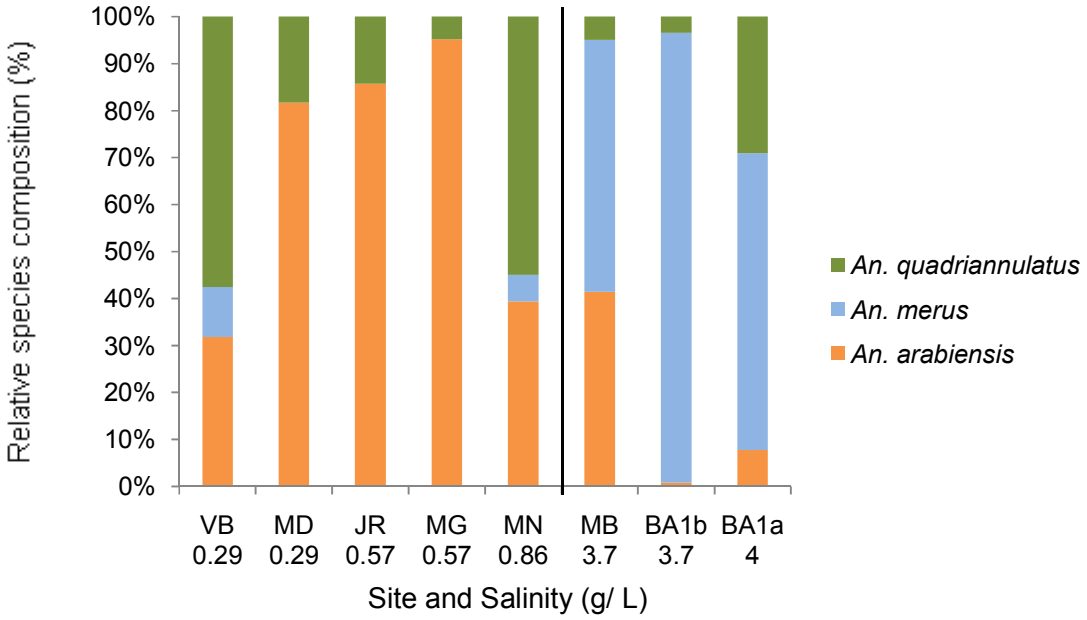

Figure 2.4 Relative species composition (%) of the three *Anopheles gambiae s. l.* species sampled at each site in the Nkomazi Municipality in eastern Mpumalanga. Sites are ordered by increasing salinity (g/ L). Sites to the right of the line have salt concentrations approximately 10 fold greater than those to the left of the line. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

The waters sampled during this study were all slightly alkaline and ranged between pH values of 7.28 and 8.88. In the three sites with highest salinity, pH differed markedly where BA1a and BA1b had values of 7.98 and 7.94, respectively, while site MB was more alkaline with a value of 8.88, the highest recorded pH of all sites (Table 2.4). The lowest pH value recorded was in site MG with a reading of 7.28.

When sites were ordered by total dissolved solids (TDS) (g/ L), sites MB, BA1b and BA1a with the highest TDS values (> 1 g/ L) also had the greatest proportion of *An. merus* (Fig. 2.5). This species was also present in small proportions in site VB where TDS was lowest. However, it was absent in sites MD, JR and MG which had low TDS values. *Anopheles merus* shows a significant positive association with TDS (Pearson's Product Moment, $r = 0.922$, $p < 0.05$), whereas *An. arabiensis* and *An. quadriannulatus* do not show a significant association with this variable. The underlying substrate of the breeding places where sites had the highest TDS values were muddy and *An. merus* was the dominant species (Table 2.3, Fig. 2.6). However, site MG with a lower TDS value was in a muddy substrate with no record of *An. merus*, instead it was dominated by the vector species *An. arabiensis*. Of the eight sites investigated, the majority were classed as floodplain (MG, MN, MB and MD); two were found in a riverbed (BA1a and VB); one on a river bank (BA1b); and one in a stream (JR) (Table 2.4).

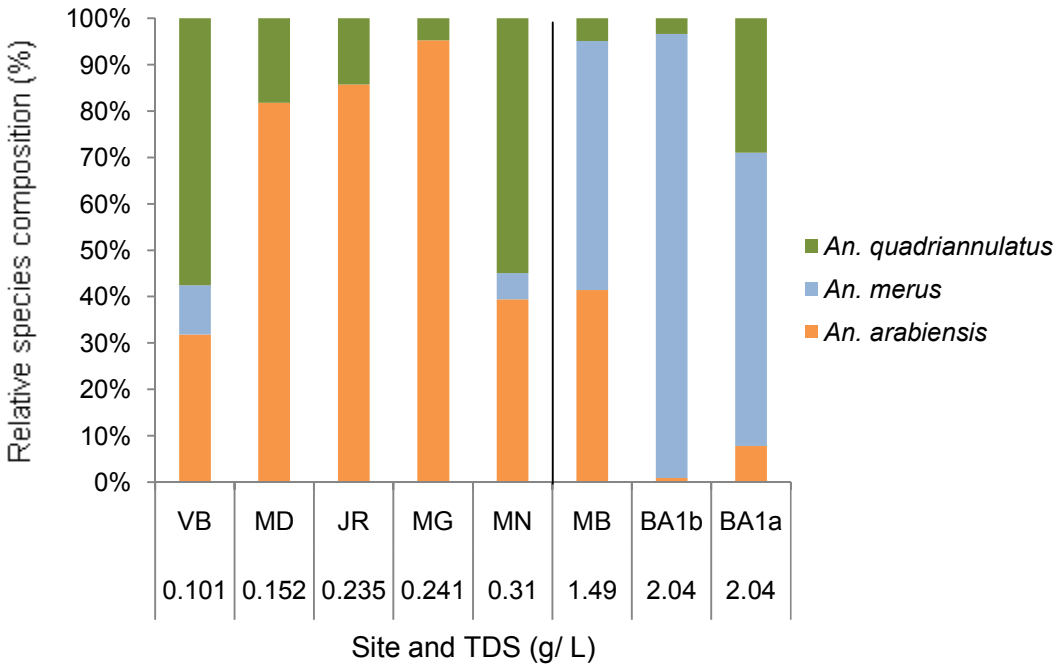

Figure 2.5 Relative composition (%) of the three *Anopheles gambiae s. l.* species sampled at each site in the Nkomazi Municipality in eastern Mpumalanga. Sites are ordered by increasing values of Total Dissolved Solids (TDS). Those to the right of the line have TDS values approximately 10 to 30 fold greater than those to the left of the line. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

Table 2.4 Summary of the habitat characteristics recorded at each site where *Anopheles gambiae* complex mosquitoes were collected in the Nkomazi Municipality in eastern Mpumalanga, South Africa. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

Characteristics	**Site**							
Habitat characteristics	**BA1a**	**BA1b**	**JR**	**MG**	**MN**	**VB**	**MB**	**MD**
Salinity (g/ L)	0.014	0.013	0.002	0.002	0.003	0.001	0.013	0.001
pH	7.98	7.94	8.29	7.28	7.32	8.22	8.88	7.57
Total Dissolved Solids	2.04	2.04	0.235	0.241	0.31	0.101	1.49	0.152
Distance to house (m)	50 - 100	50 - 100	> 150	50 -100	< 50	< 50	> 150	< 100
Distance to road (m)	50 - 100	50 - 100	> 150	> 150	< 10	< 50	> 150	< 50
Class	Riverbed	River bank	Stream	Floodplain	Floodplain	Riverbed	Floodplain	Floodplain
Substrate	Muddy	Muddy	Sandy	Muddy	Sandy	Muddy	Muddy	Sandy

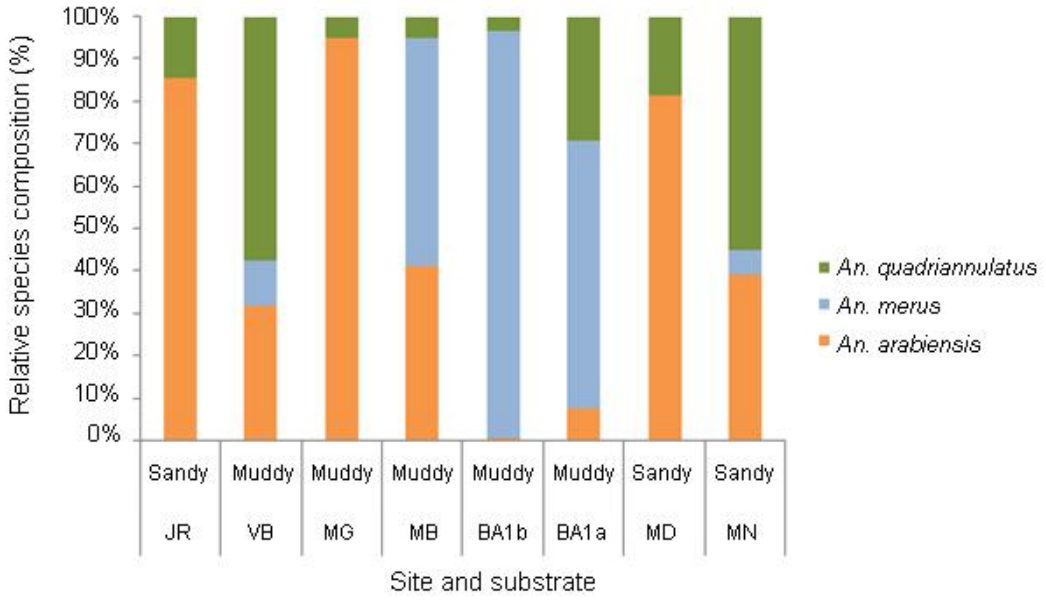

Figure 2.6 Relative composition (%) of the three *Anopheles gambiae s. l.* species sampled at each site in the Nkomazi Municipality in eastern Mpumalanga. Sites are grouped by the dominant underlying substrate. Site names: BA = Block A; JR = Jeppe's Rust; MG = Mgobodi; MN = Mzinti; VB = Vlak Bult; MB = Masibekele; and MD = Magudu.

Two larval breeding sites were recorded within 50 metres of a house, whereas three were found within 50 metres of a road. Between 50 and 150 metres from a house and from a road, four and two sites were sampled, respectively (Table 2.4). Two sites were further than 150 metres from a house whilst the remaining three sites were more than 150 metres from a road. *Anopheles quadriannulatus* contributed the greatest proportion of *An. gambiae* species within 50 metres from a house and road. This was followed by *An. arabiensis* and then by *An. merus* which contributed a small percentage in number. As the distance from these man-made features

increased, *An. merus* contributed a greater proportion to the composition of *An. gambiae* species whilst *An*. *quadriannulatus* was seen to decrease in both instances.

2.4.3. Insecticide resistance

Results of the World Health Organization (WHO 2013b) insecticide susceptibility tests on 1 – 3 day old first generation progeny of each of the species in the *An. gambiae* complex found in Mpumalanga suggest that all species from the sites surveyed during this study are completely susceptible to the Bendiocarb and Deltamethrin compounds used in vector control (Table 2.5).

Following criteria outlined by the WHO for possible resistance (< 98%), *An. merus* may show possible resistance with three out of 113 mosquitoes surviving exposure to DDT. The number of *An. merus* and *An*. *quadriannulatus* exposed to Bendiocarb and Deltamethrin, respectively, are less than the required numbers specified by the WHO (2013b) due to insufficient material (Table 2.5). The main malaria vector in the region, *An. arabiensis*, was susceptible to all three insecticides.

Table 2.5 World Health Organization insecticide susceptibility results on 1 – 3 day old members of the *Anopheles gambiae* complex (n = number of individuals) from the Nkomazi Municipality in eastern Mpumalanga. Anophelines were exposed to WHO recommended dosages of Bendiocarb, DDT and Deltamethrin. All adults exposed were reared from larvae in the Botha de Meillon Insectary at the National Institute for Communicable Diseases.

	Insecticide					
	Bendiocard (0.01%)		**DDT (4%)**		**Deltamethrin (0.05%)**	
***Anopheles* spp.**	**n**	**% mortality**	**n**	**% mortality**	**n**	**% mortality**
An. arabiensis	126	100	97	98	100	100
An. quadriannulatus	102	100	133	99	84	100
An. merus	34	100	113	97	98	100

2.5. Discussion

2.5.1. Species composition

Larval source management contributes a supplementary component to the Malaria Control Programme in eastern Mpumalanga and knowledge of the distribution and composition of vector populations is important for interventions of this type. Disregard for local vector composition and differences in feeding and resting behaviours amongst vector species, such as *An. arabiensis*, can lead to programme

failure (White 1974; Coluzzi *et al.* 1979; Coetzee *et al.* 1993; Duchemin *et al.* 2001). Complimentary vector control in the study area through targeting of larval breeding sites during winter months when rainfall is low is attainable. During this season breeding sites are discrete in the landscape and less transient which offers more targeted control and as such, is a worthy tool for malaria control in the framework of integrated vector management (Soper and Wilson 1943; Charlwood *et al.* 2000; Fillinger *et al.* 2004; WHO 2013a). The topography of the area in this study is flat to undulating, creating a relatively homogenous spatial distribution of habitat types. No permanent breeding sites were surveyed during the study which may help explain the low density in our results of some mosquito species such as *An. coustani*, known to favour swamp habitats and the absence of members of the *An. funestus* group commonly found in freshwater wetland sites (De Meillon 1951). Another reason for the absence of *An. funestus s.s.* could be due to continued DDT usage in IRS in the area and susceptibility previously reported in this species (Hargreaves *et al.* 2000) with the result that populations of this highly anthropophilic species have all but been eradicated from South Africa (Brooke *et al.* 2013; Coetzee *et al.* 2013; Maharaj *et al.* 2013). The continued presence, despite control efforts, of the vector *An. arabiensis* in Mpumalanga requires an understanding of the conditions of the larval environment as well as its outdoor resting and feeding behaviours.

In this study we have demonstrated the heterogeneous distribution of anophelines across aquatic habitats in Mpumalanga with both fresh and saline waters being exploited and we also give an indication of the possible presence/ composition of anopheline species based on certain habitat characteristics. Criteria for site selection required that they be located in an area of malaria transmission and that vector

presence is suspected mainly through previous routine surveillance operations by the malaria control programme personnel in Mpumalanga. Habitats within sites were selected based on the presence of suitable breeding places.

Small-scale variation in environmental characteristics such as salinity and total dissolved solids influenced *Anopheles* species abundance in this study. A total of twelve anopheline species were collected and identified during the April field trip to Mpumalanga. Results from PCR identifications showed that three species of the *An. gambiae* complex were present in the region while the notable vector, previously implicated in sustained malaria transmission, *An. funestus s.s.* remained absent. The most abundant anopheline was the non-vector *An. pretoriensis* followed by the major vector in the region, *An. arabiensis*, which was present in all sites. *Anopheles pretoriensis* has been reported to feed on humans, and in Zimbabwe and South Africa a ±1% sporozoite infection rate has been reported (De Meillon 1951). The larvae of this species are found in a diversity of habitats, except where shading is high, and as such this species is commonly associated with *An. gambiae* species (De Meillon 1947; De Meillon 1951). *Anopheles quadriannulatus* was found in association with the major vector in all breeding sites and may act as an indicator species for the presence of *An. arabiensis* in a breeding place. Variability in larval proportions across the sites is likely explained by interactions of habitat characteristics with the availability of food resources and spatio-temporal differences in habitat structure (Robert *et al.* 1998; Ndenga *et al*. 2012; Obsomer *et al.* 2013), as well as the current methods used for controlling adults (Coetzee *et al.* 2000; Hargreaves *et al.* 2000; Derua *et al*. 2012).

2.5.2. Habitat characteristics

Relationships between the occurrence of anopheline species and environmental variables have been reported elsewhere (e.g. De Meillon 1947; De Meillon 1951; Muirhead-Thomson 1951; Gillies & De Meillon 1968), however to ascertain the determinant conditions or preferential breeding sites has proven much more difficult; largely a consequence of the complex nature of anopheline ecology. Earlier works in vector studies and larval ecology highlighted the pivotal role climate plays in the distribution of anophelines (De Meillon 1948; De Meillon 1951; Lindsay *et al.* 1998; Craig *et al.* 1999). At the local scale however, the influence of environmental parameters is important in determining the assemblage of mosquito species present in an area.

In rural Mali around the Banambani Village, significant differences were found in the distribution of the two primary vectors *An. arabiensis* and *An. gambiae s.s.* amongst the three habitat types surveyed, however the authors could not establish the preferred breeding site of each species (Edillo *et al*. 2002). Similarly, Minakawa *et al.* (1999) found a heterogeneous distribution in the abundance of *An. gambiae* species across different habitat types in the Suba District of Kenya but were unable to identify the key site parameters characterising associations with the abundance and occurrence of larvae. Many biological (e.g. predation and interspecific competition) and physiochemical variables have been proposed as determinants of *An. arabiensis* larval occurrence and population size (Robert *et al.* 1998; Edillo *et al.* 2002; Shililu *et al*. 2007).

The association between high pH and *An. arabiensis* larval population size found by Robert *et al.* (1998) in market-garden wells in urban Dakar is mimicked in the current study where the proportion of this species was high where the pH was greater than 8, especially where the water was moderately saline. Where salinity was high and the pH low, *An. arabiensis* contributed less than 10% to the relative proportion of *An. gambiae* complex members found in the Block A sites. Only two of the environmental factors assessed in the current study showed a significant correlation with larval composition, specifically for the species *An. merus*, and as such, identifying the key environmental conditions for the remaining species is problematic. As a result it would be not be possible to use habitat characteristics as a bio-indicator of species presence. However, where waters tend toward higher values of salinity and total dissolved solids, *An. merus* dominates (Coetzee and le Sueur 1988; White *et al.* 2013).

Biotic interactions may have an important effect on mosquito population regulation and the co-existence of larvae with other species as well as predators can influence the survivorship of a species (Koenraadt and Takken 2003; Muiruri *et al.* 2013). Inter- and intra-specific competition and predation regulate larvae of the North American malaria vector, *An. quadrimaculatus*, which may lead to a reduction in numbers whilst simultaneously increasing generation time which has an effect on adult emergence and malaria incidence (Knight *et al.* 2004). In addition, small differences in conditions in the breeding place can alter the landscape in which biotic interactions, such as competition, occur. When dissolved oxygen was highest, Elono *et al.* (2010) found that the natural competitors of *Aedes* spp. exerted a stronger influence on larval numbers than when levels were lower. Apart from differences in

salinity, the abundances of anopheline larvae recorded in sites during the present study do not appear to be affected by variations in the parameters assessed. Predators were noted in the majority of sites, except where salinity was highest, however the suppressive effect that these may have had on larval composition and abundance is unknown and requires further investigation.

The dominant habitat type from which anopheline mosquitoes were collected was a floodplain (Fig. 2.7) characterised by a slow-moving flow of water and in most instances, the presence of vegetation which contributes to impeding the rate of flow. This habitat type may also be the site of animal movement, being well utilised by cattle, as it connects areas of feeding to water bodies. Thus, anopheline species which are zoophilic are likely to be more prevalent here as a food source is in close proximity to the breeding sites. The geographic sites associated with this habitat type, and from where larvae were collected, are Magudu, Mzinti, Masibekela and Mgobodi. Sites were characterised by high light intensity and where they were partially shaded in some places, this was provided by semi-aquatic vegetation, grasses and in the case of the incised streambeds, its side walls.

The underlying substrate appears to have no determinant effect on the species composition or other environmental characteristics such as total dissolved solids or pH. The temporary nature of these sites may favour proliferation of some anophelines (De Meillon 1951), however the occurrence of such sites is largely dependent on the water source from which they are borne. If these form from river overflow, sufficient rainfall is needed and the reduction in temporary breeding places during low rainfall is associated with declining malaria vector numbers and transmission (Centre for Disease Control 2007). In Eritrea, Shililu *et al*. (2003; 2007)

reported high *An. arabiensis* larval densities in streambed pools. In the current study, this habitat type was represented by the riverbed habitat class. Only one stream habitat (Jeppe's Rust) was surveyed here and this was characterised by having a steeper incline and faster flowing waters that fed into the main river channel (personal observation). This site yielded the fewest anophelines and the majority of larvae found here were sampled from outside the main stream channel where there was a decrease in the rate of flow. That increased volumes of water can lead to higher mortality in anophelines due to them being "flushed" out (Paaijmans *et al.* 2007*)* possibly explains the observations in the current study.

Figure 2.7 A residual pool left following the overflow of the river at Masibekela.

Observations on the larval breeding places of the *An. gambiae* complex mosquitoes reported here support the general opinion that favourable sites tend to be shorter lived compared to more permanent bodies such as wetlands (Patton 1905; De Meillon 1938; De Meillon 1951; le Sueur and Sharp 1988; Ndenga *et* al. 2012). Exposure to sunlight, either fully or partially, further characterises these breeding places. However, reports of occurrence in uncommon sites such as artificial containers (De Meillon 1947) and in heavily shaded sites for *An. gambiae s.l.* (Causey *et al*. 1942) have been made. Uncommon breeding places are likely to be utilised when suitable sites are not available or where an excessive number of larvae are found in a site. Minakawa *et al.* (1999) and Gimnig *et al.* (2002) have shown that for *An. gambiae s.s.* immatures, presence is most likely where the breeding site is small and the water is turbid, and where it is surrounded by emergent vegetation and algae are present. In the current study however, these factors were not able to discriminate for the productivity of breeding sites where larvae were found.

In addition to the environmental parameters, the proximity to human habitations in urban Dakar where demographic pressure is high, likely has a marked influence on *An. arabiensis* abundance (Robert *et al.* 1998). In the Mpumalanga region population density is comparatively low (~ 82 persons/ km^2, Statistics South Africa 2012), and *An. quadriannulatus* was most abundant where human dwellings were within 50 m of a breeding place. This was followed by *An. arabiensis*, unless where salinity was high. In western Kenya, although the spatial heterogeneity of larval habitats was found to be significant, no environmental parameters were found to determine the occurrence and abundance of the primary malaria vector in the region, *An. gambiae s.s.* (Minakawa *et al.* 1999). However, proximity of a larval breeding place to the

nearest house was found to be an important determinant of *An. gambiae s.s.* relative abundance. The role that distance of breeding sites to human habitations plays in the proliferation of *Anopheles* spp., has been highlighted elsewhere and these may act as notable refugia during the dry season (Minakawa *et al.* 2002; Girardin *et al.* 2004).

In the present study, *An. quadriannulatus* larvae were the most abundant of the *An. gambiae* complex when breeding sites were within 50 metres of a house. Occasional anthropophagic feeding activity by preferentially zoophagic *An. quadriannulatus* (Pates *et al.* 2001) may explain its dominance in these sites, however the proximity of domestic animals to human settlements here could also explain this observation. In all sites surveyed there was evidence of domestic animal activity and the members of the *An. gambiae* complex sampled in this area have shown plasticity in host feeding behaviour elsewhere (Pates *et al.* 2001; Pock-Tsy *et al.* 2003). The most abundant anopheline in the region was the mainly zoophilic *An. pretoriensis* suggesting that zoophily is favourable for proliferation of this species. Following this species in abundance terms was *An. arabiensis* regardless of proximity to human habitations or roads. Adult female biting preferences of *An. arabiensis* are known to show a tendency for feeding on both humans and animals (Tirados *et al.* 2006; Sinka *et al.* 2012). Thus the effect that the proximity to human habitations has on vector abundance may be less important as *An. arabiensis* abundance is independent of human habitations, an observation supported by Braack *et al.* (1994) who report a non-linear change in abundance of this vector as distance from breeding sites increases.

Studies on the mechanisms of osmoregulation have found that 5% of mosquito species are able to live in saline waters with the majority of species investigated favouring freshwater to brackish habitats (Bradley 1987). Several authors have investigated the halotolerance of *An. merus* and it is well known as a salt water breeder (Paterson 1964; Gillies and De Meillon 1968; White *et al.* 2013) and has been found to dominate field sites where salinity is between 30 and 50% seawater concentration (Mosha and Mutero 1982). To date, no field evidence has been reported for the occurrence of *An. merus* in water that is less than < 1 % seawater, although Coetzee *et al.* (1993) report the occurrence of *An. merus* larvae in a roadside pool near Skukuza, Kruger National Park, with salinity of 1.8% seawater. In the Matiovila and Mafayeni regions of the Kruger National Park, Munhenga *et al.* (2014) reported dominance of *An. merus* with a combined proportion of 98.6 % in these areas where breeding sites were moderately (3 – 10 g/ L) and highly (10 – 35 g/ L) saline, respectively. In two sites surveyed during the present study, Mzinti and Vlakbult, salinity is less than 1 g/ L and this species contributed 6% (n = 21) and 11% (n = 14) to the total proportion of *An. gambiae* complex members providing evidence for its ability to breed and survive, though at reduced numbers, under "freshwater" conditions. However, in the remaining three sites where water was reported as fresh, *An. merus* was absent. Under laboratory settings, Coetzee and le Sueur (1988) found that survival rates of *An. merus* larvae decreased from 46.4% in 25% sea water to 15.5% in distilled water, supporting dose-mortality response results reported elsewhere for larvae to varying salt concentrations (White *et al.* 2013). The phenotypic expression of salt tolerance occurs in response to exposure to salt conditions during different instar stages. Full expression occurs following hatching and decreases if exposed after 24 hr and 48 hr (White *et al.* 2013). Thus,

oviposition of eggs by adult females in fresh puddles may limit the success of *An. merus* larvae if the initial salinity dose is low. If these conditions are maintained i.e. by the influx of freshwater, the benefits of salt tolerance will be negated and the number of *An. merus* may decline. Mosha and Mutero (1982) propose that the decreasing numbers of larvae of *An. merus* observed in water that is increasingly fresh is in response to increased competition with other larvae as well as predation by organisms absent under saline conditions due to intolerance. Without the influx of freshwater from, for instance, rain, evaporation of water from the breeding site results in increasing salinity. The ability to osmoregulate and adapt the internal ionic concentration to the external salinity environment is hampered in species normally inhabiting freshwaters and when these mechanisms begin to fail, an increase in mortality is seen (Bacher and Garnham 1992; James *et al.* 2003). The effect this has on fitness has a marked impact on species diversity in increasingly saline waters, such that in highly saline sites *An. merus* dominates. It is therefore also likely that towards the end of the rains and when temperatures increase in the Mpumalanga region, leading to higher levels of evaporation, *An. merus* begins to dominate at sites that were previously recorded as slightly saline, outcompeting other anophelines.

The presence of *An. arabiensis* and *An. quadriannulatus* in the three sites where salinity was high, and the notable contribution these made to the species mix in Masibekela (n = 119 and n = 14 out of 287 *An. gambiae s.l.*) requires confirmation of whether these freshwater species can tolerate constant or intermittent dosages of salt water and thus can complete their non-adult stages when exposed to saline conditions. Coetzee and le Seuer (1988) report decreasing survival of *An. quadriannulatus* with increasing salinity, however, survival in freshwater was also low

for this species in the same study suggesting that laboratory conditions were not optimal for survival. Salinity tolerance in South African *An. rivolorum* has been reported while *An. funestus*, in the same study, showed negative responses to increasing concentrations of salt (Koekemoer *et al.* 2013). The southeast Asian species complex *An. sundaicus*, includes malaria vectors capable of breeding in both brackish and fresh water making them versatile in their breeding site requirements and contributing to malaria transmission over widespread areas (Sinka *et al.* 2011). The results reported in the current study suggest that *An. arabiensis* may be common even in areas where water is saline. Its continued presence under these conditions may therefore contribute to continued malaria transmission in an area, however, the degree of salinity is an important factor to consider and the moderate values reported here may serve to explain its presence in these sites. At higher salinity values the abundance of this vector species declines and *An. merus*, implicated in malaria transmission elsewhere, dominates. If one of the goals of control efforts was to target the potential vector *An. merus*, doing so at times when rainfall declines and evaporation is more significant, will be best for this species.

2.5.3. Insecticide resistance

Over a decade ago, Hargreaves *et al.* (2003) assessed the insecticide resistance status of *An. arabiensis* and *An. quadriannulatus* in northern Kwazulu-Natal, South Africa where DDT is used in IRS in traditional houses and Deltamethrin in western style houses. Both species showed resistance to DDT but not to Deltamethrin. The

current study assessed the status of resistance in adults from field-collected larvae from Mpumalanga using WHO bioassay kits, however the mechanisms of resistance were not elucidated as susceptibility was high. Data from this study indicates susceptibility to all the insecticides (DDT, Bendiocarb and Deltamethrin) tested with the exception of possible resistance to DDT in *An. merus* (97% mortality). Confidence in the interpretation of the results for the survival rates is good as all the exposures were conducted on 1 – 3 day old mosquitoes that were reared from larvae. It has been found that in adult *An. gambiae* older than five days, significant mortality is observed when exposed to discriminating WHO concentrations of DDT, affecting the interpretation of the results (Lines and Nassor 1991). The occurrence of possible resistance requires further investigation as the mortality rate was marginally lower than the threshold prescribed by the World Health Organization (fully susceptible = 98 - 100% mortality) (WHO 2013b).

Without an understanding of the local behavioural patterns of this species, it is difficult to suggest possible reasons for resistance. In localities where *An. merus* or other complex members are known to be largely exophilic in their resting behaviour, such as in Pemba, Tanzania (Odetoyinbo and Davidson 1968), the likelihood of exposure to insecticides indoors is very low and resistance selection pressure is more likely to have occurred at the larval stage as a result of the unwanted contamination of breeding sites with insecticides (Hargreaves *et al.* 2003). The resting behaviour of *An. merus* in the Mpumalanga area is unknown. If local populations showed endophilic resting behaviour, as was reported on the behaviour of this species in urban Dar-es-Salaam where 60% were reported to rest indoors (Muirhead-Thomson 1951), resistance may have arisen as a result of the

reintroduction of DDT for IRS in 2000. Behavioural plasticity in species' resting behaviours exists (Pates *et al.* 2001; Pock-Tsy *et al.* 2003) and has been documented in *An. arabiensis* in neighbouring Kwazulu-Natal (Sharp and le Sueur 1991). If *An. merus* is resting outdoors, which is more likely, then it will not be exposed to insecticides used in IRS and selection pressure for resistance is probably at the larval stage.

2.6. Conclusion

This study presents the results of the relative abundance and composition of anopheline species in late summer in the eastern Mpumalanga Lowveld. The physical and biological landscape is continuously changing and this snapshot provides insight into this highly dynamic system. To complement this research, longitudinal studies which cover all seasons of the year should be implemented. In addition, the study was conducted following rainfall in excess of the long term mean in the region (South African Sugar Research Institute 2014). Although the field work was carried out more than two weeks following the heaviest rainfalls, it is unclear whether the breeding sites that were surveyed are representative of those in years when rainfall was around the mean or lower. Variations in the habitat conditions assessed during this study may also occur and site selection by gravid females may be different in years when rainfall is less and the availability of breeding sites is altered either in physical or chemical structure. For instance, a change of salinity when drier conditions prevail has been reported by Mosha and Mutero (1982) which

was followed by a peak in the densities of *An. merus* when salinity increased to 50% seawater as the site dried up, with a concomitant decline in species favouring low to moderately saline waters.

The breeding site requirements for larvae of anopheline species recorded in this study are not easily delineated and as such the breeding sites are not limited to set ecological parameters. This finding is mirrored in larval ecology studies from elsewhere on the African continent, in both urban (Robert *et al.* 1998; Keating *et al.* 2004; Sattler *et al.* 2005) and rural settings (Minakawa *et al.* 1999; Minakawa *et al.* 2004; Ndenga *et al.* 2012). However, it is worth noting that breeding sites such as rain puddles, hoof prints and residual pools left over after rivers recede are examples of important breeding sites and these are characteristically short-lived, fully or partially sunlit and stagnant. Occurrence of *An. merus* in this study was found in association with relatively higher salinity levels and total dissolved solids (TDS). However, even when salinity and TDS values were relatively low, this species occurred in two of the sites surveyed albeit in much reduced numbers.

Enhancing our knowledge of the insecticide resistance status and ecology of breeding sites of anopheline species, especially malaria vectors such as *An. arabiensis*, in the Lowveld of Mpumalanga contributes important information to understanding malaria epidemiology in the region. Furthermore, this study provides a needed assessment of the resistance status of *An. gambiae* complex mosquitoes in the eastern Mpumalanga region. Investigating the ecological factors which determine anopheline composition and vector abundance provides malaria control programmes with information on their breeding sites and may assist with mapping malaria risk as well as informing control programmes on larviciding interventions. The vector, *An.*

arabiensis, in this area exploits a variety of breeding sites and occurred in all those surveyed here, regardless of ecological conditions such as elevated salinity. Thus, larval control operations should include all available breeding sites and focus efforts during the dry season when breeding places are discrete in the landscape and the extent of available breeding habitats is diminished. Elsewhere, dry season refugia of anopheline larvae are more often associated with man-made features surrounding human settlements and may be source sites of proliferation of anophelines when conditions are favourable (Minakawa *et al.* 2002; Shililu *et al.* 2003; 2007), thus identifying and targeting these sites is important in any malaria control programme.

CHAPTER THREE – COMPETITIVE BEHAVIOUR AND RESPONSE OF *ANOPHELES ARABIENSIS* AND *AN. QUADRIANNULATUS* UNDER DIFFERENT TEMPERATURE TREATMENTS

3.1. Introduction

In the north-eastern, sub-tropical region of South Africa the major vector of human malaria parasites - *Plasmodium falciparum* - is *Anopheles arabiensis* (White 1974; Braack *et al.* 1994; Govere *et al.* 2000). Occurring sympatrically with this species is the morphologically indistinguishable non-vector *An. quadriannulatus* and the minor vector *An. merus*, all three being members of the *An. gambiae* complex (Coetzee *et al.* 2000).

The larvae of these species favour transient pools exposed to sunlight (Gillies and Coetzee 1987; Gimnig *et al.* 2001) and are known to occur together in north-eastern South Africa (le Sueur and Sharp 1988), however the density of *An. merus* increases as conditions become more saline (Mosha and Mutero 1982; Kipyab *et al.* 2013; White *et al.* 2013; Munhenga *et al.* 2014). As a result of their co-existence in larval habitats, the immatures of these species will come into frequent contact and compete for space and resources (Schneider *et al.* 2000; Kirby and Lindsay 2009; Paaijmans *et al.* 2009b). Predatory organisms also interact with and exert an influence on the populations of mosquito larvae in these sites (Koenraadt and Takken 2003; Muiriri *et al.* 2013). Similarly, the influence that older instar larvae have on con- and hetero-specifics, through cannibalism and predation respectively,

significantly reduce survival in sibling species of the *An. gambiae* complex (Schneider *et al.* 2000; Koenraadt and Takken 2003). The results of these biological interactions as well as physical variables, such as temperature, on populations are important in mosquito ecology and may shift the competitive landscape in favour of one species over another, influencing species abundance and diversity (Juliano 2009).

Temperature affects many aspects of insect life parameters including adult body size, rate of development and survival from egg to adult (Russell 1986; Lyimo *et al.* 1992; Bayoh and Lindsay 2003), all of them important in mosquito population dynamics. In the absence of non-anopheline predators, inter and intra-specific competition at the immature stages has a strong influence on adult production (Schneider *et al.* 2000; Kirby and Lindsay 2009), which may be further regulated by environmental factors such as food availability and temperature. Few studies have investigated the effect temperature has on these species interactions in *Anopheles*. In one such study, when *An. gambiae s.s.* and *An. arabiensis* were reared under constant temperatures, the proportion of each species surviving to adulthood changed in the presence of the other species, regardless of density, with *An. gambiae s.s.* outcompeting its sibling species at 27°C (Schneider *et al.* 2000). Generally though, *An. arabiensis* larvae went on to pupate a day earlier than *An. gambiae* suggesting a more rapid development rate. In a different study, when immatures were reared under higher temperatures (> 30°C) the production of *An. arabiensis* adults was greater than *An. gambiae s.s.*, although overall production dropped as temperatures approached lethal limits (Kirby and Lindsay 2009).

The analyses of temperature effects on anopheline immature development and survivorship have largely been studied at mean temperatures (e.g. Craig *et al.* 1999) and under ideal laboratory conditions. Relying on extrapolations from these averages may be unrealistic and daily temperature dynamics, in addition to means, influence mosquito biology to a large degree (Paaijmans *et al.* 2009a; Lyons *et al.* 2013). Dynamic temperature ranges in the field are common across the malaria transmission landscape in Africa, where localities may experience diurnal changes in temperature from 5ºC to over 20°C (Minakawa *et al.* 2006; Pascual *et al.* 2009). Fluctuations around lower mean temperatures have been shown to speed up vector and parasite development (Paaijmans *et al.* 2009a) suggesting transmission scenarios can arise at temperatures lower than previously estimated (Mordecai *et al*. 2013). Understanding nonlinear biological responses to variable temperatures thus contributes important information to modelling malaria transmission. The relationship between fluctuating temperatures and *An. arabiensis* larval development and survival in the presence of a competitor remains unclear and information on the effects of temperature on the larvae of *An. quadriannulatus* is valuable in the light of competition with the vector. A better understanding of species interactions under fluctuating temperature conditions as well as constant temperatures is valuable as these conditions alter the development rates and survival of malaria vectors (Lyons *et al.* 2013) and results are more transferable to the situation in the field.

The outcome of interactions between immatures of the vector *An. arabiensis* and the non-vector *An. quadriannulatus* is relevant to vector control programmes as this determines the production and species composition of adults which can alter mosquito-borne disease epidemiology (Alto *et al.* 2005; Juliano and Lounibos 2005;

Bevins 2007; Alto *et al.* 2008; Beck-Johnson *et al.* 2013; Takken *et al.* 2013). Little is known about the thermal biology of *An. quadriannulatus* and, importantly, what effect its co-occurrence has on the life history of immature *An. arabiensis*. Models of malaria transmission predict an increase in the species range of the dominant vector, *An. arabiensis*, in the southern African region in response to a warming climate (Tonnang *et al.* 2010). Improving our knowledge of species interactions at the larval stage in response to increased temperature variability and temperature extremes contributes to our understanding of the current distributions and relative abundances of *An. arabiensis* and *An. quadriannulatus* in southern Africa. This also provides valuable data for forecasting future distribution changes in response to a warming climate (Githeko *et al.* 2000; Tonnang *et al.* 2010).

3.2 Objectives

The overall aim of this study was to determine the competitive outcome between *An. arabiensis* and *An. quadriannulatus*, under conditions of intra- and inter-specific competition at various temperature regimes.

Specific objectives

a. Determine whether community composition at the adult stage is regulated by competition under constant and fluctuating abiotic conditions, specifically temperature, at the larval stage.

b. Determine the effect of constant and fluctuating temperatures on the hatch rate, development rate and survival in single and mixed species scenarios.

3.3. Materials and methods

3.3.1. Mosquito colonies

Long-established colonies used in the experiments are housed in the Botha De Meillon Insectary at the Vector Control Reference Laboratory in Johannesburg, South Africa. The *An. quadriannulatus* colony, SANGWE, originated from Zimbabwe and has been reared in the laboratory since 1998, whilst the *An. arabiensis* colony, AMAL, originates from the Kruger National Park, South Africa and was established in 2009. The *An. quadriannulatus* colony used in this study represents the previously designated species A which occurs south of the Zambezi River in southern Africa, in contrast to species B from the Ethiopian highlands now known as *An. amharicus* (Coetzee 2004; Coetzee *et al.* 2013b). The insectary is kept at a constant temperature of around 25°C and relative humidity of approximately 80% with a photoperiod of 12 hours light to 12 hours dark and simulated dawn/dusk periods of 30 mins. Larval stages of all colonies are reared on a mixture of ground Beeno® dog biscuits and yeast extract, while adults are provided with a 10% sugar solution and blood fed twice weekly.

3.3.2. Experimental design

Adult cages of each colony were provided with a darkened petri dish serving as an egg plate and females were allowed to oviposit following two blood meals to ensure successful completion of the gonotrophic cycle (Clements 1963). The egg plates were left in the cages for six hours to minimise time between egg laying and the potential for variance in hatching. Between 40 and 50 eggs or larvae (comprising single species treatments, or a 1:1 split of both *An. arabiensis* and *An. quadriannulatus*) were introduced into containers with distilled water (25°C). Eggs were transferred by first collecting them onto filter paper and gently washing off into the experimental bowls to prevent damage to the chorion. For experiments beginning at the larval stage, recently hatched and unfed larvae that were less than 24 hours old of each species were transferred into containers using a pipette. Each temperature treatment consisted of five bowls for statistical purposes and to ensure a balanced design.

Larvae or eggs of the two species were placed into standard 500 mL bowls and either reared under fluctuating temperature regimes mimicking those most likely to exist under natural conditions (Gillies and Coetzee 1987) and which have been shown to be development thresholds in the laboratory (Kirby and Lindsay 2009; Lyons *et al.* 2013): (1) 20 – 30 °C and (2) 18 – 35 °C (Panasonic MIR-154 Cooled Incubator, Gunma, Japan); or at a constant temperature of 25°C in the insectary. Distilled water (the same temperature as the experimental treatment) was used to routinely wash eggs from the sides of the experimental bowls. The bowls were

marked so that the surface area was maintained at 175 cm^2, ensuring that the density of eggs/larvae was normalised across treatments. The replicates were also randomly assigned in the incubator and were switched around daily to avoid any temperature effects associated with placement in the incubator. Daily light:dark cycles in the incubator were set on a 12:12 hour regime cycling from 12am to 12pm as in the insectary. Temperatures were set to peak during the day light hours and lower temperatures set to occur during the dark cycle. Larvae in all treatments received optimal amounts of food according to their instar stage. Monitoring of egg hatch and emergence of adults was done at three intervals, six to eight hours apart depending on developmental stage. Adults were collected and killed using ethyl acetate, and the individuals stored in eppendorf tubes with silica for preservationand identification.

As the two species are morphologically identical, PCR techniques (Scott *et al.* 1993) were used to distinguish between adults from treatments where there was interspecific competition 9see Chapter 2.3.3.4 for detailed methodology). DNA from the leg or wing from the preserved adults was isolated following Scott *et al.*'s (1993) protocol in a Bio-Rad Thermal Cycler C1000 (Hercules, U.S.A.). DNA isolates of *An. arabiensis* (AMAL) and *An. quadriannulatus* (SANGWE) were used as controls.

3.3.3. Statistical analysis

Survivorship to the adult stage was measured as the proportion of adults that emerged from the initial number of eggs or larvae used in each treatment. Time to hatch of eggs and development rate analyses were conducted on the time it took for 50% of the eggs to hatch and 50% adult emergence, respectively. This approach effectively removed late egg hatch and adult emergence outliers. Rates were calculated as rate ($days^{-1}$) = 1/ (t to 50% life stage/24). No distinction could be made between the hatch times of the two species when in mixed species treatments as the larvae are morphologically identical.

Shapiro-Wilk's and Levene's tests were used to assess assumptions of normality and homogeneity of variances, respectively, in SPSS version 20 (IBM Inc., Chicago, U.S.A.). When both assumptions were met, a parametric ANOVA was conducted to test the effect of constant and fluctuating temperatures on mean time to hatch, survivorship and development rate in adult mosquitoes under scenarios of intra- and inter-specific competition. The significance level for statistical tests was set at an alpha level of 0.05. The Tukey HSD post-hoc test was used to determine where the differences in means lie following the ANOVA. In cases where the assumption of homogeneity of variances was rejected, a Welch test was employed using the Games-Howell post-hoc test for unequal variances (Field 2013).

3.7. Results

3.4.1. Hatch rate

The mean hatch rate measured as time in hours to 50% of the first-instar larvae in response to the different temperature treatments is presented in Fig. 3.1. As expected, temperature significantly affected time to 50% hatch ($F_{2,36}$ = 16.704, $p < 0.05$). When reared alone, the response to temperature treatments in hatch rate were similar between the two species (Fig. 3.1) and for both species was significantly faster at 25°C in intraspecific treatments (Tukey HSD, $p < 0.05$) (Fig. 3.1). In interspecific treatments there was no significant difference at 25°C (0.536 $days^{-1}$) in hatch rate compared to 18 - 35°C (0.556 $days^{-1}$). Eggs took slightly longer to hatch at 20 - 30°C (Tukey HSD, $p < 0.05$) (Fig. 3.1). The temperature treatment 20 - 30°C resulted in the longest hatch time in all treatments regardless of competitive scenario (Fig. 3.1). Under the mixed species treatments it was not possible to distinguish between the two different species at the immature stages, and for this reason, they were lumped together for analysis of hatch time purposes.

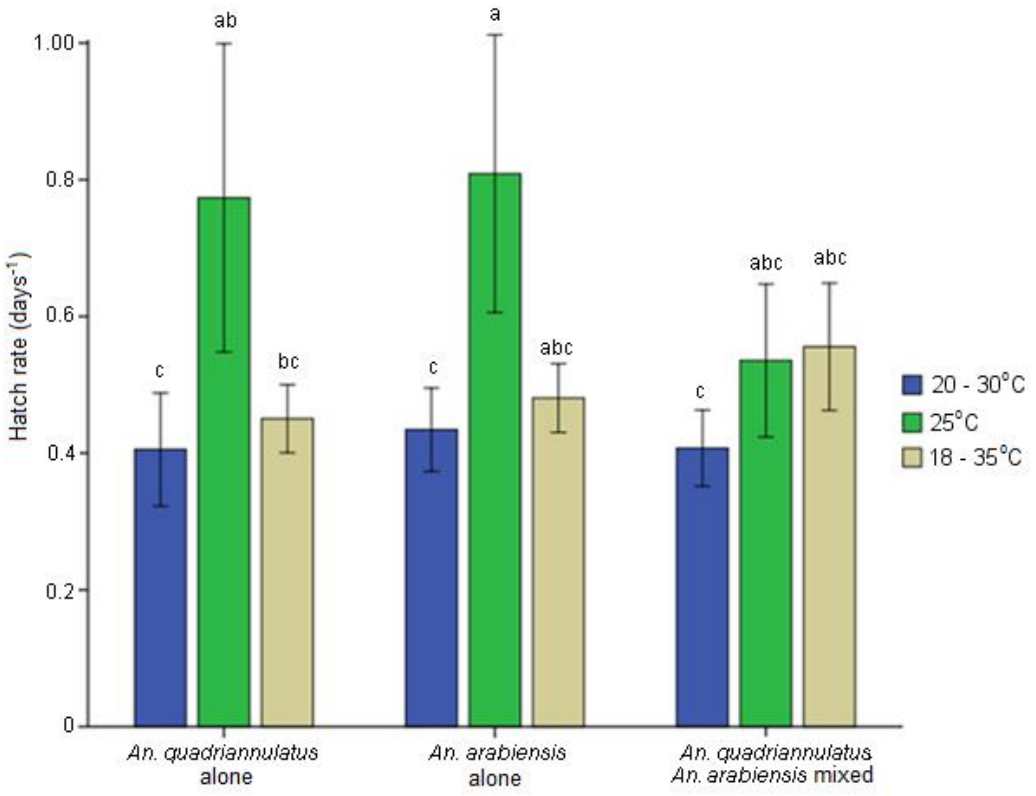

Figure 3.1 Hatch rate (50%) of the larvae (days^{-1}± 95% CI) in single species and mixed species treatments of *An. arabiensis* and *An. quadriannulatus* at the two fluctuating temperatures (20 - 30°C and 18 - 35°C) and a constant temperature of 25°C. Lower case letters indicate where significant differences in mean hatch rate for each group lie (Tukey HSD, $p < 0.05$). Both species developed fastest at 25°C in intraspecific, single species treatments.

3.4.2. Survivorship

3.4.2.1. Larval treatments

A comparison of the relationship between mean survivorship (%) and temperature from first-instar larvae to adult in each species treatment is presented in Fig. 3.2. There was a significant effect of temperature ($F_{2,48}$ = 35.122, $p < 0.05$) and species ($F_{3,48}$ = 6.650, $p < 0.05$) treatment on mean survivorship. There was a significant interaction effect of temperature and species treatment on survival from larvae to adults ($F_{6,48}$ = 43.966, $p < 0.05$). The effect of temperature on survivorship from egg to adults is thus dependent on the species treatment.

The temperature treatment 18 - 35°C resulted in a significantly lower survival for *An. quadriannulatus* and *An. arabiensis* when reared alone. *Anopheles quadriannulatus* survival at 18 - 35°C was below 50% in mixed species treatments (Tukey HSD, $p < 0.05$). Survival of *An. arabiensis* was also significantly reduced (55%) at 25°C (Tukey HSD, $p < 0.05$) in the presence of its heterospecific. *Anopheles quadriannulatus* survived as well in mixed species treatments as they did when in reared alone at 25°C.

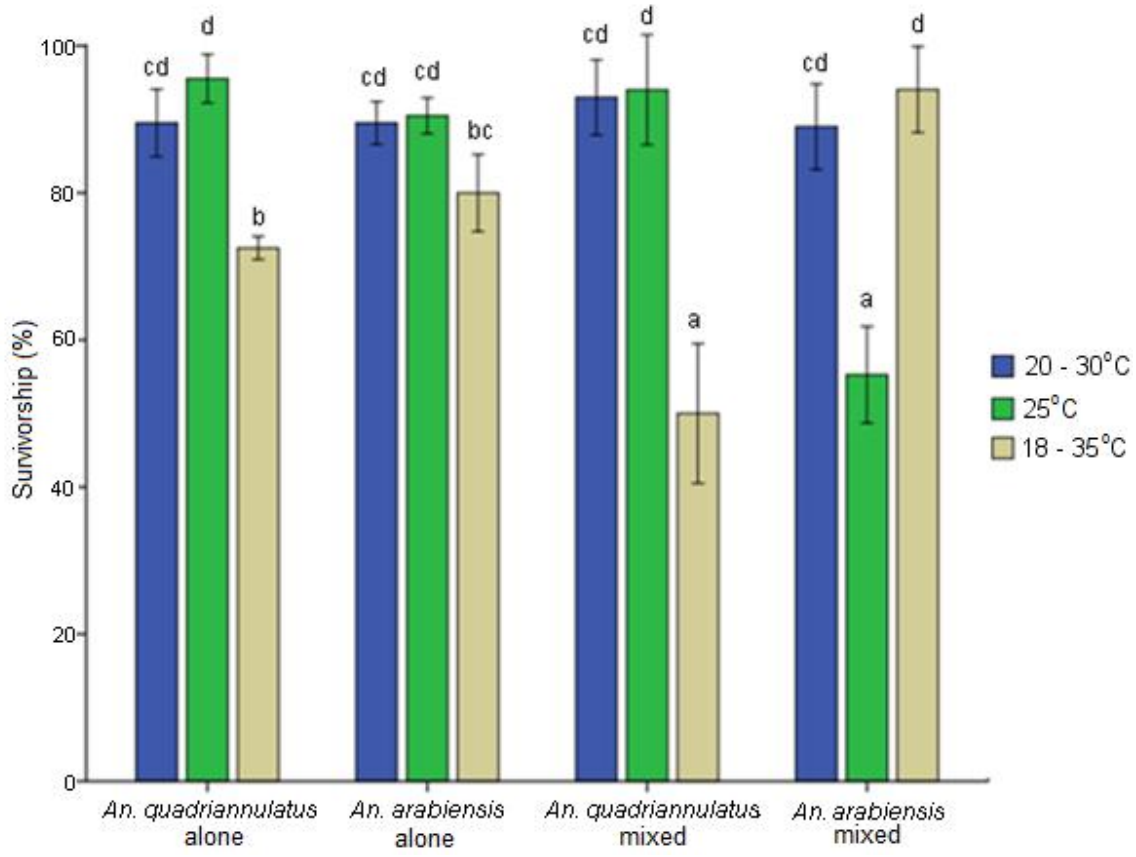

Figure 3.2 Survivorship (% ± 95% CI) of first-instars to adults in single species and mixed species treatments of *An. arabiensis* and *An. quadriannulatus* at the two fluctuating temperatures (20 - 30°C and 18 - 35°C) and a constant temperature of 25°C. Lower case letters indicate where significant differences in mean survival for each group lie (Tukey HSD, $p < 0.05$). *Anopheles arabiensis* tolerated higher temperatures better, and *An. quadriannulatus* survival was lower when in the presence of its sibling species at the more extreme temperature treatment.

3.4.2.2. Egg treatments

The mean survivorship from eggs to adult in each species treatment, expressed as a percentage, in response to temperature treatments is presented in Fig. 3.3. There was a significant effect of temperature ($F_{2,48}$ = 17.940, $p < 0.05$) and species ($F_{3,48}$ = 16.199, $p < 0.05$) treatment on mean survivorship from eggs to adults. A significant interaction was observed between the temperature and species treatment ($F_{6,48}$ = 22.047, $p < 0.05$) and survivorship. When *An. arabiensis* and *An. quadriannulatus* were reared alone the number of adults emerging from the initial cohort of eggs was lowered in the temperature treatment 18 - 35°C. In interspecific treatments, survival was higher for *An. arabiensis* at 18 - 35°C and for *An. quadriannulatus* at 20 - 30°C. Survival rates were similar for both species at 25°C in interspecific treatments when reared from egg to adult. Survival of *An. arabiensis* in mixed species treatments at 18 - 35°C and 20 - 30°C was similar.

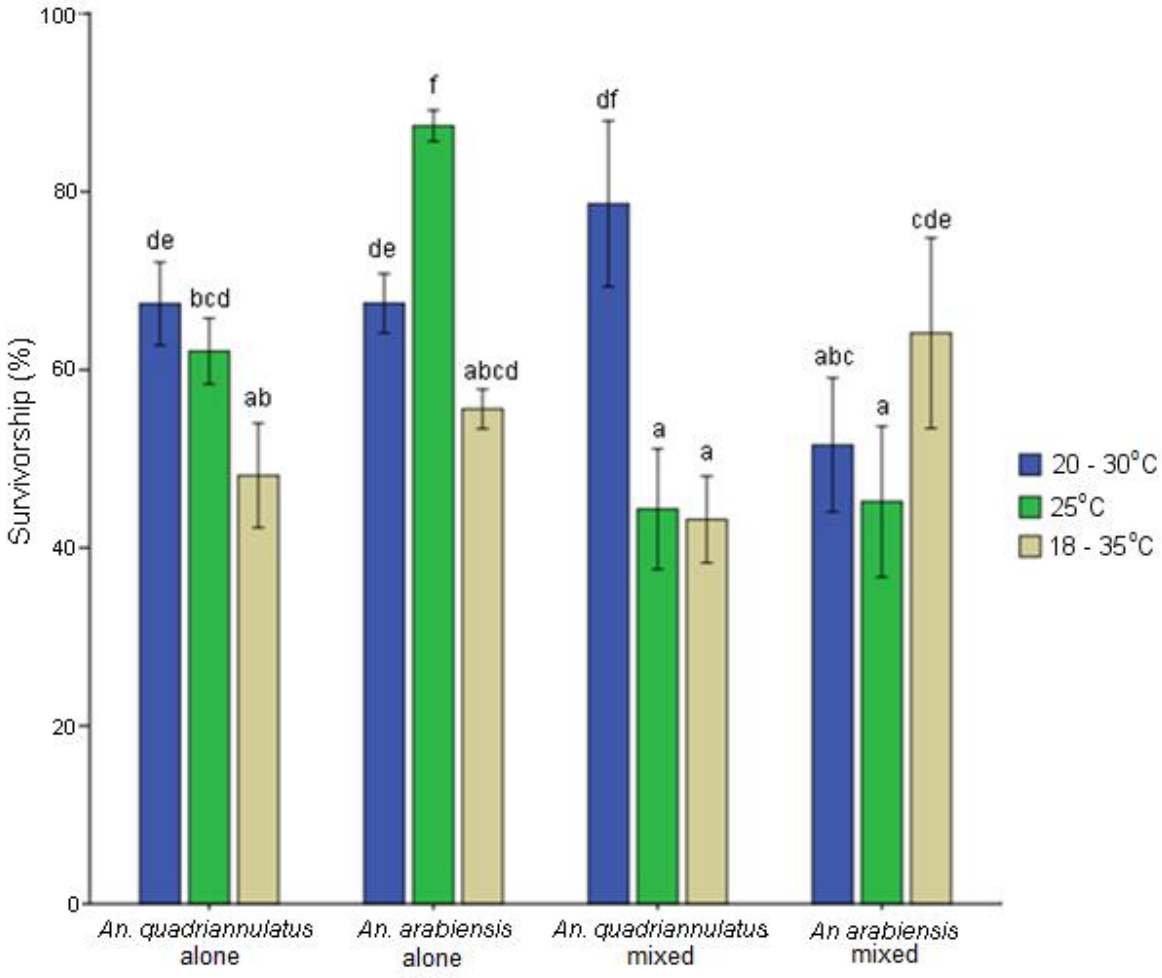

Figure 3.3 Survivorship (% ± 2S.E, 95% CI) of eggs to adults in single species and mixed species treatments of *An. arabiensis* and *An. quadriannulatus* at the two fluctuating temperatures (20 - 30°C and 18 - 35°C) and a constant temperature of 25°C. Differences in lower case letters indicate significant differences in survivorship for each group (Tukey HSD, $p < 0.05$).

3.4.3. Development rate

3.4.3.1. Larval treatments

A comparison of the development rate from first-instar larvae to 50% of the total adult production in each species treatment, expressed as a percentage, in response to different temperature treatments is presented in Fig. 3.4. A significant interaction was observed between the effects of temperature and species treatment ($F_{6,48}$ = 51.342, $p < 0.05$) and the effect of one main factor is not the same at different levels of the other factor. There was a significant main effect of temperature ($F_{2,48}$ = 181.181, $p < 0.05$) and species ($F_{3,48}$ = 25.097, $p < 0.05$) treatment on development rate from larvae to adults; however in some temperature treatments the development rate profiles were similar.

The temperature treatment had no effect on the development rate of *An. arabiensis* larvae to adult when reared in mixed species treatments. When in single species treatment, the mean outcome of *An. arabiensis* survival did not differ markedly between fluctuating temperatures but at the 25°C level the mean outcome was higher. Mean survival was highest when species were reared with their conspecifics and at a constant 25°C. The wider fluctuating temperature treatment (18 – 35°C) had the greatest effect on *An. quadriannulatus* survival, reducing it across species treatments and leading to a significant reduction in the development rate when in mixed with *An. arabiensis* (Tukey HSD, $p < 0.05$).

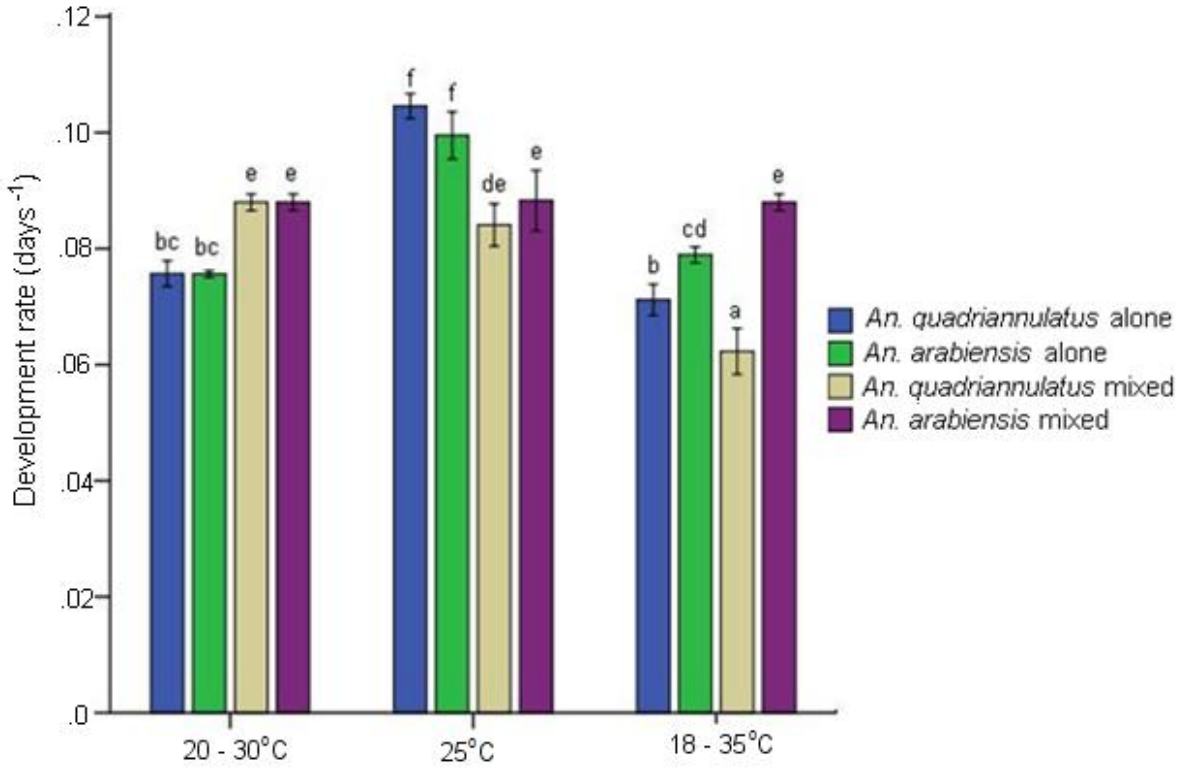

Figure 3.4 Development rate (days^{-1} ± 2SE, 95% CI) of first-instar larvae to 50% of the adult population in single species and mixed species treatments of *An. arabiensis* and *An. quadriannulatus*, at three temperature treatments (20 - 30°C, 25°C and 18 - 35°C). Differences in lower case letters indicate significant differences in survivorship for each group (Tukey HSD, $p < 0.05$).

3.4.3.2. Egg treatments

There was a significant difference in the development rate from eggs to 50% of the adult population in single and mixed species across temperature treatments (Welch

ANOVA $F_{11,18.683}$ = 13.653, p = 0.026) (Fig. 3.5). The development rate of *Anopheles arabiensis* in mixed species treatments was significantly faster and the adults emerged sooner across all temperature treatments. The development rate of *An. quadriannulatus* was slowest when reared alone at 25°C and 18 - 35°C (Tukey HSD, $p < 0.05$). Generally, development rates were slower, regardless of competitive scenario, at 18 - 35°C and at 20 - 30°C, while the mean development rates of each species was highest at a constant 25°C.

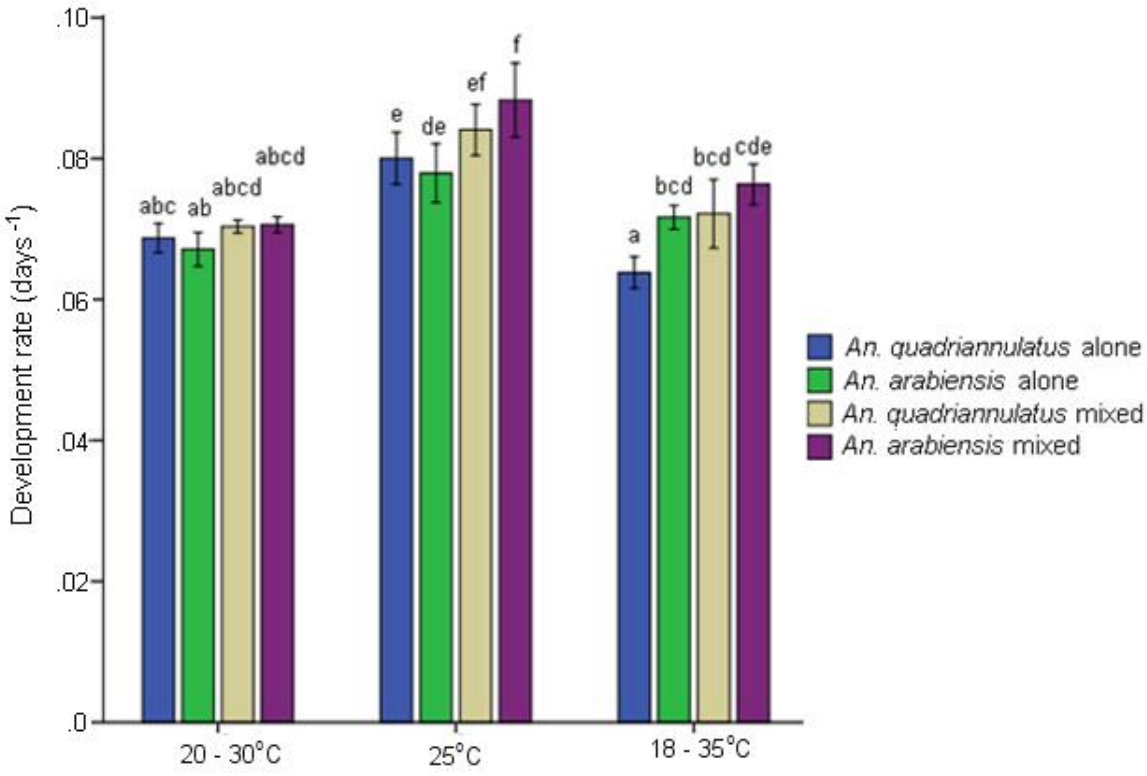

Figure 3.5 Development rate (days^{-1} ± 2SE, 95% CI) of the eggs that survived to adulthood (50% of the adult population) in single species and mixed species treatments of *An. arabiensis* and *An. quadriannulatus* at three temperature treatments (20 - 30°C, 25°C and 18 - 35°C). Differences in lower case letters indicate significant differences in survivorship for each group (Tukey HSD, $p < 0.05$).

3.8. Discussion

Comparing the hatch times, survivorship and development rates of immatures to adults of the vector, *An. arabiensis* (AMAL strain), and non-vector, *An.*

quadriannulatus (SANGWE strain), different responses to both fluctuating and constant temperatures in single and mixed species treatments were found. Importantly, these effects were evident when these species co-occurred, with responses in the variables measured being influenced by the presence of a heterospecific. Generally, overall survival of adults of both species was highest at 25°C and 20 - 30°C. The exception to this was lower survival in *An. arabiensis* which declined in the presence of *An. quadriannulatus*.

In larval treatments of a single species, fluctuating temperatures led to lowered survival rates, however this decrease was only significant for *An. quadriannulatus* at 18 - 35°C (18 – 25% decrease in survival). In mixed species treatments, *An. quadriannulatus* survival was decreased by as much 40% at 18 - 35°C compared to the other two temperature treatments. *Anopheles arabiensis* survival was significantly lowered in the presence of its heterospecific at 25°C, however, the two fluctuating temperatures appeared to have no significant effect on its survival in mixed species treatments. No other studies are available that consider the response of *An. quadriannulatus* to various water temperature regimes; however its sympatric occurrence with *An. arabiensis* over its distribution range (Gillies and Coetzee 1987; Coetzee *et* al. 2000) suggests that this species is likely able to tolerate similar conditions, and interspecific competition is likely to occur.

3.5.1. Hatch rate

The shorter hatch time found in treatments of a constant 25°C suggest that this is the optimal hatching temperature, which may be a possible artefact of these being long established laboratory colonies. The presence of a species' heterospecific prolongs the time to hatch. Although hatching typically took place within three days, some eggs only hatched after a week. However, the majority of the larvae that hatched did so within the first four to five days, a finding supported in *An. arabiensis* by Yaro *et al.* (2006) and Kaiser *et al.* (2010) in *An. gambiae s.s*. When these species occur with their conspecifics, the time to 50% hatch increases at 25°C. Prolonged time to hatch was also observed in treatments where temperatures fluctuated. These variations result in staggered hatching amongst egg batches which may be beneficial when the conditions at the breeding site are unpredictable, or when predators/pathogens are present that could be detrimental to the entire egg batch (Yaro *et al.* 2006; Kaiser *et al.* 2010).

The fluctuating temperatures more closely resemble field conditions and the increased time to 50% hatch suggests that temperature variation results in a longer time for eggs of *An. arabiensis* and *An. quadriannulatus* to hatch. Development of the larva in the egg may be prolonged as thermal fluctuations increase in magnitude, as described by Bayoh and Lindsay (2003) for the eggs of *An. gambiae s.s.* at temperatures above 28°C. Thus it would be expected that the greater temperature range, incorporating extremes, investigated here would have a greater effect on development and thus time to hatch. This was not observed in the current study

where fluctuations of 20 to 30°C and 18 to 35°C had similar time to hatch profiles. When eggs are exposed to more extreme temperatures for longer periods (relative to the temperature for the rest of the regime), development of the larvae inside the eggs might be constrained whereas exposure to brief periods of these same extremes would not be as detrimental (Impoinvil *et al.* 2007).

3.5.2. Survivorship

Survivorship values differed greatly in the current study, with 80 – 90% of the first-instar larvae of *An. arabiensis* surviving to adulthood across the temperature treatments and only falling below 60% when in mixed treatments at 25°C. Survivorship of *An. arabiensis* was highest when reared in mixed species treatments at 20 - 30°C and 18 - 35°C. Comparable results were reported by Kirby and Lindsay (2009) at a constant temperature of 35°C. At this temperature, survival of *An. arabiensis* was 20 – 24% higher than its heterospecific *An. gambiae s.s.* (Kirby and Lindsay 2009). However, overall survival was lower as this was near the temperature limit of the species where survival and development is drastically reduced (Lyons *et al.* 2013). Similar results for survival from eggs to adult as reported in the current study have been reported for *An. arabiensis* by Lyons *et al.* (2013). The high survival rate of larvae to adults in mixed species treatments of *An. arabiensis* (mean = 94%) and *An. quadriannulatus* (mean = 50%) at 18 - 35°C suggest the superior competitiveness of the former species over the latter species at more extreme temperatures. This observation reflects our current understanding of at least *An.*

arabiensis in Africa which is known to increase in abundance, relative to *An. gambiae s.s.*, during the months of the year with the highest maximum air temperatures and lower humidity (Lindsay *et al.* 1998).

A similar trend was observed when survival was measured from egg to adult (Fig. 3.4) with the rates comparable to those found by Schneider *et al.* (2000) and Kirby and Lindsay (2009) in studies on larval competition. At a constant 25°C, *An. quadriannulatus* is the superior competitor (mean = 94%) over *An. arabiensis* (mean = 55%) in mixed species, larval experiments. In comparison, survivorship was equal when reared at 25°C from eggs. Lower survival values were also reported in *An. arabiensis* single and mixed species (with *An. gambiae s.s.*) treatments at this constant temperature (Kirby and Lindsay 2009). At 20 - 30°C larval survival rates did not differ markedly across species treatments in this study. However, survival from egg to adult was higher in *An. quadriannulatus* than *An. arabiensis* at this temperature regime. Higher development rates have been reported in *Aedes* in the laboratory (Haufe and Burgess 1956) and in *An. arabiensis* and *An. gambiae s.s.* under semi-field conditions (Paaijmans *et al.* 2009b) with similar means to the current study. The density of larvae may also play a factor in the high larval survival rates observed in this study. Schneider *et al.* (2000) reported reduced survival when density increased from 0.5 – 1 larva/cm^2 to 2 larvae/cm^2 and competition for resources was higher. The comparatively low density of 0.25 larvae/cm^2 in the current study may translate to less competition and more food for each larva. This would favour nutrient accrual and growth, thus decreasing mortality directly or indirectly related to food deprivation.

The survival of individuals of these *Anopheles* species in the wild may be much reduced compared to rearing immatures under ideal laboratory conditions. Pathogens, predators and food availability are some of the factors affecting survivorship among wild populations (Service 1973, 1985). If space were a limiting factor we could expect cannibalism (in intraspecific treatments) and predation (in interspecific treatments) to both be factors that could result in a reduction in survivorship in the larval treatments (Koenraadt *et al.* 2004). The high survival rates reported here suggests that space was not a limiting factor. In species treatments in the current study, a mix of instar stages was often observed. Koenraadt and Takken (2003), report that the presence of fourth-instar larvae had a negative effect on survival of first-instars, regardless of food and space availability, suggesting cannibalism is an obligatory process in *An. arabiensis*. We found a 10% reduction in survivorship of *An. arabiensis* at 18 – 35°C in larval treatments when reared alone compared to in mixed treatments. Koenraadt and Takken (2003) reported as much as a 60% loss in the number of *An. arabiensis* larvae which was attributed to cannibalism. We also observed no significant reduction in survivorship in single species larval treatments at 25°C and 20 – 30°C in *An. quadriannulatus* and *An. arabiensis*. In mixed species larval treatments a loss of up to 40% of the larvae of *An. arabiensis* in the presence of *An. quadriannulatus* was observed at 25°C and vice versa at 18 – 35°C. These observations suggest that the loss of larvae in larval treatments, may be attributed to predation which occurred as a facultative process rather than a necessity to food deprivation. Koenraadt and Takken (2003) report *An. quadriannulatus* fourth-instars consuming *An. gambiae s.s.* first-instars regardless of food availability. This may be explained by increasing stress under more extreme

temperatures which require increased nutrient uptake not available through normal means. Predation on heterospecifics is likely to have taken place in mixed species larval treatments, with *An. quadriannulatus* reducing *An. arabiensis* survival at 25°C, and *An. arabiensis* reducing *An. quadriannulatus* survival at 18 – 35°C. Survivorship, regardless of species treatment (single vs. mixed species), in all instances was not 100%. Although mortality through failed pupation or eclosion was common, it did not account for all reductions in survival, and cannibalism likely played a role in single species treatments. The effects of predation and cannibalism do however require further investigation.

Where *An. arabiensis* and *An. quadriannulatus* were reared alone, the significant decline in survival rates at 18 – 35°C may also be attributed to increasing environmental stress on *An. arabiensis*, and likely *An. quadriannulatus*, as the temperatures that the larvae are exposed to for a significant proportion of the time are not favourable for survival (Lyons *et al.* 2013). Temperature variations in the small, often temporary, water bodies in which the larvae of these species exist (Gillies and Coetzee 1987; Gimnig *et al*. 2001), may show significant temporal variation and are generally not stratified by depth (Paaijmans *et al.* 2008). Exposure to extreme temperatures in species that inhabit temporary, small sites is common. As such, tolerance of higher temperatures compared to anopheline species that occupy larger, more permanent sites, such as *An. funestus*, is important (Lyons *et al.* 2012). At this temperature regime, deaths were increasingly common when fourth-instar larvae pupated as well as in failed eclosion of pupae into adults (pers. obs.), an observation supported by Bayoh and Lindsay (2003) in *An. gambiae s.s.* above 30°C. One explanation for the failed metamorphosis to pupae and then to adult is

that raised temperatures heighten the development rate (Bayoh and Lindsay 2003; Lyons *et al.* 2013) demanding more rapid uptake of nutrients and a quicker metabolism (Korochkina *et al.* 1997), requirements which may be physiologically demanding, resulting in insufficient mass being accumulated for eclosion (Chambers and Klowden 1990; Lassiter *et al.*1995; Agnew *et al.* 2000).

3.5.3. Development rate

We have demonstrated that under different temperature scenarios, interspecific and intraspecific competitive interactions between *An. arabiensis* and *An. quadriannulatus* larvae alter development rates and thus the time to adult emergence. The development rates at 25°C and 20 - 30°C were significantly different between species when reared alone and in mixed species from larvae and eggs. Earlier emergence was reported at 25°C and at 20 - 30°C in single species treatments. No difference in development rate was seen between species in mixed treatments. The effect of temperature was more pronounced at 18 - 35°C with *An. arabiensis* developing faster under both competitive scenarios and *An. quadriannulatus* slower, especially when in the presence of its competitor. When reared from eggs, development rates at the two fluctuating temperatures were generally similar.

Kirby and Lindsay (2009) and Paaijmans *et al.* (2009b) report notable differences in time to eclosion between *An. arabiensis* and *An. gambiae s.s.* under competition and different water temperatures, with the latter species consistently emerging sooner.

These findings have been attributed to the larger adult size of *An. arabiensis* which requires greater mass accumulation and thus a longer time spent acquiring resources as immatures (Schneider *et al.* 2000; Kirby and Lindsay 2009). The comparable development rates in the current study, suggest the requirements for growth are similar for each species under the constant 25°C and the fluctuating 20 - 30°C temperature regimes. At 18 - 35°C, environmental temperature may have a significant stress effect on the time to adult emergence especially for *An. quadriannulatus* regardless of the presence of the competitor species studied here. The observation in *An. arabiensis* that there is no significant negative effect of extreme temperatures on development time (Lyons *et al.* 2012) likely explains the occurrence of this species in drier and warmer conditions, such as northern Botswana, where *An. quadriannulatus* is absent (Coetzee 2004).

Habitat sharing by the larvae of these two species would not be detrimental on the development rate of either at moderate temperature conditions, and in fact, may benefit immatures of each as they emerge sooner compared to when reared alone, avoiding the risk that the aquatic habitat will dry up. However, as adults tend to be smaller when emerging sooner (Lyimo *et al.* 1992) their fitness as adults is diminished (Ameneshewa and Service 1996). As the temperature difference increased and became more extreme, approaching lethal limits (Lyons *et al.* 2012), *An. arabiensis* displayed a faster development rate in single and mixed species treatments. The potential advantages for mosquito larvae to developing faster is an earlier release from threats from competitors, predators and pathogens (Service 1985) and potential loss of habitat through habitat flushing from excessive rainfall (Paajmans *et al.* 2007) or drying up (Berrigan and Charnov 1994). In addition, the

effects of competition on development rate in *An. arabiensis* and *An. gambiae s.s.* are prominent where density is a factor. Schneider *et al.* (2000) and Gimnig *et al.* (2002) showed that when the density of larvae increased, development times also increased, with the effect more apparent in mixed species treatment. Longer development times as immatures at higher densities may be an advantage as the larvae survive up to a point where competition is diminished. However, the increased risk of mortality through predation and cannabilism increases (Schneider *et al.* 2000). The current study was designed so that density was not a factor.

3.6. Conclusion

The effects of competition on development rates and survival may only be apparent in the sibling species investigated here when temperatures fluctuate between extremes. *Anopheles arabiensis* will reach adulthood sooner in single and mixed species habitats at higher temperatures. Interference competition, leading to insufficient nutrient acquisition, and predation are possible causes of reduced survival and slow development of *An. quadriannulatus* at high temperatures (Chambers and Klowden 1990; Lassiter *et al.*1995; Schneider *et al.* 2000). Interestingly, the more rapid development of *An. arabiensis* at high temperatures did not lead to reduced adult emergence either through failed pupation or eclosion, as has been hypothesised by Chambers and Klowden (1990) in *Aedes* and used to explain similar observations in *An. gambiae s.s.* by Bayoh and Lindsay (2003). At a constant 25°C, *An. quadriannulatus* appears to exert a predatory effect on *An.*

arabiensis survival but not development rate. Predatory behaviour by *An. quadriannulatus* is reported by Koenraadt and Takken (2003) as fourth-instars were observed to prey on *An. gambiae s.s.* first-instar larvae. A mix of different instar stages in a treatment may thus result in predation by one species and this appears to be a facultative process in *An. quadriannulatus,* dependent on temperature.

The current distributions and relative abundances of *An. arabiensis* and *An. quadriannulatus* in southern Africa are unlikely to be influenced by changing species interactions in response to increased temperature variability and temperature extremes associated with a warming climate (Githeko *et al.* 2000; Tonnang *et al.* 2010). If high temperature extremes, such as the 18 - 35°C investigated here, were to become commonplace and extend over a number of weeks, the increased survival and negative effect *An. arabiensis* has on *An. quadriannulatus* where these two occupy the same breeding sites, would favour the former's survival and could alter the vector borne disease burden. Survivorship of both species at 20 - 30°C was not significantly impacted and the adult production was high across species treatments. However, the complexities of the effects of changing local conditions, such as temperature, on vector abundance do not only extend to the interactions between these two sympatric species and are an interplay between the influences of the human and ecological settings.

CHAPTER FOUR – GENERAL CONCLUSION

Prior to the advent of wide scale DDT usage during the 1940s, larval source management played a pivotal role in the elimination and eradication of malaria vectors in certain areas. Subsequently, vector control interventions targeting the adult vectors of malaria gained increased popularity and came to the fore as the primary method made use of in an attempt to eliminate and eradicate anopheline species which transmit malaria. According to the WHO, vector control interventions, such as indoor residual spraying, have led to a significant decline in global malaria morbidity and mortality (WHO 2014). In saying so, the rising costs and logistical constraints experienced with expanding adult vector management, as well as increasing resistance to the insecticides used, supplementary methods such as larval control (seeing a re-emergence) have been incorporated into control programmes as outlined in the WHO Global Framework on Integrated Vector Management (2004).

Research findings in malaria vector control explicitly state the need to understand anopheline ecology which has been, and will continue to be, an essential prerequisite to any malaria vector control programme (Ferguson *et al.* 2010). Insofar as larval control is concerned, a lack of knowledge of local larval bionomics has the potential to hinder efforts to reduce vector numbers and, accordingly, the ultimate aim to eradicate and eliminate malaria vectors. The consensus amongst larval ecologists is that the habitat requirements differ among anopheline species and that an understanding of the influence of environmental conditions on vector distribution

aids control programmes, especially in respect of targeted larval control. Well-established examples of differential habitats include the major African malaria vector *Anopheles funestus*, known to favour more permanent water bodies, as well as members of the *An. gambiae* complex which are known to exploit breeding sites with a diverse set of characteristics, being largely those of a transient nature (De Meillon 1947; 1951; Sinka *et al.* 2012).

Malaria is endemic in north-eastern Limpopo, eastern Mpumalanga and northern Kwazulu-Natal provinces in South Africa and transmission is low risk, largely occurring during the wet summer months when conditions are more conducive for development of the vectors as well as the parasites (Maharaj *et al.* 2013). The main vector in the aforementioned areas is *An. arabiensis* of the *An. gambiae* complex, co-occurring with it is the sibling species *An. merus* and *An. quadriannulatus* (Coetzee *et al*. 2000). Having alluded to the habitual requirements and environmental conditions above, it follows that the efficacy of larval source management and larviciding is dependent on knowledge of the larval ecology in a given area, as well as the levels of insecticide resistance in the local populations of mosquitoes.

This thesis investigated the local larval bionomics in eastern Mpumalanga province as well as their spatial distribution; the associated insecticide resistance of *An. gambiae* complex members; and, in a controlled environment, competition between two closely related complex members, namely, *An. arabiensis* and *An. quadriannulatus*. Broadly, the following research hypotheses were explored:

1. Larvae of anopheline vectors and non-vectors in Mpumalanga associate with certain, determinable conditions in the breeding site:

Having conducted field collections of larvae and assessed associated breeding site parameters, it was found that there is a heterogeneous distribution of anophelines across aquatic habitats in eastern Mpumalanga with both fresh and saline waters being exploited. In addition, *Anopheles* species composition and abundance was influenced by small-scale variation in the environmental characteristics: salinity and total dissolved solids. No definitive environmental conditions could, however, be ascertained to describe presence of the notable vector in the region, *An. arabiensis*. *Anopheles quadriannulatus* was found with the major vector in all breeding sites surveyed. The similarities in breeding site preference between these two species suggest that where *An. quadriannulatus* is found, *An. arabiensis* is likely to occur. Where collections from larval habitats yield *An. quadriannulatus*, control programme operators should monitor the breeding site closely to establish the abundance of the vector.

Where salinity and the levels of total dissolved solids are higher, *An. merus* tended to dominate the species mix. At minimum, this finding in relation to salinity supports the known habitat preference of *An. merus* larvae (Kipyab *et al.* 2013; Munhenga *et al*. 2014). The presence of this species in freshwater field sites, where salinity is less than 1% seawater, adds to the existing body of knowledge relating to its larval bionomics (Coetzee *et al.* 1993). In addition, findings from the field study reported in this thesis suggest that *An. arabiensis* and *An. quadriannulatus* can tolerate constant or intermittent dosages of salt water and thus, can complete their non-adult stages when exposed to saline conditions. Coetzee and le Seuer (1988) have reported low

survivorship of *An. quadriannulatus* in increasingly saline waters in a laboratory setting. Tolerance levels of *An. arabiensis* require further confirmation and laboratory studies will contribute to our understanding of the ecology of this species.

2. There is no insecticide resistance present in populations of members of the *Anopheles gambiae* complex in Mpumalanga:

Following the WHO (2013) protocol, insecticide susceptibility bioassays conducted on members of the *An. gambiae* complex collected during the field study indicate potential low level resistance to DDT in *An. merus,* a secondary vector in neighbouring Mozambique (Cuamba and Mendis 2009). Furthermore, the dominant vector, *An. arabiensis*, showed continued susceptibility to the three classes of insecticide tested. This information contributes to the growing knowledge of insecticide susceptibility tests relating to the aforementioned species and is essential for continued susceptibility monitoring, especially so considering the unprecedented worldwide spread of insecticide resistance in *Anopheles* species (Ranson *et al.* 2009). These data add to the short-term pillar of the Global Plan for Insecticide Resistance Management in Malaria Vectors by monitoring resistance and preserving susceptibility (WHO 2012). The findings of this study support the continued use of the insecticides employed for Indoor Residual Spraying in the region.

3. Under different temperature conditions, there is no difference in the outcome of intra-specific and inter-specific competition in the larvae of *An. arabiensis* and *An. quadriannulatus*:

Conditions in the breeding site are known to influence the species present. Furthermore, biotic interactions have an important effect on species survival and

development in mosquito populations. The outcome of competition between the two sympatric species investigated in the study at hand, under conditions that mimic field conditions in one variable, temperature, has implications for the vector-borne disease burden in an area. For this reason, the effect of constant and fluctuating temperatures on the development rate and survival of larvae of the dominant vector in the region, *An. arabiensis*, and its co-occurring sibling species, *An. quadriannulatus* was investigated in this study. The treatments conducted in this study were carried out under scenarios of interspecific and intraspecific competition, resulting in the ability to test whether or not community composition of anophelines at the adult stage was regulated by conditions at the larval stage.

Constant and fluctuating temperatures were found to affect the immatures of the vector, *An. arabiensis* (AMAL strain), and non-vector, *An. quadriannulatus* (SANGWE strain) in this study. Survival of both species in larval treatments was highest at 25°C and 20°C - 30°C and lowest for *An. quadriannulatus* at 18°C - 35°C. Presence of a competitor species had an influence in the response variable measured, with *An. arabiensis* survival significantly reduced at 25°C in the presence of *An. quadriannulatus*, and *An. quadriannulatus* survival significantly reduced at 18°C - 35°C in the presence of *An. arabiensis*. As the temperature difference increased and became more extreme, approaching higher limits (Lyons *et al.* 2012), *An. arabiensis* displayed a faster development rate in single and mixed species treatments. Reduced survival and slow development of *An. quadriannulatus* under the higher fluctuating temperature regime investigated here, are likely a result of interference competition, leading to insufficient nutrient acquisition, and/ or predation (Chambers and Klowden 1990; Lassiter *et al.*1995; Schneider *et al.* 2000).

Anopheles arabiensis survival was not affected in the same way as *An. quadriannulatus* by the higher fluctuating temperature treatment, suggesting it is able to tolerate greater fluctuating temperatures than its sibling species in this study.

In instances where the two species occurred together the faster development time recorded, and thus earlier emergence of adults, may benefit both species. In the field, other biotic interactions, such as with predatory organisms and exposure to pathogens, will have a negative effect on larval survival (for example, Service 1977; 1993). Earlier emergence of adults could be beneficial as the immatures are released from these risks at an earlier stage. Fluctuating temperatures between 20°C - 30°C are unlikely to significantly influence species composition where these two species occur alone or together. When the temperature fluctuates between 18°C - 35°C, however, the number of emerging adults of *An. quadriannulatus* was lower when reared alone and when in the presence of *An. arabiensis*.

The information gathered here is crucial in understanding the drivers of vector abundance and composition and as such, can be used by the malaria control programme in the Mpumalanga province to identify key breeding sites, with known vector densities, for larval control purposes. In addition, this information can contribute to hotspot mapping of transmission zones wherein vectors make up the greater part of the species composition and/or such vectors are most abundant. This study found that the major vector in the region, *An. arabiensis*, was abundant in Mzinti, Masibekela and Magudu. Moreover, it was found that the minor vector, *An. merus*, was the dominant species at both Block A sites, and in Masibekela. Accordingly it is suggested that based on the results of this study, these productive sites, as well as the surrounding malaria incidence, should be monitored by larval

control programmes in the region. In saying so, by virtue of the wide occurrence of the vector *An. arabiensis* and the minor vector *An. merus* it follows that the control programme should continue to focus their efforts on all available breeding sites. Habitat drying during the dry, winter months offers control programmes an opportunity to target over-wintering refugia of the anopheline vectors in the region.

The niches of species are a complex interplay of both physical and biological variables. Physiological tolerance of mean monthly temperatures, humidity, rainfall and other physical variables contribute to determining a species' fundamental niche (Kearney *et al.* 2009). In creating spatial distribution models, environmental data are important inputs which link to species distribution (Elith and Leathwick 2009). The narrow and widely fluctuating temperature regimes studied herein and the influence of such on the larvae of two members of the *An. gambiae* complex provides additional species data for distribution mapping and predictions of vector occurrence. In addition, the outcome of biological interactions under different temperature regimes was considered. Although the outcome of these species interactions may not be range limiting processes, they may well have an effect on vector abundance, as physical conditions in a breeding site favour the production of one species over another (Juliano 2009). The results of this study show superior competitiveness in the malaria vector, *An. arabiensis*, over its sibling non-vector species *An. quadriannulatus*, at widely fluctuating temperature regimes (18°C - 35°C). Increases in mean temperatures under various climate change scenarios have been predicted across the African continent and in southern Africa (Tonnang *et al.* 2010). These increases may promote the conditions favourable to vector productivity. In addition,

these data provide important biological information which can be incorporated into malaria risk assessments.

The surveyed anopheline breeding habitats in the Mpumalanga region are discrete compared to other regions such as the extensive flood plains of rivers in the Gambia (Majambere *et al.* 2008). As such, larviciding offers an effective tool for the well-defined larval breeding sites in Mpumalanga. It is suggested that the findings reported herein may be of interest to the malaria control programme in Mpumalanga and may further contribute to our knowledge on the larval bionomics of anophelines. Future research may wish to consider the change in vector composition and abundance in each site over time as environmental characteristics may change seasonally and correlate with a change in species composition. Information arising from future research such as this will further enhance the effectiveness of larval control operations as it would allow for the identification of peaks in vector productivity. Furthermore, this will also contribute to the knowledge on vector demographics for *An. gambiae s.l.* in South Africa as well as the rest of Africa where they can be found.

Differences in breeding habitat preferences between Africa's major malaria vectors exist across the continent. The information gathered herein contributes to the growing literature on the larval bionomics of the vector, *An. arabiensis*, in the Mpumalanga region and is by no means exhaustive. There is potential for further research to be conducted to understand the influence of, amongst other things, biological interactions such as food source and availability, the effect thereof on competition between species and the manner in which this may affect species composition. It is important to note at this juncture that these effects are context

dependent and that smaller, transient pools may have higher rates of competition compared to larger water bodies where predators may have a greater impact on community organisation of adult *Anopheles* species (Juliano 2009). Moreover, investigations on larval interactions may wish to determine the influence these conditions have on malaria epidemiology by studying transmission probabilities and other characteristics of anopheline vectors such as adult body size. Generally, more field experiments are required to investigate the effects of competition and how this changes under different conditions. While such recommendations for future research are admittedly broad, the findings of this study nonetheless give us an insight into the outcome of biotic interactions between closely related species, important in community ecology terms, and vital for understanding vector-borne disease epidemiology in a given region.

CHAPTER FIVE – REFERENCES

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