The effects of low temperatures on metabolic rates in *Anopheles quadriannulatus*

Duduzile K. Ngwenya

Student Number: 937185

Supervisor: Professor Frances Duncan

A Research submitted to the Faculty of Science, University of the Witwatersrand,

Johannesburg,in partial fulfilment of the requirements for the degree of Master of Science in

Environmental Science.

19 February 2018

Abstract

The effects of low temperatures on the metabolic rates of *Anopheles quadriannulatus* were investigated by measuring the rate of carbon dioxide emission (VCO_2) at different temperatures using a closed respirometry system. In general, the metabolic rates of *A. quardiannulatus* decreased with decreasing ambient temperatures. However, the decreases were not uniform over the temperature range of 30 °C and 10 °C. Mean VCO_2 measured at 30 °C and sometimes at 25 °C, were statistically different from those measured at other temperatures. Surprisingly, at 15 °C and 10 °C, the mean VCO_2 were statistically similar. It was found that metabolic rates of the mosquitoes were affected differently if a 5 °C reduction in temperature occurred at 30 °C and when it occurred at 15 °C. Thus, a 5 °C drop in temperature at 30 °C and 15 °C did not yield similar percentage decrease in metabolic rates. Age was found not to impact on the metabolism of mosquitoes except at 10 °C and 15 °C in blood fed mosquitoes (both mated and unmated). Lack of a correlation between age and metabolic rates in these mosquitoes was a result of constant body masses as mosquitoes aged. Mean VCO_2 of four days old mosquitoes in different mating and feeding states were found to be significantly differently only at 10 °C. Mated and blood fed mosquitoes from this age group retained constant metabolic rates despite changes in temperature. The finding that *A. quadriannulatus* is not capable of significantly dropping its metabolic rates at low temperatures (15 °C-10 °C) is an indication that the species most probably lacks a physiological overwintering mechanism. Consequently, its survival and longevity is highly compromised during winter. Thus, it can be concluded that if adult *A. quadriannulatus* can overwinter in cooler parts of sub-Saharan regions, it does so using non-physiological mechanisms. Otherwise, this species and related members of *Anopheles gambiae* overwinters in developmental
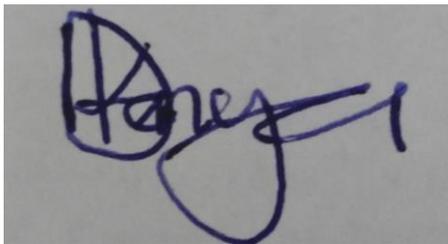
stages other than adulthood. In this way, these mosquitoes are able perpetuate their populations post winter season.

Declaration

I, Duduzile K Ngwenya declares that apart from the contributions mentioned in the acknowledgements, this work is entirely my own. It has not been previously presented for examination at any other institution for any other qualification or conference. I have referenced other people's work referred in the report.

Signed on this day 19 of February 2018 at University of Witwatersrand

Signature …………………………………………………………………

Date ……19 February 2018…………………………………..

Acknowledgement

Table of contents

List of Tables

List of Figures

x

Definition of terms / Abbreviations

NICD	National Institute for Communicable Diseases
CO_2	carbon dioxide
VCO_2	rate of carbon dioxide emission
$nl.min^{-1} mg$	nanolitres of carbon dioxide per minute per milligram
ppm	parts per million
DDE	Dependent Delayed Differential Equations
PCR	Polymerase Chain Reactions
KZN	KwaZulu Natal
NRF	National Research Foundation
PMA	Postgraduate Merit Award
TW	Transmission Window
WNV	West Nile Virus

xi

Chapter 1

1.0   Introduction

Historically, vector-borne diseases like malaria and yellow fever were responsible for more human disease and death in the 17th through the early 20th centuries than all other causes combined (Gubler and Casta-Valez, 1992). Malaria, a disease transmitted by mosquitoes, is responsible for more deaths and sickness than any other vector-borne disease (Gubler, 1998). This makes mosquito population dynamics an interesting and important subject within the medical community as well as for policy administrators.

It was first discovered that mosquitoes transmit diseases to humans in 1877 (Coetzee *et al.*, 2013). Ever since then, medical entomologists have been studying the life cycle of the mosquito, hence its once cryptic life cycle is now well documented (Gubler, 1998). Though notorious for vectoring diseases like malaria, dengue fever and West Nile Virus (WNV), mosquitoes have other useful roles in ecosystems. For example, they feed on nectar and in doing so they help in flower pollination (Russell *et al*., 2013) and they also make up part of the diet of different bat species (Russell *et al*., 2013).

The ability of mosquitoes to thrive in different climatic conditions is a key factor for their success as a species. They are extensively distributed, occurring in all ecosystems except in permanently frozen areas (Capinera, 2008). Their large geographical range has seen them being responsible for death and sickness in all continents except Antarctica (Capinera, 2008). This ecological success is facilitated by the ability of their various developmental stages to inhabit different environmental niches. The egg, larvae and pupal stages are confined to stable aquatic habitats whilst the adult stage is terrestrial. This separation of niches reduces intra-specific

competition for ecological resources, ensuring greater chances of success for different developmental stages (Lehmann and Diabate, 2008).

Even though mosquitoes are ubiquitous, the exact species composition, abundance and diversity vary across different geographical areas. Seventy-five percent of mosquito populations occur in tropical and subtropical areas where some species transmit diseases such as malaria and dengue fever (Capinera, 2008; Gillett, 1971). The remainder of the mosquito populations occurs in temperate and colder regions such as the northern hemisphere and African highlands. For example, *Culex pipiens* and *Culex tarsalis* are the dominant vectors of the WNV in North America (Gray, 2013). *Anopheles quadrimaculatus* was once a principal vector for human malaria in North America. Despite the successful eradication of malaria in that region, *A. quadrimaculatus* remains a major pest and potential malarial vector in the region (Wallace and Merritt, 1999).

The global health hazards associated with mosquitoes are of interest to medical entomologists and hence the need to understand the biology, distribution and population dynamics of the associated vectors (Capinera, 2008). Studies have shown that on a local level, the long-term perpetuation and efficacy of mosquitoes as pathogen vectors is dependent on external factors such as climate, with temperature and humidity being the most important (Gillett, 1971). Global climate change is expected to result in the increase of average temperatures and rainfall; hence some areas may get wetter and warmer than they are currently (Thomson, 2010). It has been modelled that these changes will result in extension of the areas endemic to most disease vectors, including mosquitoes. Boundaries and surrounds of endemic areas are likely to be invaded by the vectors. Thus some areas currently free of mosquitoes and associated diseases will get infested by the vector if predictions are true since the ecological success of mosquitoes is positively correlated

with temperature and humidity (Denlinger and Armbruster, 2014). These predictions are of great relevance to medical entomologists and policy makers alike.

Changes in mosquito distributions mediated by a warming climate will result in mosquitoes infesting areas that are currently too cold or dry for them. As highlighted above, temperature and humidity play an important role in determining population dynamics of most vectors by impacting on their physiological processes and survival (Dao *et al*., 2014; Denlinger and Armbruster, 2014; Huestis *et al*., 2012; Lehmann *et al*., 2014; Lyons *et al*., 2012). It follows that warming or humidifying zones adjacent to infected areas will be conducive to vector breeding and perpetuation. It is well known that a decrease in temperature as altitude increases lessens the risk of malaria transmission in higher altitude areas (Maxwell *et al*., 2003; Reisen *et al*., 2006; Sanburg and Larsen, 1973). A global increase in temperatures therefore means areas at high altitudes and once free from mosquitoes may get warm enough for mosquitoes to proliferate. Global warming may also result in changes in the duration of seasons. It is predicted that the length of summers will increase; hence the duration of winter will decrease (Yang *et al*., 2010). This will have dire consequences for areas in the tropics where malaria epidemics are seasonal (Coetzee *et al*., 2013; Roca-Feltrer *et al*., 2009). In most parts of Africa, for instance, malaria cases spike during the hot, wet summers and decline in winter. Lengthening of the summer season will increase the transmission window (TW) for malaria and other vector-borne diseases.

During the 19th and 20th centuries, vector-mediated diseases prevented the development of vast areas of the tropics, especially in Africa (Gubler, 1998; Philip and Rozeboom, 1973). It can be postulated that the envisaged increase in malaria prevalence will mainly affect third world countries, many of which have poor health delivery and surveillance systems. Africa currently accounts for 90 % of world malaria related deaths (World Health Organization, 2016). Malaria's

historic impact is evidenced *inter alia* in malaria antigen found in Egyptian remains dating from 3200 and 1304 BC (Miller *et al*., 1994). In South Africa, cases of deaths from malaria were initially recorded in 1837 and 1838. The disease claimed the lives of twenty members of the Louis Trichardt trek to Maputo (Coetzee *et al.*, 2013). More severe cases of malaria deaths were record in the years that followed. However, better medical care and effective vector programs has drastically reduced death tolls from malaria to about 0.01 percent of reported infection cases (Diseases *et al*., 2017). Currently, there has been a surge in malarial cases over the past months in 2017 as compared to 2016 in South Africa (Diseases *et al*., 2017). In total, there were 675 recorded cases of malaria in April 2016 and 472 in May 2016 in South Africa. An upsurge of malaria cases was experienced in 2017 whereby 3463 and 2783 cases were recorded in April and May respectively. However, cooler temperatures experienced in June 2017 marked the end of malaria season as the number of malarial cases decreased (Diseases *et al*., 2017). It is speculated that the drought experienced in 2016 could have played a major role in reducing the number of malarial cases during that year (Diseases *et al*., 2017).

Despite such a high percentage in malarial incidences, some parts of South Africa have been spared the impacts of mosquitoes because they are too dry, too cold or both for mosquitoes to survive (Afrane *et al.*, 2012). Spread of malaria with raised temperature and humidity will put more pressure on already economically and administratively stretched governments. The discussion above clearly shows why there is concern over the future distribution and prevalence of malaria in light of the predicted increases in temperature and rainfall (Lyons *et al.*, 2012).

In Africa, the vectors of malaria are mainly female individuals of the *Anopheles gambiae* species complex and to a small extent females of *A. funestus* (Coetzee *et al.*, 2000). In sub-Saharan Africa, *A. gambiae* species complex is studied more than any other because of its greater role in

malaria epidemics, wider spatial distribution and most importantly its ability to easily colonise new areas (Huestis *et al.*, 2012; Lanzaro and Lee, 2013). This group is found extensively in Africa where it infests mostly desert-like areas with conditions characterised by hot days and cold nights (De Meillon, 1951). Although the population dynamics of mosquitoes are a function of temperature and humidity (Bayoh, 2001; Beck-Johnson *et al*., 2013; Craig *et al*., 2004; Denlinger and Armbruster, 2014; Lehmann *et al*., 2014), the exact physiological responses responsible for this dependency are not well understood for some species of mosquito. Studies on this topic have been carried out in North America and Europe, but little research has been done in Africa. Nelms *et al.,* (2014) indicated that adult *Culex spp,* responsible for the WNV in North America overwinter as female adults in reproductive arrest. However, this cannot be taken as true across all species considering that different species are known to have different strategies for surviving harsh conditions (Reisen and Brault, 2007). This is particularly true considering that winters in North America are harsher than in Africa and as such species existing in these environments may have evolved different coping strategies.

Temperature and humidity do not only influence distribution patterns of mosquitoes but also their physiology and behaviour too. The development of most immature stages depends on the presence of water. Breeding sites occur in temporal or permanent water bodies because juvenile stages are aquatic (Denlinger, 2002). If the surroundings are too dry, oviposition, embryonic development, egg hatching and larval development ceases (Kaiser *et al*., 2014). Temperature can slow down or speed up the rates of biological processes undertaken by mosquitoes such as embryonic and larval development, flight activity and reproduction. Understanding the physiological and behavioural changes that accompany variations in ambient temperature is critical in managing the vector (Denlinger and Armbruster, 2014). For example, in countries such

as South Africa where malaria control is carried out mainly in summer (Coetzee *et al.*, 2013), these could be shifted to winter if it is found that mosquitoes have physiological characteristics which make control measures more effective at this time.

This study was therefore motivated by the fact that mosquitoes and mosquito mediated diseases are absent during winter seasons and in areas with cool temperatures but are present once temperatures increase. A trend has been observed in places endemic to malaria and whose seasons comprise hot and wet summers, paired with cold and dry winters (Diseases *et al*., 2017). During the cold seasons, it has been observed that adult mosquitoes are scarce and the incidence of malaria is low (Afrane *et al*., 2012; Coetzee *et al*., 2013; De Meillon, 1951; Leeson, 1931; Stuckenberg, 1969) compared to the wet and warm seasons. Another striking feature is that these mosquitoes proliferate quickly at the onset of spring. The refuge of mosquitoes during the cold season which enables population proliferation immediately after the first rains has not been properly identified. Any attempt to explain this observation is inherently speculative. It can be speculated that due to constrained movement of mosquitoes arising from low temperatures, mosquitoes may be concentrated in microhabitats leading to uneven distribution in the ecosystem. Thus, their abundance and density could be perceived as low by observers (Lehmann *et al.*, 2014). Or else adults die during winter and the populations which proliferate in the next season do so via overwintered embryonic stages that quickly develop once conditions become hospitable. However, this does not explain why the populations increase by ten-fold in a short time.

Results of this study will shed light on how mosquitoes physiologically respond to lowered temperatures in their natural environment. These findings will provide baseline information on how mosquitoes manage to proliferate soon after the spring rains. If mosquitoes used in the experiment respond to reductions in temperature in a manner similar to that displayed by Culex

mosquitoes this could highlight the possibility that they can survive winter temperatures, hence re-appear at the advent of spring. Most importantly is to know if lowered metabolic rates will sustain the mosquitoes throughout the winter period. The death of the mosquitoes under cold laboratory conditions could indicate that adult mosquitoes cannot cope with cold temperatures in their natural environment. Therefore, other overwintered life stages of mosquitoes would be responsible for the surge in mosquito population after winter. Knowledge on the exact temperature levels responsible for specific changes in physiology and behaviour of mosquitoes will explain how mosquitoes can manage to quickly re-appear on the onset of the wet, warm season (Koenraadt *et al.*, 2003) preceded by a period of inhospitable conditions. Future studies can then do further research to characterise the survival mechanisms assisting mosquitoes to quickly re-establish after winter and persist within ecosystems.

1.1 Response of mosquitoes to adverse conditions

Adult mosquitoes are subjected to unpredictable and highly variable terrestrial surroundings (Gray, 2013). Unlike homeotherms, they cannot maintain a constant body temperature by internal mechanisms. If ambient surroundings are too cold and dry the continued survival of adult mosquitoes is threatened (Denlinger and Armbruster, 2014). Thus, a survival mechanism is required to see them through the winter temperatures. For heterodynamic insects such as mosquitoes (Roberts, 1978), behavioural change which is synchronised with that of the surroundings is required to facilitate longevity during adverse conditions.

Dormancy and long-distance migration have been proposed as the survival strategies used by mosquitoes in adverse conditions by a variety of authors such as Roberts (1978), Nelms *et al.* (2013) and Denlinger and Armbruster (2014). It is reported that only female mosquitoes adopt

survival mechanisms and males die soon after copulation (Russell *et al*., 2013). The survival mechanisms will be dealt with further in the following chapter.

The life history of mosquitoes and many other insects is adaptive and allows them to exist in altered morphology at different times and in a variety of habitats (Roberts, 1978). It allows vulnerable juvenile stages to occupy more stable environments (larvae in aquatic sites) to increase their chances of survival, yet mature stages face adversities in more variable terrestrial habitats. Factors affecting each developmental stage will influence the survival of the following stage. For example, the dynamics of the development of immature stages impact on the physiology and population structure of the adults. The conditions in which juveniles are reared influence the characteristics of the adults (Beck-Johnson *et al.*, 2013; Gray and Bradley, 2003; Kaiser *et al.*, 2014). For example, the time taken to hatch eggs in some *Anopheles* mosquitoes influence the body size of the adult (Gray and Bradley, 2003; Kaiser *et al*., 2014). Therefore, the physiology of an adult mosquito can be understood better if the dynamics involved during maturation of juvenile stages are known.

1.2   The life cycle of mosquitoes

The life cycle of mosquitoes is divided into four major stages comprising adult, egg, larva and pupa. These developmental stages require different resources for their survival. For instance, unlike adult mosquitoes, most juvenile stages depend on aquatic habitat for their development. Depending on the species, the breeding sites can be on temporary or permanent water bodies. In *A. gambiae,* the selected larval sites are temporary waterbodies such as tyres and hoof prints (Gillies and Coetzee, 1987). Besides differences in habitats, the developmental stages of mosquitoes also vary significantly in morphology, modes of locomotion and food sources.

Adult mosquitoes usually emerge from the pupal stage in a few days' time (Gillett, 1971) depending on the species and prevailing temperatures. Most female *Anopheles* mosquitoes begin to develop oocytes a few hours after emergence (Gillett, 1971). These species are non- autogenous (Gillett, 1971; Robert *et al*., 2011) and require a blood meal to mature and ripen their eggs otherwise maturity of eggs is arrested until such a time when a blood meal has been consumed (Gillett, 1971). Upon consuming a blood meal and depending on the ambient temperature, female mosquitoes take two days to mature eggs on warm days (Muirhead-Thomson, 1951) and 3-4 days (Kaiser *et al*., 2014; Muirhead-Thomson, 1951) when cold (Muirhead-Thomson, 1951). In natural environments, blood-fed females direct nutritional resources towards oogenesis when they are inseminated (Baldini *et al.*, 2013).

Female mosquitoes acquire sperm from males during copulation. The female then stores the sperm in its spermathecae waiting to fertilise matured eggs during oviposition (Lehmann *et al.*, 2010). The molecular interaction between males and females during copulation affects the reproductive behaviour and physiology of female mosquitoes (Baldini *et al.*, 2013). The mating-dependent pathway of egg production in mosquitoes is regulated by a steroid hormone which is secreted by males during copulation (Baldini *et al.*, 2013).

Fertilized eggs are oviposited in selected sites such as aquatic bodies, stagnant water sites and damp surfaces individually or as a group. Muirhead-Thomson, (1951) reported that *Anopheles* mosquitoes tend to lay their eggs singly on the surface of the water. Under optimal conditions, the eggs of *A. gambiae* hatches into larvae after 2-3 days' post oviposition (Kaiser *et al.* 2014). Embryonic development can be delayed up to 18 days or more if the conditions are inhospitable (Kaiser *et al.*, 2014). It has been demonstrated that even though embryonic development proceeds at the same rate in *Anopheles* eggs, a small proportion can delay hatching by entering diapause

(Kaiser *et al.*, 2014) which is an important strategy for bridging inhospitable conditions. The larvae undergo four developmental stages before maturation into the pupae after 5-7 days (Kaiser *et al.*, 2014), the latter develops into an adult after 2-3 days.

After emerging from the pupal stage, the young adult rests for a few hours and dries its wings before it can fly to the surrounding areas. Adults of female *Anopheles* mosquitoes can disperse up to 3 km from their breeding site (Russell *et al*., 2013). The female mosquitos spend their entire lives away from water sources but occasionally fly back to lay eggs or search for mating partners. On average, the adult female survives less than two month (Beier *et al.*, 1990; Gillies and De Meillon, 1968; Koenraadt *et al.*, 2003; Lehmann and Diabate, 2008) in the terrestrial environment which can include anthropogenic structures. Males do not disperse as far as females and they are usually found within a 200-metre radius of a breeding site (Russell *et al*., 2013). The male lives for a short period ($\leq$ 7 days), and dies soon after copulation which takes place a few days past emergence, thus leaving behind inseminated females (Denlinger and Armbruster, 2014).

Due to their vector role in malaria, longer lifespan, vulnerability to unpredictable weather conditions (Coetzee *et al.*, 2000; Gray, 2013) and higher risks of mortality, adult female mosquitoes have been widely used in population dynamic models (Beck-Johnson *et al.*, 2013). Again, concern over climate change has made them epidemiologically significant for modelling and forecasting mosquito population dynamics (Afrane *et al.*, 2012). What is surprising about adult mosquitoes is that, despite exposure to highly variable conditions, they survive for more days than their immature stages which live in relatively stable environments.

1.3   Rationale

Information on the life cycle of mosquitoes mainly applies to warm environments conducive for their survival and where each development stage leads to the next. However, there is limited information on how the cycle and time frame is adjusted during adverse conditions. Thus, information from life cycle does not make provision on how populations of mosquitoes persist in the absence of breeding sites and how development of different life stages is staggered due to adverse environmental conditions. This raises questions such as: "How do adult mosquitoes cope with high variability in temperature and manage to survive up to two months?" and "Where do adult mosquitoes which emerge at the end of summer disappear to during winter?". It would also be interesting to know what happens to the juvenile stages developed at the end of summer, as this could explain sources of mosquitoes at the start of the new breeding season. Clearly, those mosquitoes which emerge at the end of summer and are present at the advent of the first spring rains must have survived up to three or more months of the cold weather. This would need to be explained by a special survival mechanism (Lehmann *et al*., 2014). Since winter seasons in cooler parts of southern Africa can last 3-4 months (Diseases *et al*., 2017), one would expect no mosquitoes to survive this lengthy period. Most mosquitoes would have exceeded their life span by that time and it would be too cold for them to search for food. Yet as previously indicated, mosquitoes become abundant soon after the first rains. Lehmann *et al.* (2014) suggests that newly developed adult mosquitoes emerge from overwintered juvenile stages a few days before the first rains, and or overwintered adult mosquitoes reappear.

This study seeks to gain insight into possible overwintering strategies of *A. quadriannulatus* species. The metabolic rates themselves are influenced by multiple factors such as age, body size mating and feeding status of the mosquitoes, and hence these factors need to be accounted for. In

the current study, we hypothesize that *A. quadriannulatus* will lower its metabolic rate in response to reduction in ambient temperature. In this way, the mosquito will be able to conserve sufficient food reserves to survive winter. Therefore, the findings of this study will point to the possible effects of global changes in temperature on mosquito species abundance, distribution and the incidence of malaria.

The questions which prompted the investigations forming the basis of this study are:

1. Can adult female mosquitoes survive winter?
2. How are their metabolic rates impacted by low temperatures?
3. Do female mosquitoes respond similarly to effects of reducing temperature when they are in different mating and feeding states?

This study sets out to investigate the temperature-metabolic rate relationship in *A. quadriannulatus* using proven methodology which indicates physiological changes in organisms (respirometry). This species was chosen because it is a member of the *A. gambiae* complex and does not transmit malaria.

1.4 Aims and objectives

The aim of this study was to determine the effect of temperature on the metabolic rate of laboratory bred *A. quadriannulatus*. The metabolic rate of female *A. quadriannulatus* were measured at temperatures ranging from 30 °C to 10 °C. Factors which influence metabolic rates such as age, mating, feeding and mass were also taken into consideration during the study. The study was conducted on female mosquitoes whose age ranged from 2 to 4 days.

1.4.1   Specific Objectives

1. To determine the effects of lowering temperature on metabolic rates of unmated and sugar fed female mosquitoes. This was the control of the experiment and it gave reference information on the general effects of low temperatures on metabolic rates without any additional physiological conditions of the mosquito.

2. To determine the effects of lowering temperature on mated sugar fed female mosquitoes. The results of this objective were compared with the control to see if mating influences metabolic rates when the temperatures are reduced.

3. To determine the effects of lowering temperature on metabolic rates of unmated females who have been given a blood meal. The objective seeks to find out if blood feeding female mosquitoes will influence their metabolic rates at lowered temperatures. A blood meal is likely to impact on metabolic rates due to blood digestion and development of eggs.

4. To determine the effects of lowered temperatures on mated and blood-fed female mosquitoes. Mated mosquitoes which are blood-fed are most likely to develop and lay eggs. Eggs will however only be laid in the presence of a suitable oviposition site. If mated mosquitoes could overwinter in gravid state, then populations of *A. quadriannulatus* are most likely to recover very soon after the first spring rains.

Chapter 2

2.0   Literature Review

2.1   *Anopheles gambiae* species complex

In sub-Saharan Africa, the *A. gambiae* complex is the most widely studied and vital group of mosquitoes due to its profound contribution towards malaria transmission (Huestis *et al*., 2012; Lanzaro and Lee, 2013). This species complex consists of both vector and non-vector species (Coetzee *et al*., 2000). The most important and widespread malarial vectors of the *A. gambiae* complex have been reported from most African countries and their distribution reflects dependency on regional and seasonal climatic conditions. Breeding sites are usually along riverbeds, temporal pools, pans and anthropogenic stagnant water sources (Gillies and Coetzee, 1987). An estimated 90 % of the one million malarial deaths per year occur in sub-Saharan Africa from transmissions associated with *A. gambiae* (Baber *et al*., 2010).

Before 1962, *A. gambiae* species complex was regarded as a single species. This changed when Hugh Paterson demonstrated that at least three fresh water breeding species of *A.gambiae* could not mate naturally (Coetzee *et al*., 2013). In 1964, additional salt water breeding species were identified, bringing the total number of subspecies in the complex to five. A sixth species was later discovered in Uganda by Davidson and Hunt at a date not stipulated (Hunt *et al*., 2000). Up to that date, the complex was made up of the following six species; *A. gambiae*, *A. arabiensis*, *A. quadriannulatus*, *A. bwambwe A. merus* and *A. melas* (Coetzee *et al*., 2000). The last two are East and West African salt water breeding species. In 1998, another species formerly named *A. quadriannulatus* species B from Ethiopia was discovered (Coetzee *et al*., 2000; Hunt *et al*., 2000). This was renamed *A. amharicus* and resembles the same polytene chromosomal arrangement as that of the southern African *A. quadriannulatus* despite the large distance between their geographic

location (Coetzee *et al*., 2013). The two locations differ in amount of annual rainfall, with greater than 1000 mm falling in southern Africa and approximately 700 mm falling in Ethiopia (Coetzee *et al*., 2000). The southern African *A. quadriannulatus* was described and named by F Theobald in 1911 (Coetzee *et al*., 2000) as a non-malarial vector, normal outdoor resting and cattle feeding (Bayoh, 2001; Coetzee *et al*., 2013, 2000; Lanzaro and Lee, 2013). According to Hunt *et al*. (2000), *A. quadriannulatus* is zoophilic and comprises allopatric taxa in Ethiopia and southern Africa. All species in this complex are morphologically indistinguishable and can only be characterised using the Polymerase Chain Reactions (PCR) (Lanzaro and Lee, 2013). The high genetic plasticity of *A. gambiae* complex has aided their wide geographic distribution, high tolerance and persistence throughout Africa (Huestis *et al*., 2012) creating a huge challenge in the control and eradication efforts of malaria within Africa. However, some areas in this region remain free of this complex.

2.2   Physiological and behavioural survival strategies in mosquitoes

Possible overwintering strategies employed by mosquitoes can be grouped into two main categories. They could be physiological or physical (behavioural) adaptations (Denlinger and Armbruster, 2014; Roberts, 1978). Exiting habitats with adverse conditions and resuming a normal life elsewhere or finding a suitable microhabitat to occupy are examples of behavioural adaptations. In contrast, physiological survival strategies usually depend on the manipulation of metabolic rates to alter the speed at which some biological processes proceed, thereby effectively conserving energy and prolonging the life span of the organisms, hopefully until condition become more favourable for foraging. Mosquitoes could adopt any of the following physiological and behavioural methods to overcome effects of low temperatures.

2.2.1 Cold torpor

Torpor is defined as a temporal response induced by sudden short spells of adverse weather such as daily temperature changes (Roberts, 1978). The low temperature experienced may not kill the insect, but can halt its activity and development. Due to inactivity, the metabolic rates during this period become lower than usual. Cold torpor can occur at any time of the year and at any stage of the life cycle (Roberts, 1978). Torpor is therefore non-seasonal and thus can be regarded as a daily form of survival which enables the mosquitoes to overcome short-term adversities. This could be a survival strategy for mosquitoes that inhabit areas whose winters are interspersed with warm days. It means during times when it is very cold, the mosquitoes could then enter torpor, but as soon as temperatures pick up, normal activity is resumed.

2.2.2 Quiescence

This is a form of dormancy and persists longer than torpor. It is seasonal and is a result of extrinsic factors which determines its onset and termination (Roberts, 1978). Quiescence is initiated and terminated in response to the temperature fluctuations of the surroundings and the response is lowering of the metabolic rates. Nelms *et al*. (2013) describes quiescence as a period of temperature-induced inactivity which does not require a preparatory phase as is the case in diapause. This dormancy is, however, terminated as soon as conditions becomes favourable; hence it lacks the fixed latency and early programming found in the case of classical diapause (Denlinger and Armbruster, 2014). Depending on the severity of the cold, some mosquito species only use this type of dormancy to bridge cold seasons (Denlinger and Armbruster, 2014). Records of overwintering by quiescence have been documented for *Culex annulirostris* and *C. tarsalis* from southern parts of Australia and California respectively (Nelms *et al*., 2013). Another southern hemisphere member of the *Culex* complex called *C. quinquefasciatus* was found to overwinter using a temperature-

induced quiescence (Eldridge, 1987). In temperate South African Highveld which experience change in day length, quiescence is reported to occur in *Culex. pipiens* and *Culex theileri* (Russell *et al.*, 2013).

2.2.3 Diapause

To survive winter temperatures, some mosquito species undergo diapause which lowers their metabolic rates (Denlinger and Armbruster, 2014; Nelms *et al.*, 2013). The difference between this strategy and the other two outlined above is that lowering of metabolic rates is triggered by external cues to which the insect is "programmed" to respond. Cues that indicate the arrival of winter, like shortening of day length are commonly used by insects to go into diapause (Denlinger and Armbruster, 2014). According to Eldridge (1987), the programming can originate from the immature stages of the adult mosquito. For example, short day-length and cooling water temperatures experienced by immature stages induce diapause in adult *Culex* females. Diapause is pre-determined hormonally and its duration is also programmed independent of what is happening in the surroundings. Thus, it does not terminate immediately when conditions become hospitable. Diapause is a well-known survival strategy typically found in mosquitoes endemic to temperate areas (Denlinger and Armbruster, 2014; Lehmann *et al.*, 2010; Russell *et al.*, 2013). Diapause can occur in stages linked to different behaviours namely pre-diapause, diapause and post-diapause.

Pre-diapause is initiated at the end of autumn / beginning of winter and prepares the mosquito for diapause by prompting storage of energy reserves. Consequently, the mosquito becomes less active but bigger in size. Low temperatures and short daylengths are believed to be responsible for the initiation of diapause by enhancing photoperiodic response except in obligatory diapause (Denlinger and Armbruster, 2014).

Diapause is the phase when development is halted; hence the mosquito becomes unresponsive to environmental signals. Metabolic depression is the key characteristic of diapause (Denlinger and Armbruster, 2014), although reduced metabolic rates do not always mean that the organism is in diapause. Instead this could be a torpor or quiescent response to a brief cold spell (Gray and Bradley, 2005). In most instances, diapause is specific to a single life cycle stage at a time and occurs for a restricted amount of time (Roberts, 1978). The period spent in diapause is reported by Denlinger (2002) to be determined by an internal clock which detects daylength and temperature. It is however still disputable whether temperature and daylength are the only inducers of diapause (Denlinger, 1974) or not. This is after discovering diapause in flesh flies living in East Africa at a latitude of 1° south despite the absence of photoperiodism in that area. These flesh flies experience a robust pupal diapause initiated by low temperatures during July and August (Denlinger, 1974).

In mosquitoes species which undergo diapause, only females diapause and males die after mating, leaving behind inseminated females to overwinter (Denlinger and Armbruster, 2014). During this time, the females refrain from taking a blood meal and do not develop eggs until diapause has been completed. The termination of diapause is followed by post diapause quiescence which is indistinguishable from diapause except that the mosquito can respond to environmental signals that prompt development (Denlinger and Armbruster, 2014). However, suppression of development by low temperatures results in the build-up of individuals all primed to start developing. Thus when temperatures rise above a certain threshold, the whole population can initiate development simultaneously, leading to synchronous springtime emergence (Denlinger and Armbruster, 2014).

Zhou and Miesfeld, (2009) used radioactive tracers to track the metabolic fate and flux of glucose in *C. pipiens* adults reared under diapause and non-diapause conditions. Incubation temperatures and day lengths were used to differentiate between diapause (18 °C, short day) and non-diapause (27 °C long day) conditions. The study initially investigated the pre-diapause phase and it found that metabolic patterns of mosquitoes were not altered in both rearing conditions. The only difference noted was in lipid assimilation. The study conducted on one-day old mosquitoes found that after 72 h of labelling glucose, diapause-destined mosquitoes had converted 46 % more glucose into lipid than mosquitoes reared under non-diapause conditions. Sugar-feeding in adults could have initiated a metabolic shift that activated lipogenesis (Zhou and Miesfeld, 2009). The nulliparous and inseminated adults were discovered to enter diapause in response to reduced day length and low temperatures experienced during larval and pupal development (Zhou and Miesfeld, 2009). It was revealed that mosquitoes in diapause were capable of sparing lipids generated during pre-diapause to enhance egg production. This was demonstrated by the ability to detect 33 % of the lipids synthesized during pre-diapause in newly developed eggs. According to, Mitchell and Briegel (1989), *C. pipiens* reserves 1.7 times more lipids and 2.3 times more carbohydrates in preparation for diapause. During this period of overwintering, females do not take blood meals and cannot transform the blood to generate lipid reserves for survival (Mitchell and Briegel, 1989),but increase their sugar feeding during the first few weeks (Bowen, 1992; Robich and Denlinger, 2005).

Despite the widely studied insect diapause ecology in the temperate regions, there is lack of information on the underlying principles occurring in sub-Saharan mosquitoes during the cooler seasons (Canzano *et al*., 2006). The existence of diapause in *A. gambiae* is yet to be confirmed even though eleven *Anopheles* species have been discovered to diapause as adults, three as larvae and one as eggs (Denlinger and Armbruster, 2014).

2.2.4   Aestivation

Aestivation is a more general type of dormancy usually utilised by mosquitoes in warmer regions to survive aridity during the dry season (Denlinger and Armbruster, 2014). Many studies (Denlinger and Armbruster, 2014; Lehmann *et al*., 2010; Roberts, 1978) repeatedly refer to aestivation as a summer dormancy typically characterized by suppressed reproduction. In addition, Roberts (1978), Yaro *et al*. (2012) and Huestis *et al*. (2012) refer to aestivation as summer or dry season diapause. Suppressed reproduction in aestivating mosquitoes is indicated by failure of the adult females to develop eggs despite feeding on blood in the early dry season (Lehmann *et al*., 2010). Aestivation has recently been discovered in M-form *A. gambiae* renamed *A. coluzzi* during the fourth to eighth month dry season in the Sahel (Denlinger and Armbruster, 2014). Using a Mark-Release-Recapture method, Lehmann *et al*. (2010) showed that one of the female mosquitoes used in their research was able to survive seven dry months after moving only 570 m from where they were released. The short distance between the release and recapture points indicate that the mosquitoes did not undergo any long-distance migration and as such aestivation was proposed as the most plausible explanation of the survival strategy used by the recaptured mosquito.

2.2.5   Long distance migration

Instead of being concealed locally, mosquitoes can escape inhospitable places by adopting long distance migration. This is common in some African adult mosquitoes which tend to fly to favourable regions. Such a move is beneficial to the insect as it enables continuous growth and development (Russell *et al*. 2013). For example, *A. arabiensis* is understood to disperse up to 20 km away from the Nile valley during the dry season (Russell *et al*., 2013) Mosquitoes are reported to be able to migrate several kilometres a day (Nelms *et al*., 2013; Reisen and Reeves, 1990; Robert

et al., 2011) and can be transported even further by wind. Movement over short distances, viz 2 km, have been long known for *A. gambiae* although it was not tested between seasons (Baber *et al*., 2010). However, the effectiveness and feasibility of migration as a seasonal survival strategy is questionable, because it is influenced by a variety of factors such as proximity of favourable microclimates, flight range, strength of prevailing wind and dispersal scale (Roberts, 1978). Russell *et al*. (2013) showed that only 20 % of *A. funestus* can disperse further than 0.8 km and only few females of *A. gambiae* can disperse further than 3 km. Logically, these distances are too short for these mosquitoes to escape adversity unless they can hide in microclimates. Migration will most probably be possible in species such as *A. pharoensis* which can disperse 6 km and even 100 km if aided by wind (Russell *et al*., 2013).

Evidence of migration in mosquitoes has been found in a study conducted by Baber *et al.* (2010) on the population size and movement of *A. gambiae s.s.* between two villages located in Mali. One of the villages called Fourda is a fishing area adjacent to the Niger River and has medium to high densities of *A. gambiae s.s*. The other village named Kenieroba is an agricultural site 12 km inland and experiences strong seasonal fluctuations in *A. gambiae* densities. Baber *et al*. (2010) used a Mark-Release-Recapture survey to observe population size and migration of *A gambiae s.s.* during the wet and dry seasons. Mosquitoes collected in Fourda, were marked and then released to the surroundings in March (dry season) and July (rainy season). The marked mosquitoes were searched for in the Keneiroba village during the same periods. No marked mosquitoes were captured in March while two were captured in July. Migration was expected to occur in March rather than July since March is a dry season in that area. The presence of a water body in Fourda could have been the reason why mosquitoes did not move in March, but they remained in Fourda close to water. Although this comprises proof of migration between the two areas, the areas are too

close (2km apart) to simultaneously experience different weather conditions. Hence the movement of those marked mosquitoes recaptured in Keneiroba was not necessarily driven by adverse conditions in Fourda (Baber *et al*., 2010).

A different investigation highlights the evidence of long distance migration in *A. gambiae s.s* as a survival strategy. Dao *et al*. (2014) investigated signatures of aestivation and migration in Sahelian malarial mosquito during the long dry season. The population growth of three malarial vectors including *A. gambiae s.s.* was observed using time series analyses to estimate their seasonal cycles. Unlike *A. coluzzii* which displayed population growth consistent with aestivation, long distance migration was deduced for *A. gambiae s.s.* and *A. arabiensis*. This was derived from the duration of population growth post the dry season. It was found that the population of *A. coulozzii*, grew immediately after the first rains yet in *A. gambiae s.s.* and *A. arabiensis*, there was a two-month delay in population growth. The delay better fitted a long-distance migration explanation as opposed to local persistence from aestivating mosquitoes as it shadowed a window period for travelling. However, the findings of long distance migration in *A. gambiae* appear to flout the flight range highlighted previously (3 km). Based on this flight range, one would assume that wind facilitated the migration of these mosquito. Adamou *et al*. (2011) hypothesised that wind (jet streams) aid long distance migration in S-form *A. gambiae*. although it is not clear how far these mosquitoes can travel without the aid of jet streams.

2.2.6   Hiding

Wallace and Grimstad (2002) investigated physiological ecology of overwintering *Anopheles* mosquitoes in south-western Michigan. Their findings showed that *A. quadrimaculatus* had the capability to survive winter temperatures as low as -15 °C. This was achieved by hiding in anthropogenic and natural protected areas during winter, with feeding restricted to warm days. The

study did not specify the temperatures of the hiding places; however, it is assumed that the microhabitats in this case were warmer than the ambient surroundings. It is also not known whether the mosquitoes survived by merely hiding, or if they also adopted other physiological techniques during that time.

2.3   The gonotrophic cycle in *Anopheles* mosquitoes

Gonotrophic cycle of mosquitoes has a greater impact on their population dynamics. At the same time, its dependency on ambient temperatures has led to the season and climate related trend in their distribution and abundance. Several studies have been carried out on the life cycle of the mosquito. Researchers like Baldini *et al*. (2013), Gillett (1971), Klowden and Russell (2004), Mala *et al*. (2014) and Muirhead-Thomson (1951) have contributed a lot to our understanding of the reproductive or gonotrophic cycle of the *Anopheles* mosquito in relation to changes in temperature. Their research has focused on how temperature influence the gonotrophic cycle of the mosquito (Gillett, 1971; Muirhead-Thomson, 1951). Gonotrophic cycle of a mosquito is simply defined as the events that take place from the time a mosquito start seeking a blood meal until viable eggs are laid (Muirhead-Thomson, 1951). General, it takes about 48 h for some mosquito species to mature eggs once they acquire a blood meal (Gillett, 1971; Klowden and Russell, 2013; Muirhead-Thomson ,1951). However, this duration can vary depending on the surrounding temperatures and species. For example, it takes 4.1 days for *A*. *gambiae* to mature eggs when average temperatures range between 7.59 °C and 23.65 °C but three days when average temperatures range between 12.5 °C   and 28.4 °C (Mala *et al*., 2014). Despite the effects of ambient temperatures on the gonotrophic cycle of the mosquitoes, other factors such as the presence of a blood meal and sperm could interfere with the duration of the cycle. Thus, the interacting effects of age, status or sex of the mosquito can influence their response towards temperature changes (Lyons *et al*., 2014). For

example, for non-autogenous mosquitoes, egg development is arrested until a blood meal has been consumed (Gillett, 1971). Also, for the eggs to be laid, the mosquito needs to be inseminated so that fertilisation can take place (Mala *et al*., 2013). Because metabolic rates are an integral response to multifactorial variables, it was imperative for the present study to account for factors such as age, gonotrophic and mating status of the mosquitoes when assessing the effects of temperature on metabolic rates.

2.4   Temperature and mosquitoes

Temperature influences the population dynamics of mosquitoes in space and time in different ways (Beck-Johnson *et al*., 2013). Firstly, it determines their ecological and seasonal distribution based on the geographic and climatic conditions. For example, in South Africa the abundance of mosquitoes is seasonal and vector mosquitoes are endemic to low lying areas such as Mpumalanga, Limpopo and KZN (Diseases *et al*., 2017). Secondly, temperature has a direct impact on the physiology of mosquitoes and as such it can enhance or hinder their survival (Lyons *et al*., 2014). Since temperature is a function of both season and geography, mosquito populations are usually unevenly distributed across both temporal and spatial scales. This therefore explains why there are low mosquito densities in east African highlands or during colder months (Beck-Johnson *et al*., 2013). Thirdly, in general temperature directly affects the duration of the entire life cycle of most insects including mosquitoes. The development of both the embryo and larva depends on ambient temperatures. These stages may cease or delay development if ambient temperatures are unfavourable (Bayoh, 2001) and mortality rates of immature stages will be affected. This may consequently determine the number of larvae that successfully develop into adult mosquitoes, thus influencing the population structure of the adult mosquitoes.

Adult mosquitoes have the most pronounced ecological effects compared to other stages in the life cycle. This is because adult mosquitoes are responsible for the transmission of diseases and are easily detected macroscopically in the ecosystem. The physiology and survival of adult mosquitoes is dependent on temperature just as in juvenile stages. For example, the number of times an individual adult mosquito can lay eggs in its lifetime (gonotrophic age) depends on the ambient temperatures (Gillett, 1971). This has an impact on the total number of eggs a mosquito can lay before it dies, thereby influencing mosquito abundance at any time (Klowden and Russell, 2004).

Different developmental stages of the mosquito respond to changes in ambient temperature differently (Bayoh, 2001; Beck-Johnson *et al*., 2013). Immature stages usually occupy more thermally stable aquatic environments, so they tend to be buffered from changes in ambient temperatures of the surroundings. In contrast, adult mosquitoes are subjected to more thermally variable terrestrial environments. Thus, mortality rates of juvenile stages are lower than adults' over smaller temperature changes (Beck-Johnson *et al*., 2013). Bayoh (2001) discovered an interesting relationship between temperature and survival of both larvae and adult mosquitoes. It was reported that larvae of *A. gambiae sensu stricto* survived for less than seven days if they were reared at 10 °C, 12 °C, 38 °C and 40 °C but survived for more than five weeks without developing into adults if reared at temperatures between 14 °C and 18 °C. The larvae could only develop into adults if the temperatures were above 16 °C but lower than 34 °C and optimal larval development took place at 28 °C.

Beck-Johnson *et al*. (2013) developed simulations of parameterised models (dependent delayed differential equations (DDE)) to predict the temperatures at which adult *Anopheles* mosquitoes could survive the best. Their model took into consideration the realisation that temperature relations are complex, nonlinear and persist throughout the life cycle of the mosquito.

Therefore, it accounted for all the developmental delays such as time to hatch and mortality rates which may impact on the physiology of adult mosquitoes to make the predictions. The models were performed at temperatures ranging from 16 °C to 40 °C and predicted that the abundance of mosquitoes will be greater than one at temperatures between 17 °C and 30 °C. Adult survivorship showed a sharp decrease at temperatures lower than 17 °C (Beck-Johnson *et al*., 2013). According to Bayoh (2001), optimal survival of adult mosquitoes occurs at temperatures ranging from 15 °C to 25 °C and humidity of 60 % - 100 %. It can be deduced from these studies that the population dynamics of mosquitoes strongly depends on ambient temperatures. Due to the correlation between temperature and seasons, the distribution of mosquitoes assumes a seasonal related pattern.

2.4.1   Seasonality and distribution of mosquito populations

Roberts (1978) found that insects can be classified into two distinct groups as per response to seasonality within their life cycle. Homodynamic insects can survive all seasons throughout the year without difficulties whereas heterodynamic insects must synchronize their behaviour and physiology with changes in seasons to survive. Thus, population sizes of heterodynamic insects such as malarial vectors fluctuate throughout the year as observed in most biomes of sub-Saharan Africa which experience strong seasonal climatic variability (Baber *et al*., 2010). The biomes of the sub-Saharan region vary climatically from extremely dry to wet and hot to cold. The vast of sub-Saharan regions in Africa have seasons which alternate between wet and dry (Roberts, 1978). Only a small part of the region are temperate and experience periods of low and high temperatures. Surprisingly, *gambiae* s.l. is widely distributed throughout this region despite the high environmental variation across its range (Huestis *et al*., 2012). Even though this attribute of *A. gambiae* mosquitoes is understood to originate from their high genetic plasticity (Huestis *et al*., 2012), this does not explain why then some regions are free of certain species of mosquitoes. For

example, for other unknown reasons, anopheline mosquitoes are reported to be absent in the Seychelles (Robert *et al.*, 2011). According to Lanzaro and Lee, (2013), exposure to environmental variation can lead to adaptive radiation of populations in their local conditions resulting in speciation and differentiation of niches.

Temperature and moisture vary with varying seasons (Lehmann *et al.*, 2014) and as such population dynamics of mosquitoes are seasonal (Diseases *et al.*, 2017). This has been confirmed by observations made over the years by several researchers such as Leeson (1931), Afrane *et al.* (2012) and Lehman *et al.* (2014). Climate is considered important in shaping observed mosquito distribution trends in sub-Saharan Africa (Huestis *et al.*, 2012; Lehmann *et al.*, 2010) and elsewhere. For example, malaria distribution exhibit both seasonal and regional patterns in response to prevailing temperature and rainfall in sub-Saharan region (Craig *et al.*, 1999) including KwaZulu Natal (Craig *et al.*, 2004). Low-lying areas such as Mpumalanga, KwaZulu Natal and Limpopo have higher populations of malarial vectors which are absent in the Highveld areas such as the Drakensberg Mountains and most parts of Gauteng Province (Disease *et al.*, 2017). For those areas where malaria is endemic, malaria incidences have been found to be higher during warmer seasons than cold seasons (Coetzee *et al.*, 2013, 2000; Craig *et al.*, 2004). There are more malarial incidences during the rainy season (September to May) than in cold months (June-August) (Disease *et al.*, 2017). Also, pointing to the influence of rainfall, *A. gambiae s.s.* could not be detected in the Sahel during the long dry season (Dao *et al.*, 2014). Therefore, the spatial and season related temperature changes strongly impact on the distribution and abundance of mosquitoes. Variations in temperature and humidity influence the availability of breeding sites, behaviour and physiology of the mosquitoes resulting in seasonal population fluctuations (Coetzee *et al.*, 2000; Roberts, 1978).

Several authors such as Denlinger and Armbruster (2014), Huestis *et al*. (2011) and Wallace and Grimstad (2002) have noted that conditions most threatening to the survival of mosquitoes are low temperatures in temperate regions and long dry summers in the tropics. Aridity and low temperatures have been found to impede the continuous development of blood feeding insects such as non- autogenous female mosquitoes (Denlinger and Armbruster, 2014). Under these conditions, food is either scarce and energy sources of the mosquito are limited leading to reduced fitness which in turn threatens their survival. The ability of mosquitoes to survive these circumstances strongly depends on their capability to accordingly adjust their behaviour and physiology (Roberts, 1978).

Previous studies such as those undertaken by Craig *et al*. (2004), Canzaro *et al*. (2006), Barber *et al*. (2010), Lehman *et al*. (2014) and Dao *et al*. (2014) have all provided evidence of seasonal survival mechanisms in mosquitoes. According to Russell *et al*. (2013), female mosquitoes survive colder climates by hibernating or remaining quiescent in safer areas. Wallace and Grimstad (2002) and Denlinger and Armbruster (2014) suggest that mosquito species found in the northern hemisphere such as *C. tarsalis* and *C. pipiens* overwinter by undergoing diapause.

 Strategies to survive adverse conditions have been reported in several mosquito species and specific strategies vary with respective species. A few factors such as species, geographical location, and stress tolerance could possibly influence the nature of a survival strategy employed by mosquitoes. A good example is the difference in stress tolerance levels in *A. quadriammalatus* and *Anopheles gambiae sensu stricto* mosquitoes. The former can survive temperatures as low as -15 °C in the northern hemisphere (Capinera, 2008) whereas the latter survives in temperatures from 15 °C and 25 °C (Bayoh, 2001). Consequently, the two species employ different strategies to survive adverse conditions. For example, *C. pipiens* (North America) refrain from blood feeding and seek dark humid hibernaculum at the onset of diapause but *A. gambiae s.s* aestivates in the

Sahel (Denlinger and Armbruster, 2014). The seasonal distribution and abundance of adult *A. gambiae* and *A. arabiensis* in the dry tropical areas is often justified by desiccation tolerance and aestivation (Gray and Bradley, 2005). In temperate regions, *Culex* mosquitoes are understood to overwinter using diapause (Nelms *et al*., 2013). Long distance migration, hibernacula, reduced activity and alteration of metabolic rates are some of the strategies found to be employed by mosquitoes to overwinter (Adamou *et al*., 2011; Dao *et al*., 2014; Denlinger and Armbruster, 2014; Huestis *et al*., 2011; Lehmann *et al*., 2010; Mitchell and Briegel, 1989; Wallace and Grimstad, 2002).

There is a dearth of information with regards to how *Anopheles* mosquitoes in cooler sub-Saharan areas survive winters. Most of the work by researchers such as Huestis *et al*. (2011; 2012), Dao *et al*. (2014) and Lyons *et al*. (2012; 2014) focused more on how the mosquitoes addressed desiccation problems associated with the long dry and hot season. The general limitation of breeding sites during winter raises the question of how mosquitoes persist in ecosystems during and past this season. Logically, one expects minimal / no breeding to occur if there is a limitation / lack of breeding sites. Consequently, one would expect, at best, a slow increase in the population of mosquitoes the next rain season. Surprisingly, a ten-fold mosquito population within five days after the first summer rains in sub-Saharan Africa (including temperate areas) has been reported (Dao *et al*., 2014; Huestis *et al*., 2012; Lehmann *et al*., 2010). It remains to be explained how this dramatic increase in adult *A. gambiae* populations occurs despite low populations and low malarial incidences in winter (Denlinger and Armbruster 2014). However, since each individual female mosquito can lay multiple eggs at a time, this can then account for the observed population explosion. Similar observations have been made in South Africa (per personal communication Maria Kaiser from National Institute for Communicable Diseases). The time taken for the

population to re-establish is usually too short to accommodate a window period for development of a new generation of young adults which would take at least nine days under optimum conditions (Dao *et al*., 2014).

2.5 Metabolic rates in mosquitoes

Metabolic rates have been measured in mosquitoes for over a decade (Table 2.1) and are used to monitor the physiological changes in organisms as they give an indication of an animal's behaviour and fitness (Gray and Bradley, 2003; Hahn and Denlinger, 2011; Huestis *et al*., 2011) which influences the energy expenditure of an organism per unit time. In general the lower the metabolic rate, the less energy is being spent (Canzano *et al*., 2006). Based on the outcome of the studies conducted by several authors (Table 2.1) who have investigated metabolic rates of *Anopheles* mosquitoes, it appears as if their metabolic rate measurements are relatively similar (Table 2.1). According to Denlinger 1979 the metabolic rates of mosquitoes are similar regardless of their species name. This is partly supported by the findings of Huestis *et al*. (2011) and Gray and Bradley (2005). Both authors found no statistically significant difference in the resting metabolic rates of *A*. *gambiae* and *A*. *arabiensis*. For this reason, the findings of the present study, could be used to infer other members of the gambiae complex especially the malarial vectors since they are closely related.

One of the crucial factors influencing metabolic rates investigated in this study is temperature. Denlinger and Armbruster (2014) showed that metabolic rates of mosquitoes are positively correlated to ambient temperatures especially in areas which experience negative daily temperatures. Lowering of metabolic rates in response to decreased temperatures is common in these mosquitoes during diapause. However, lowered metabolic rates do not always infer that an insect is in diapause (Denlinger and Armbruster, 2014; Gullan and Cranston, 2010). For example,

in the Sahel, although *A. gambiae* and *A. arabiensis* were not in diapause, it was found that their metabolic rates were lower at 25.1 °C than they were at 32.4 °C (Huestis *et al*., 2011). This is an indication that change in temperature affects the metabolic rates of the mosquitoes in the absence of diapause. Changes in behaviour, flight and feeding activity of the mosquito due to temperature differences were found to have contributed towards the outcome of the study (Huestis *et al*., 2011). Other physiological states that result in lower metabolic rates include quiescence, cold torpor or general inactiveness in response to falling external temperatures for example *Culex* mosquitoes from temperate southern hemisphere were found to lower their metabolic rates using quiescence not diapause (Eldridge, 1987; Eldridge and Bailey, 1979; Reisen, 1995).

Other than temperature, factors such as body size, blood digestion, egg development and larval acclimation could impact on the nature of metabolic rates of mosquitoes (Gray, 2003, 2013; Lancheincht *et al*., 2010; Niehaus *et al*., 2012; Terblanche *et al*., 2015). Blood digestion increases the metabolic rates of mosquitoes due to extra energy spent digesting a blood meal (Gray and Bradley, 2003, Houk *et al*., 1979).

Table 2. 1.　　　Measurements of metabolic rates from different species of female mosquitoes from previous studies.

Mosquito species	Mosquito status	Age (days)	Temperature (°C)	Mass (mg)	Rate of CO_2 production $nl.min^{-1}$	Reference
A. gambiae	sucrose fed female	0	28		23.33	Gray and Bradley (2005)
		2	28		28.33	
		4	28		36.67	
		8	28		18.33	
		10	28		30	
M-form *A. gambiae*	unfed female		25.1-32.8		16	Huestis et al., 2011
	unfed female		25.1-32.8		35	
	unfed female		25.1-32.8		20	
A. arabiensis	unfed female		25.1-32.8		15	Huestis *et al.*, 2011
	blood fed female		25.1-32.8		40	
	gravid		25.1-32.8		25	
	sucrose fed female	0	28		23.33	Gray and Bradley (2005)
		2	28		28.33	
		4	28		41.67	
		8	28		26.66	
		10	28		31.67	

Species						Reference
C. tarsalis		4	25		55.5	Gray and Bradley (2003)
	unmated female	4	25	1.73	53.79	Gray and Bradley (2005)
	unmated female	8	25	1.92	52.76	
	unmated female	12	25	2.18	60.39	
	unmated female	16	25	2.23	58.51	
	mated female	4	25	1.8	53.34	
	mated female	8	25	1.85	51.34	
	mated female	12	25	2.11	54.63	
	mated female	16	25	2.12	51.72	
A. quadriannulatus		4			59-70	Lanciami and Anderson (1993)

Based on the findings of relevant studies described above, *A. quadriannulatus* was expected to respond in a similar way to temperature changes. Since it has been stipulated that metabolic rates of different mosquito species are relatively similar, at constant temperatures, measurements of metabolic rates of *A. quadriannulatus* should be comparable to those measurements summarised in Table 2.1. Discrepancies in the measurements of metabolic rates of *A. quadriannulatus* are however anticipated due to differences in methodology used by the previous studies.

One of the important questions this investigation seeks to answer is whether adult *A. quadriannulatus* is capable of overwintering in the Sub- Saharan region. In the northern hemisphere, temperatures regularly fall below 0 °C (Capinera, 2008; Wallace and Grimstad, 2002) and *Culex* mosquitoes in these areas overwinter by diapause, quiescence, hibernation and sometimes migration. The exact survival mechanism adopted by respective species differs and probably depends on latitude which dictates environmental cues like ambient temperatures and photoperiod (Reisen *et al*., 1995). For instance, the temperate southern hemisphere *Culex* overwinter by temperature-induced quiescence, yet their northern counterparts undergo diapause (Eldridge and Bailey, 1979; Reisen *et al*., 1995).

For vector control purposes, similar research is yet to be undertaken for *A. gambiae* mosquitoes, given their importance as potential malarial vectors in temperate parts of Africa. The projected effects of climate change require the need for investigating physiological effects of temperature on mosquitoes, especially in areas which are currently free of malarial vectors, as warming up those places could become conducive for invasion by vectors. According to Russell *et al*. (2013), mosquitoes in the subtropics and adjacent areas of warmer temperate regions respond to low temperatures by lowering their biological processes. For example, ovarian development may take 10-14 days under low temperatures as opposed to 2-3 days which occurs in optimum conditions

and it may take up to 2-3 months for the eggs to develop into adults (Russell *et al*., 2013). Otherwise they simply escape areas of adversity (Russell *et al*., 2013) by flying off to areas with more tolerable environmental conditions. However, because *A*. *gambiae* mosquitoes can only disperse three km at most (Russell *et al*., 2013). The population size of overwintered mosquitoes influences their abundance in the following wet season since they act as reservoirs for propagating the next generations (Denlinger and Armbruster, 2014).

Suppose mosquitoes migrate long distances to evade adverse conditions as they do in S-form *A*. *gambiae* (Adamou *et al*., 2011), one would expect a window period before they can recover once the conditions become ideal (Dao *et al*., 2014; Lehmann *et al*., 2014). For example, in the Sahel, *A*. *arabiensis* immigrated back from places of refuge, two months after the first rains (Dao *et al*., 2014). In this way, mosquito populations will not be expected to increase rapidly within five days after the first rains unless the mosquitoes were locally concealed in overwintered forms either as adults or other immature stages. The ability of mosquito populations to reappear within the first five days of spring infers an adult or pupal overwintering. This is because only the pupae can emerge into adults within that time frame.

In cases where mosquitoes overwinter as adults, the gonotrophic state of those mosquitoes could strongly influence their abundance in the following season. If non-autogenous mosquitoes such as *A*. *quadriannulatus* overwinter in a gravid state, these individuals will be ready to lay viable eggs immediately when the conditions become sufficiently favourable. Thus, at the advent of spring rains, the 10-fold population structure could possibly be a result of overwintered adults together with newly emerged adults developed from the eggs laid by overwintered adults. In instances where juvenile forms are capable of overwintering e.g *A*. *gambiae* embryonic diapause, additional adults could emerge from the development of overwintered juvenile forms which were generated at the

end of the previous breeding season. If these assumptions are true, this could explain the 10-fold multiplication in populations of mosquitoes soon after the spring rains. Contrary, if non-autogenous female mosquitoes overwintered as virgins and without blood meals, one would not expect a population explosion at the beginning of spring but rather a gradual increase as the population builds up from a much smaller founder population.

Chapter 3

3.0   Methods and Materials

3.1   Study species

Laboratory reared adult female mosquitoes of *A. quadriannulatus* were used for all measurements of metabolic rates. The species was chosen based on the reasons described in section 1.3. Male adult mosquitoes were only used for mating purposes. These mosquitoes were collected from colonies maintained in the Botha de Meillon Insectary at the Vector Control Reference Unit (VCRU) in Johannesburg. The laboratory strains were established from the Sangwe field colonies originally collected from Zimbabwe and colonised in 1998. At the insectary, the colonies are maintained at 25 °C ± 1 °C and 75 - 80 % relative humidity.

3.2   Maintenance of mosquito samples

Pre-arranged dates for collection of mosquitoes were organised. Newly emerged adult mosquitoes were collected from the insectary on their day of emergence. Once they had emerged, mosquitoes were sorted into males and females in separate plastic cages. Mosquitoes used for testing the effects of blood feeding were given a blood meal before they could be transported to research laboratory at the University of Witwatersrand, Johannesburg. At the research laboratory, the mosquitoes were fed with 10 % sugar solution. This was done by placing cotton wool buds dampened with 10 % sugar solution on top of the mosquito cages. The cotton wool buds were moistened every three days.

3.3   Experimental treatments

The four treatments were:

Treatment 1 = control group was unmated and sugar fed

Treatment 2 = mated and sugar fed

Treatment 3 = unmated and blood fed

Treatment 4 = mated and blood fed

3.4   Mating mosquitoes

Female mosquitoes were transferred into a cage with male mosquitoes for mating to take place. This was done at the research laboratory on the day of collection before dusk because mosquitoes mate at dawn or dusk (Muirhead-Thomson, 1951). It is difficult to mate *Anopheles* mosquitoes under laboratory conditions (Muirhead-Thomson, 1951). Mixing / swarming male and female mosquitoes together in cages therefore does not guarantee mating of all female mosquitoes (Muirhead-Thomson, 1951). Thus, at the end of the experiment, possibly mated mosquitoes were stored in eppendorf tubes and kept in a freezer. They were later taken back to the VCRU for dissection to confirm mating. Mating was confirmed by the presence of a sperm in female spermathecae after dissections. If found unmated, the results were added to the results of corresponding unmated groups.

3.5   Blood feeding mosquitoes

Female mosquitoes used in treatment three and four were fed with human blood on their day of emergence at the VCRU before collection. Each blood fed mosquito was checked for presence of blood in the abdomen using naked eye to confirm feeding. Those mosquitoes that had not blood fed or were not fully fed were discarded and not used for measurements.

3.6   Aging of mosquitoes

Age of the mosquito was determined by the number of days past their emergence day. I took the day of emergence for mosquitoes as day one. For this experiment, I only used mosquitoes

aged two, three and four days. Younger mosquitoes were preferably used to reduce experimental variability.

3.7   Respirometry

A closed respirometry system was used to indirectly determine the metabolic rates of mosquitoes. This was done by estimating the rates of carbon dioxide emitted by the mosquitoes (VCO_2). Thus, VCO_2 is used as a proxy for metabolic rates. This method was more feasible for this study than the open respirometry system.

The amount of CO_2 released by mosquitoes was measured using a CO_2 analyser (Li – 7000 CO_2 / H_2O Analyzer, Li – Cor, Licoln, NE, USA) in a way similar to that described by Kambule *et al.* (2011) and Kaiser *et al.* (2014). Three 30 ml glass syringes (Becton Dickson, Franklin Lakes, NJ, USA) were used as respirometry chambers (Kambule *et al.*, 2011). Each of the syringes had a small hole drilled through its wall closer to the top end (plunger's side). The other end of the syringes was attached to a needle which was secured by a three-way stopcock. The first syringe was left empty and served as a control i.e. to detect CO_2 leakage into the system. Individual mosquitoes were each inserted into the remaining two syringes. After inserting the test mosquitoes, the syringes were flushed one at a time through the drilled hole with humid CO_2 free air for three minutes. Humid carbon dioxide free air was obtained by pumping room air through a soda lime scrubber and humidifier. The soda lime scrubber removed carbon dioxide present in pumped air. To humidify the air, it was bubbled through distilled water in a flask. After flushing, the plunger of the syringe was brought down to a 25-ml mark, thereby flushing out all the purged air through the needle and blocking the hole. The stopcock was closed and the three syringes were incubated at a desired temperature (10°C, 15°C, 25°C, 30°C) inside a labcon low temperature incubator (Model L.T.I.E, LABEX, Orange Grove) for 20 minutes.

Air scrubbed of carbon dioxide and water using soda lime and magnesium perchlorate was drawn through the analyser at a flow rate of 100 ml. min $^{-1}$. The flow of air was controlled using a mass flow meter (SABLE - SYSTEMS 2-CHANNEL MASS FLOW CONTROLLER v1.0). After incubation, respiratory chambers were connected to the air system through ports before the water scrubber. Air boluses of 10 ml were consecutively released from the syringes into the air system and recorded one at a time. This was repeated twice i.e. two measurements per syringe. The amount of CO_2 injected into the air stream as measured by the CO_2 analyser was recorded using Expedata software (Sable Systems, Las Vegas, USA). The software recorded the amount of CO_2 in ppm together with the time lapse between consecutive air injections. On completion of the measurements, the mosquitoes were taken to the deep freezer for 10 -15 minutes to make them inactive before they were weighed using a mass scale (Libror AEG-45SM).

Measurements of metabolic rates were conducted randomly on selected days, temperature and treatment group. Each experimental measurement was replicated four times and used two mosquitoes at a time. Individual female mosquitoes were randomly selected for each experiment and used only once.

3.8   Data analysis

3.8.1   Calculations

The amount of CO_2 produced was transformed from parts per million (ppm) to ml. min^{-1} using equation (3.1).

VCO_2 (ml. min-$^{-1}$) in bolus of air = (C1 / 1 000 000) x 100 ml min^{-1})   (3.1)

where: C1 is ppm CO_2

100 ml min^{-1} is the flow rate

To remove leakage from the system, we used the following equation:

ml CO_2 = volume of CO_2 recorded from test syringe - volume CO_2 recorded from control syringe

(3.2)

Integration was used to measure the area under the CO_2 curve using the expedata software. This gave the volume of CO_2 (ml) in each bolus of air sent to the analyser.

The volume of CO_2 in each bolus of air was scaled up to the volume of the chamber and the total CO_2 emitted (ml) was calculated using the following equation:

Total CO_2 (ml) = ml CO_2 x (volume of container (ml) / Volume injected (ml))   (3.3)

Volume of container (ml) = volume of syringe in which the mosquito was confined = 25 ml

Volume injected (ml) = volume of bolus injected = 10 ml

The emission rate of CO_2 in $ml.min^{-1}$ was calculated as following:

Emission rate CO_2 ($ml.min^{-1}$) = Total CO_2 emitted ÷ Time enclosed (minutes)

Total CO_2 emitted was calculated using equation   (3.4)

Time enclosed = incubation time before injection

The units of volume were then converted to nanoliter per minute ($nl.min^{-1}$). To calculate mass specific CO_2 emission rate ($nl.min^{-1}mg^{-1}$), the result was divided by the mass of the mosquito.

3.9   Statistical Analysis

Statistical tests were performed using the SPSS program. I randomized the experiments and assumed normal distribution hence there was no need to transform the data. The effects of the

variables on the response factor were tested at 95 % confidence interval and considered significant if the *P* value was less than or equal to 0.05 (Nunes *et al.*, 2015). To test effects of continuous explanatory variables (temperature and age) on rate of carbon dioxide emission (response), linear regression analysis was used to detect a relationship between variables and response. Thereafter, if a significant correlation was detected, analysis of variance (ANOVA) was run to compare if the mean VCO_2 released at different temperatures was significantly different. If the effects of the explanatory variables (mean VCO_2) differed significantly, a multiple mean comparisons test (Bonferroni) was conducted to determine at which temperatures the mean VCO_2 were significantly different (Kao and Green, 2008). Because status or treatment type of the mosquitoes is categorical data, one-way ANOVA was used to compare the mean VCO_2 for treatment groups. Two-way and three-way factorial ANOVA were used to compare the significance of the interactions of independent factors used in this experiment.

Metabolic rates (VCO_2) are given as mean ± standard error for different temperatures. To compare the reduction of metabolic rates between temperature ranges, I determined percentage decreases using mean differences. Percentage decrease was calculated as follows:

Percentage decrease = (Mean $_{T2}$ – Mean $_{T1}$ / Mean $_{T2}$) x 100

where Mean $_{T2}$ – Mean $_{T1}$ is mean difference in VCO_2 between two temperatures

Chapter 4

4.0   Results

4.1   Effects of temperature on VCO_2 without accounting for age, feeding and mating status of mosquito

As temperature increased, the volume of carbon dioxide produced by mosquitoes also increased after pooling all groups of age and state ($r^2 = 0.34$, $F = 17.4$, $P = 0.0001$, Fig. 4.1). Performing, an ANOVA indicated that changes in temperature significantly varied metabolism of the mosquitoes in all age and status groups ($F = 87.47$, $P = 0.0001$, Table 4.1). Post hoc comparisons using the Bonferroni test indicated that the mean VCO_2 (rate of CO_2 emission) measured at 30 °C (mean = 151.6 ±SE 10 nl.min^{-1} mg $^{-1}$) were significantly higher (approximately an 88 % increase) than that measured at 25 °C (mean = 80.4 ±SE 4.4 nl.min $^{-1}$mg $^{-1}$). The two means were also significantly different from the rest of the means even though very high variance was recorded at 30 °C. Surprisingly, this was not the same at cooler temperatures. The metabolic rate of carbon dioxide produced by mosquitoes at 15 °C and 10 °C were statistically similar (Table 4.1). These observations indicate that the impacts of temperature on corresponding metabolic rates were not uniform from 30 °C to 10 °C. Instead, the decreases in VCO_2 became smaller as temperatures were lower (Table 1).
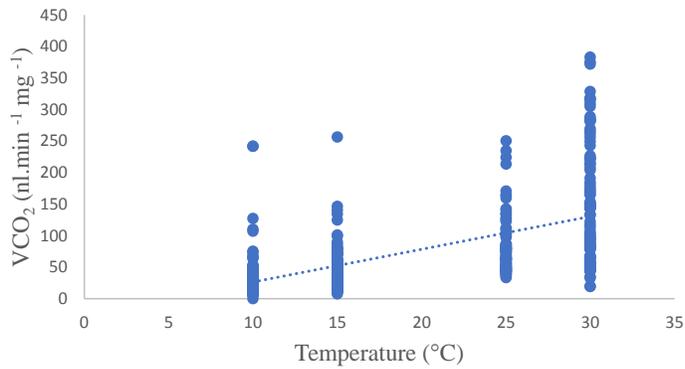
Figure 4.1.   Change in mass specific VCO_2 with an increase in temperature ($r^2 = 0.34$; $F = 17.4$; $P = 0.0001$; $VCO_2 = 5.20$ temperature $- 25.56$).

Table 4. 1.   Mean VCO_2 of all mosquitoes measured at different temperatures.

Temperature (°C)	Mean mass ±SE(mg)	Mean VCO_2 ±SE (nl.min^{-1} mg^{-1})
30 °C	1.28 ±0.03	151.62[a] ±10
25 °C	1.26 ±0.03	80.43[b] ±4.37
15 °C	1.46 ±0.04	48.35[c] ±3.42
10 °C	1.44 ±0.03	33.91[c] ±3.12

Means with different superscript letters are significantly different using Bonferroni test

4.2   The effects of temperature on VCO$_2$ from unmated and sugar fed mosquitoes (Treatment 1)

Unmated sugar fed mosquitoes were considered the control group. After pooling all ages in the group, a linear regression indicated that these mosquitoes responded positively to increases in temperature ($r^2 = 0.39$, $F = 50.9$, $P = 0.000$). After performing a two - way ANOVA, it was discovered that age alone did not impact VCO$_2$ ($F = 0.19$, $P = 0.83$) neither did its interaction with temperature ($F = 1.056$, $P = 0.389$).

Since age had no effects on metabolic rates of unmated-sugar fed mosquitoes, the metabolic rate of mosquitoes for all three age groups measured at each temperature were averaged (Table 4.2). A one-way ANOVA indicated a statistical significant difference in mean VCO$_2$ produced by mosquitoes when measured at different temperatures ($F = 17.35$, $P = 0.00$, Table 4.2). At lower temperatures (15 °C, 10 °C), the mosquitoes ceased to significantly alter their metabolic rates like they did at higher temperatures (30 °C, 25 °C, Table 4.2). Even though mean VCO$_2$ at 15 °C was at par with the mean VCO$_2$ at 10 °C, the percentage decrease in metabolism between the two temperatures was higher than when the temperature had decreased from 30 °C to 25 °C (Table 4.7).

Table 4. 2.   Mean VCO$_2$ of unmated sugar fed mosquitoes at different temperatures.

Temperature (°C)	Mean mass ±SE (mg)	Mean VCO$_2$ ±SE (nl. min $^{-1}$ mg $^{-1}$)
30 °C	1.24 ±0.07	141.700[a]±22.00
25 °C	1.17 ±0.04	94.00[b] ±2.41
15 °C	1.60 ±0.09	47.62[c]±6.44
10 °C	1.40 ±0.05	24.24[c] ±2.68

Means with different superscript letters are significantly different using Bonferroni test

4.3   The effects of temperature on volume of carbon dioxide when mosquitoes were mated and sugar fed (Treatment 2)

Metabolic rates of mated sugar fed mosquitoes decreased with decreasing temperatures (r^2 = 0.42, F = 76.78, P = 0.000). This trend was the same regardless of the age of mosquitoes (2 day old: r^2 = 0.40, P = 0.0001; 3 days old: r^2 = 0.57, P = 0.0001; 4 days old: r^2 = 0.33, P = 0.0003). Therefore, the mean VCO$_2$ was averaged across all age groups for each temperature (Table 4.3). The mosquitoes significantly changed their metabolic rates as temperature dropped from 30 °C to 25 °C (Table 4.3). Thereafter, further reductions in temperature did not result in significantly different metabolic rates. Surprisingly, a five-degree Celsius reduction in temperature at both cooler (10 – 15 °C) and hotter (30 – 25°C) extremes yielded a relatively similar percentage decrease in metabolic rates i.e. 59.20 % and 51.50 % (Table 4.7). Compared to the control group, mated sugar fed mosquitoes indicated a smaller difference in percentage decreases between hotter and cooler temperatures (Table 4.7). However, the trend in percentage decreases was similar in the

two treatments. Thus, both treatments had higher percentage decreases in metabolic rates at lower extreme temperature intervals.

Table 4. 3.   Mean VCO_2 of mated and sugar fed mosquitoes at different temperatures.

Temperature (°C)	Mean mass ±SE (mg)	Mean VCO_2 ±SE (nl.min^{-1}mg^{-1})
30 °C	1.16 ±0.05	174.07[a] ±20.17
25 °C	1.14 ±0.03	84.37[b] ±6.74
15 °C	1.56 ±0.07	52.22[b,c] ±10.44
10 °C	1.31 ±0.07	21.36[c]±1.83

Means with different superscript letters are significantly different using Bonferroni test

4.4   The effects of temperature on emission rates of carbon dioxide when mosquitoes are unmated and blood fed (Treatment 3)

Metabolic rates for unmated blood fed mosquitoes decreased with decreasing temperatures (r^2 = 0.24, F = 30.37, P = 0.00). After comparing the mean VCO_2 measured at different temperatures, it was found that mean VCO_2 recorded at 30 °C was significantly higher than the rest of the measurements (Table 4.4). This was true for all ages except in three-day old mosquitoes. The metabolic rates of unmated blood fed three-day old mosquitoes were independent of temperature (r^2 = 0.08, P = 0.10). No correlation between age and mean VCO_2 was detected after pooling results from all temperatures (r^2 = 0.03, F = 3.00, P = 0.09). However, it was found that only at 10 °C was there a positive correlation between age and metabolic rates (30 °C: r^2 = 0.09, P = 0.0; 25 °C: r^2 = 0.00, P = 0.94; 15 °C: r^2 = 0.04, P = 0.60; 10 °C: r^2 = 0.17, P = 0.02). At this temperature, mean VCO_2 increased with the age of mosquitoes (Fig. 4.2).

Dropping the temperature from 30 °C to 25 °C resulted in 53 % reduction in metabolic rates whilst reducing the temperature from 15 °C to 10 °C yielded a 5.48 % reduction in mean VCO_2. Unmated blood fed mosquitoes reduced their metabolic rates more (53 %) than unmated sugar fed (33.7 %) at warm temperatures. However, the trend was reversed at low temperatures. At low temperatures, unmated blood fed mosquitoes reduced their metabolic rates by 5.48 % whilst unmated sugar fed mosquitoes reduced their metabolic rates by 49.2 % (Table 4.7).
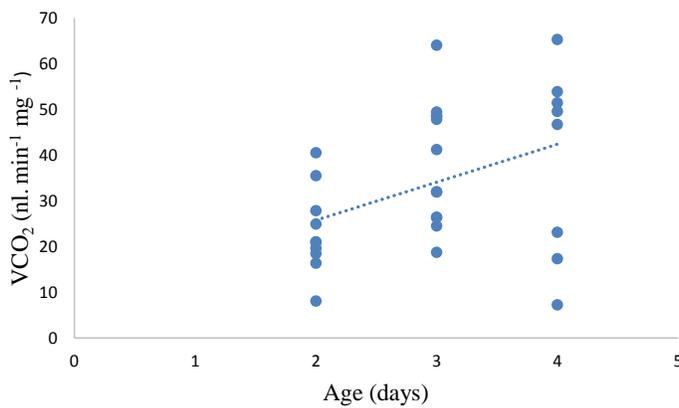
Figure 4. 2.   Change in mass specific VCO_2 for unmated blood fed mosquitoes measured at 10 °C ($r^2 = 0.17$, $F = 5.62$; $P = 0.02$; $VCO_2 = 8.31$ temperature + 9.14).

Table 4. 4.   Mean VCO$_2$ of unmated and blood fed mosquitoes at different temperatures.

Temperature (°C)	Mean mass ±SE (mg)	Mean VCO$_2$ (nl.min^{-1} mg^{-1})
30 °C	1.40 ±1.34	141.72^a±20.00
25 °C	1.46 ±1.46	66.64^b ±5.00
15 °C	1.27 ±1.27	49.23^b±4.49
10 °C	1.48 ±1.48	46.55^b±9.46

Means with different superscript letters are significantly different using Bonferroni test

4.5   The effects of temperature on emission rate of carbon dioxide released when mosquitoes were both mated and blood fed (Treatment 4)

Lowering of temperature significantly reduced metabolic rates for two and three-day old mated blood fed mosquitoes (Table 4.5). For two and three-day old mosquitoes, metabolic rates were significantly different only at 30 °C and 25 °C (Table 4.5).

Mated and blood fed fours day old mosquitoes maintained constant metabolic rates regardless of ambient temperatures (F= 5.03, P = 0.21). Compared to results from other groups, four-day old mated and blood fed mosquitoes had lowest metabolic rates at 30 °C (Table 4.9). At 10 °C, this group also had the highest metabolic rates if compared to all other groups except for three-day old unmated blood fed mosquitoes (Table 4.9). Diet and mating status of the mosquitoes impacted metabolic rates of fours day old mosquitoes only at 10 °C (F = 5.45, P = 0.037, Table 4.9). Age had no effects on metabolic rates of mated and blood fed mosquitoes except at 15 °C whereby metabolism increased with age (r^2 = 0.37, P = 0.08, Figure 4.3).
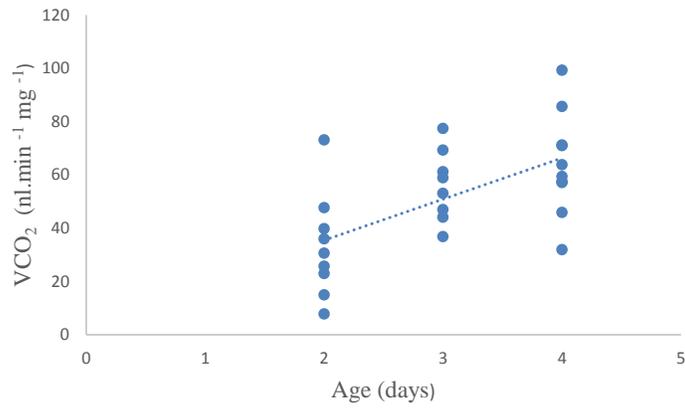
Figure 4. 3.   Change in mass specific VCO_2 of mosquitoes measured at 15 °C ($r^2 = 0.37$, $P = 0.008$; $VCO_2 = 15.42$ temperature $+ 4.61$).

Table 4. 5.   Mean VCO_2 for mated and blood fed mosquitoes at each age and temperature
group.

Age (days)	Temperature (°C)	Mean mass ±SE (mg)	Mean VCO_2 ±SE (nl.min^{-1} mg^{-1})
2	30	1.33 ±0.11	100[a] ±22.7
	25	1.15 ± 0.06	66.6[b] ±5.7
	15	1.29 ±0.14	33.2[c] ±6.5
	10	1.43 ±0.06	30.2[c] ±3.6
3	30	1.46 ±0.07	213.2[a] ±35.7
	25	1.44 ±0.13	84.8[b] ±24.3
	15	1.42 ±0.10	57.7[c] ±4.47
	10	1.69 ±0.12	48.1[c] ±12.8
4	30	1.32 ±0.13	87.3[b] ±19.0
	25	1.36 ±0.07	76.4[b] ±10.2
	15	1.36 ±0.13	63.5[b] ±5.5
	10	1.57 ±0.09	54.4[b] ±10.5

Means with different superscript letters are significantly different

4.6   Comparison of metabolic rates across treatments, age and temperature

Overall, it was discovered that change in temperature was the only factor which had a significant effect on the metabolic rates of *A. quadriannulatus*. Changing the ambient temperature did not impact the metabolic rates of mosquitoes of different ages or status. However, differences were noted if all the three factors acted on an individual mosquito (Table 4.6).

Table 4. 6.   Effects of interacting age, temperature and status of the mosquito on VCO_2

Source of variation	d.f	S.S	F	P
Age	2	2149.25	0.198	0.821
Temp	3	955065.1	58.611	0
Mosstatus	3	4656.298	0.286	0.836
Age*Temp	6	35059.56	1.076	0.376
Age * Mosstatus	6	22340.86	0.686	0.661
Temp* Mosstatus	9	42846.53	0.876	0.546
Age*Temp* Mosstatus	18	161934.5	1.656	0.045

S.S = sum of squares, d.f = degrees of freedom

4.7   Comparison of percentage decreases in metabolic rates across treatments

As previously mentioned, mosquitoes decreased their metabolism unevenly based on the level of ambient temperature. Comparing percentage decreases of metabolic rates in all treatment groups (Table 4.7) indicated a diet dependant trend whereby blood fed mosquitoes (both unmated and mated) decreased their metabolism more when the temperatures decreased from 30 °C to 25 °C than when temperatures were reduced from 15 °C to 10°C. Sugar fed mosquitoes did the opposite. They reduced their metabolism more at cold temperatures than at warm temperatures even though there was very little difference.

In four-day old mosquitoes, sugar fed females (both mated and unmated) reduced their metabolism in a similar way regardless of where the temperature was reduced from (Table 4.8). Mated and blood fed four days old mosquitoes indicated a similar trend, only that its percentage

decrease was the least in both temperature interval. Unmated and blood fed mosquitoes had a huge percentage decrease in VCO_2 at warm temperatures and far less at cold temperatures. Blood fed mosquitoes (both mated and unmated) had the lowest percentage decreases at cold temperatures.

Table 4.7.　　Average percentage decreases in metabolic rates for different treatments with the ages combined.

Change in Temperature (°C)	Percentage decrease (%) in mean VCO_2			
	Treatment 1	Treatment 2	Treatment 3	Treatment 4
30 – 25	33.7	51.5	53	43.3
15 - 10	49.2	59.2	5.48	15.7

Table 4. 8.   Percentage decrease in metabolic rates of fours day old mosquitoes from all treatments.

Change in Temperature (°C)	Percentage decrease in mean VCO_2 (%)			
	Treatment 1	Treatment 2	Treatment 3	Treatment 4
30 -25	56.9	55.6	68.1	12.5
15-10	55	53	29.1	14.5

A summary of the results is presented in Table 4.9. The table shows emission rates calculated as an average of all the replicates per age group per temperature per state of mosquito. Across the table is a comparison of mean VCO_2 with state of the mosquitoes per age group at different temperatures. A temperature - VCO_2 relationship per age and status is indicated down the table.

Table 4. 9.　Mean VCO_2 measured at different temperatures for mosquitoes varying in age and status.

Age (Days)	Temperature (°C)	Non-Blood fed		Blood Fed		Test values	
		Unmated	Mated	Unmated	Mated	F-Value	P-value
2	30 ℃	161.6 ±35.0	160.9 ±33.4	116.8 ±23.2	100 ±22.7	0.94	0.45
	25 °C	96.1 ±18.2	98.9±10.1	66.6 ±12.2	66.6±5.7	0.95	0.43
	15 °C	50.2 ±7.0	64.0 ±27.3	35.9 ±5.8	33.2±6.5	0.91	0.45
	10 °C	25.4 ±3.6	19.3 ±3.4	23.3 ±3.0	30.2±3.6	1.8	0.16
F value		8.64	8.38	11.10	11.58		
P value		0.0002	0.0003	0.0001	0.0001		
3	30 °C	139.5±17.5	217.0±34.8	118.3 ±26.2	213.±35.7	2.52	0.08
	25 °C	90.9±15.6	82.1 ±13.4	67.53 ±7.3	84.8 ±24.3	0.33	0.8
	15 °C	45.2±10.8	38.1±9.1	56.8 ±7.2	55.7 ±4.47	0.7	0.56
	10 °C	23.0 ±4.1	18.5 ±3.0	67.3 ±20.0	48.1 ±12.8	2.55	0.07

Mean VCO_2 ±SE (nl.min $^{-1}$mg $^{-1}$)

F value		8.73	22.28	1.78	12.59	
P value		0.0002	0.0001	0.17	0.0001	
4	30 °C	157.40 ±34.8	150.3±35.3	206.1±51.4	87.3 ±19.0	1.24	0.31
	25 °C	67.8 ±4.9	66.7 ±10.1	65.8±8.9	76.4±10.2	0.21	0.89
	15 °C	52.5 ±13.6	54.9±12.1	55.9±8.7	63.6±5.5	0.27	0.84
	10 °C	23.6±3.2	25.8±2.7	39.3±7.3	54.4 ±10.5	5.45	0.037
F value		7.8	7.13	10.19	1.60	
P value		0.0004	0.0008	0.0002	0.21	

Mean ± SE across rows is a comparison of mean VCO_2 from different states of mosquitoes at a specific age and temperature

Mean ± SE vertical down is a comparison of VCO_2 as temperature decreases at each age and status

Test values indicate difference in means

Chapter 5

5.0   Discussion

5.1   Effects of temperature on metabolic rates without accounting for age, mating and feeding status.

This study found evidence of a direct effect of temperature on the metabolic rates of *A. quadriannulatus* mosquitoes which resembled the general trend found in most insects (Canzano *et al*., 2006; Denlinger and Armbruster, 2014; Reisen *et al*., 2006; Roberts, 1978). In addition, the metabolic rates of mosquitoes were affected differently during a 5 °C drop in temperature at both hot and cold temperature levels. These findings concur with measurements made by researchers on mosquitoes, such as Huestis *et al*. (2012), Lehman *et al*. (2014), Yaro *et al*. (2012), Lyons *et al*., (2012) and Denlinger and Armbruster, 2014. Changes in ambient temperatures affect the metabolic rates of mosquitoes resulting in change in their behaviour and fitness (Bayoh, 2001; Beck-Johnson *et al*., 2013; Denlinger and Armbruster, 2014; Lehmann *et al*., 2014).

The metabolic rates of *A. quadriannulatus* measured at 30 °C and 25 °C differed significantly from each other and the rest of the measurements. This was not the same at colder temperatures, whereby there was less variation in metabolic rates as temperature dropped from 15 °C to 10 °C. According to, Beck-Johnson *et al*. (2013), mosquito populations are predicted to persist between 17 °C and 33 °C and they are more abundant in ecosystems at temperatures between 20 °C and 30 °C. Bayoh (2001) discovered that adult mosquitoes survive better when ambient temperature prevails between 15 °C and 25 °C and are most abundant in areas where temperatures range from 22 °C to 26 °C. These findings are in the range predicted by simulation

models conducted by Beck-Johnson *et al*. (2013). The findings of the present study display a trend similar to the outcomes of the two studies above.

The metabolic rates of *A. quadriannulatus* were always highest at temperatures between 25 °C and 30 °C except in mated and blood fed four-day old mosquitoes (Table 4.9). Due to the high metabolic rates at 25 °C and 30 °C, development and survival rates of the mosquitoes at those temperatures are high too. Thus, there are more mosquitoes in summer than in winter. Even though high metabolic rates associated with high temperatures could explain why there are more mosquitoes in summer than winter they do not explain the short recovery time of mosquitoes at the beginning of spring (Lehman *et al.*, 2014). However, the reason why there are few mosquitoes in winter is probably due to low metabolic rates recorded at 10 °C and 15 °C. The metabolic rates of mosquitoes recorded at 10 °C and 15 °C were always statistically similar and lowest in all treatment groups (Table 4.9). Personal observations indicated that the test mosquitoes were very inactive at these temperatures. This was expected since it is common in most insects to lower their metabolic rates in response to low temperatures (Russell *et al*., 2013). It was however surprising for the metabolic rates measured at 10 °C to be found statistically similar to those measured at 15 °C. Based on relevant literature such as Wallace and Grimstad (2002), Gray and Bradley (2003, 2005), Zhou and Miesfeld, (2009) and Denlinger (2014), mosquitoes that can overwinter must be able to significantly lower their metabolism in response to a decline in temperatures. Because Table 2.1 shows that the intrinsic metabolic rates of different species of mosquitoes are relatively similar at equal temperatures, it was expected that *A. quadriannulatus* would respond to lowered temperature in a manner displayed by *Culex* mosquitoes. *Culex* mosquitoes found in northern hemisphere survive winter temperatures by significantly reducing their metabolism during

diapause. If *A. quadriannulatus* was able to overwinter physiologically, metabolic rates recorded at 10 °C were expected to be significantly lower than those measured at 15 °C.

According to Beck-Johnson *et al*., (2013) population densities of *Anopheles* mosquitoes fall sharply when temperatures drop below 17 °C because low temperatures reduce their activity and development. Due to this inactivity, the distribution of mosquitoes becomes uneven and their abundance in ecosystems could be perceived as minimal especially during winter (Lehmann *et al*., 2014). Prolonged mosquito inactivity during periods of low temperatures hinders the mosquitoes from accessing food. Thus, the survival of the mosquito at the time is highly threatened especially in the absence of an energy conserving mechanism. This could mean that the mosquitoes die in the cold and hence fewer mosquitoes, if any, are found in cooler areas like the East African highlands and in winter (Beck-Johnson *et al*., 2013).

Findings of this study concur with this suggestion. The failure of the mosquitoes to reduce metabolic rates when temperatures dropped from 15 °C suggests that they lack a physiological adaptation to cope with low temperatures. Since metabolic rates were similar at 15 °C and 10 °C, it means the mosquitoes will need to find food to maintain their metabolism. This may not be possible if there is prolonged flight inactivity caused by reductions in temperatures therefore the mosquitoes will most probably die. However, if the winter is interceded by days of warmer temperatures, mosquitoes could probably fly out to replenish their food reserves. Leeson (1931) reported that in Zimbabwe, *A. gambiae s.l*, disappear completely from the ecosystem when absolute minimum temperature falls below 5 ° C. The present study however, did not investigate metabolic rates below 5 °C, because winter temperatures in South Africa rarely reach 5 °C in the mosquito's habitat.

Under optimal circumstances, it would be expected that a minimum of nine days will be required to generate new adults from supposedly overwintered adults, but the *A. gambiae* populations proliferate within five days of the onset of rains (Lehmann *et al.*, 2010). Lack of a window period for reestablishment of mosquitoes in those areas has raised questions on sources of mosquitoes at the advent of spring. Mosquito infestations come earlier than expected i.e. before a new generation can be produced or immigrants could arrive. It is therefore important to decipher the source of this founder population for the effective control of the vector. The speculation of lack of a physiological overwintering mechanism in *A. quadriannulatus* gathered from the findings of the current study is however not conclusive due to a variety of reasons. Firstly, the study subjects were exposed to cold temperatures for only 20 minutes under laboratory conditions which could not have been adequate for the mosquito to fully express its intended response to lowered temperatures. Twenty minutes is too short for the mosquito to substantially show the signs of overwintering due to change in temperature. Thus, further experiments could look at the effects of exposing *A. quadriannulatus* to low temperatures for longer periods. Secondly, diapausing insects are hormonally "preprogrammed" to enter into diapause by some environmental cues. For instance, shortening of days signalling the arrival of winter is a reliable cue for many insects which go into diapause (Denlinger and Armbruster, 2014). This factor was not accounted for in the current experiment and hence it was not possible to detect the ability of the mosquito to enter diapause. There is little information about diapause in sub Saharan *A. gambiae* species. Lehmann *et al*. (2010) suggested that aestivation is used in *A. gambiae* species as a survival mechanism in the long dry and hot areas.

Winter seasons in temperate Africa are not as cold as in the northern hemisphere and are usually interrupted by periods of warm temperatures. It follows that *A. gambiae* mosquitoes in

sub-Saharan Africa could physiologically overwinter by mechanisms other than diapause. Cold torpor could be utilised to counteract non-life threatening daily reductions in temperature (Roberts, 1978) since it is short termed. Because quiescence is seasonal, lasts longer than cold torpor (Roberts, 1978) and is associated with lowered metabolic rates in the cold (Nelms *et al*., 2013), it follows that *A*. *gambiae* species could overwinter by quiescence in temperate African highlands. The findings of the current study however do not infer on this mechanism either. This is because *A*. *quadriannulatus* failed to significantly reduce its metabolic rates at cold temperatures. This leaves the gap in the characterisation of overwintering strategies in *A*. *gambiae* unresolved.

Even though some of these possibilities are somehow not supported by the findings of my study, I would think that temperate *A*. *quadriannulatus* and other members of *A*. *gambiae* species are thermally opportunistic. That is, depending on the levels of temperature during winter, they can adopt a combination of non-physiological strategies to meet the high unpredictability of ambient temperature. For example, during short spells of low temperatures, *A*. *quadriannulatus* could maintain their metabolic rates, until the time when temperatures are warm enough for them to replenish their energy sources. Similarly, the mosquitoes could hide in microhabitats when it is cold and occasionally fly out on warmer days (Russell *et al*., 2013). In this way, by adopting any of these possibilities, *A*. *quadriannulatus* and its *A*. *gambiae* relatives can manage to overwinter as adults in temperate regions of Africa. However, more research is required to explain the sources of mosquitoes and short recovery periods experienced soon after the first spring rains.

To compare whether mosquitoes dropped their metabolism by a similar magnitude in cold and warm temperature ranges, we determined the percentage decreases in metabolic rate over a 5 °C range. Huestis *et al*. (2011) reported that a 1 °C change in ambient temperature between 25.1 °C and 32.8 °C altered metabolic rates of Sahelian *A*. *gambiae* by six percent. In this study, we

found an overall 30 % percent over a 5 ℃ drop at the cooler temperatures of 15 – 10 ℃ while over a 5 ℃ drop at the warmer temperatures, 30 – 25 ℃, there was an overall 47 % percent decrease in metabolic rate. This is a clear indication that a 1 °C change in temperatures does not yield equal percentage drop in metabolic rates.

The physiological state of mosquitoes can have significant impacts on their metabolic rates. For this reason, it was imperative for the present study to assess if mosquitoes responded similarly to changes in temperature when their age, mating and feeding status were manipulated. Most important is knowing how the physiological state of a mosquito affects its metabolic response to changes in temperature. This will highlight whether the mosquito is capable of overwintering in that state or not. The physiological state in which the mosquito overwinters as, could have significant impact on the population dynamics of the following season. For example, adult mosquitoes which overwinter in gravid state, will lay their eggs as soon as the breeding sites are available. In this way, the population of mosquitoes could proliferate quickly.

5.2   Effect of age on metabolic rates

Both unmated and mated female mosquitoes which did not receive a blood meal (sugar fed only) did not show any correlation between age and metabolic rates at all temperatures. A correlation was however detected in some blood fed mosquitoes at specific temperatures. Lack of an age-metabolic rates correlation in sugar fed mosquitoes suggests the mosquitoes did not undergo any form of tissue development as they aged four days past emergence. Gray and Bradley (2003) found that the resting metabolic rates of *C. tarsalis* measured at 25 °C increased as the mosquitoes aged from emergence to four days and ceased thereafter. This could be explained by the fact that *C. tarsalis* is autogenous and therefore *C. tarsalis* commenced egg development without a blood meal (Gray and Bradley, 2003). In *A. gambiae* a blood meal is required to mature

and rippen eggs even though oogenesis commences a few hours after emergence (Gillett, 1971). It is possible that in the sugar fed sample of *A. quadriannulatus* oogenesis did not take place during the period highlighted by Gillett (1971) but it only commenced when the mosquitoes had taken a blood meal. Again, without taking a blood meal, the changes in growth due to oogenesis were most likely too small to impact on the metabolic rates of unmated and mated sugar fed mosquitoes.

Age played a role in two cases in my study. Both blood fed mated and unmated mosquitoes produced more CO_2 as they aged from two to four days old at 15 °C and 10 °C respectively. Although the temperatures were different, the positive correlation observed in both unmated and mated blood fed mosquitoes suggest that blood feeding impacts on the physiology of the mosquito regardless of its mating status. Even though Klowden and Russell (2004) found the presence of small post emergence growth (between 0-60 h) in both unmated and mated blood fed female mosquitoes, the difference in size of their accessory glands was only significant at 36 h after emergence. Because the 0-60 h growth was more rapid in mated than unmated mosquitoes, follows that mated mosquitoes were undergoing egg development at that time which was optimal at 36 h. According to Gillett (1971), distention of the abdomen of a mosquito after consuming a blood meal, causes the mosquito to develop ovaries beyond resting stage. Therefore, a blood meal was sufficient to trigger maturation of oocytes in mated *A. quadriannulatus* at 15 °C. Because unmated blood fed females also indicated correlation between age and metabolic rates at 10 °C, it follows that either there was post emergence growth as indicated by Klowden and Russell (2004) or the increases in metabolic rates was a mere result from blood digestion. According to Gray and Bradley (2003), at 25 °C blood digestion commenced 20-24 h post blood feeding and lasted for approximately 55 h. The 20-24 h duration coincides with the time taken by *C. tarsalis* to develop its peritrophic matrix (Houk *et al*., 1979). This could have been the reason why unmated

mosquitoes increased their metabolic rates with age at 10 °C. The rate of blood digestion could have been slower though at this temperature and hence the process could have taken more than 55 h. Because blood digestion and egg development are dependent on time and temperature , (Gillett, 1971, Klowden and Russell, 2004, Mala *et al.*, 2014), it can be deduced that these processes were underway at the time of measurements in either of the treatments.

5.3   Effects of mating and blood feeding on metabolic rates at constant temperature and age

5.3.1   Mating only

Sugar fed only mosquitoes (both unmated and mated) test mosquitoes responded to changes in temperature differently. Dropping the temperature by 5 °C from 30 °C, yielded a percentage reduction in metabolic rates which were lower than if temperature was reduced from 15 °C to 10 °C (Table 4.7) in both treatments. Even though such a trend was observed, sugar fed mosquitoes yielded relatively similar percentage decreases in metabolic rates at both cold and warm temperature changes (Table 4.7). Because the mosquitoes were not given a blood meal to prompt blood digestion or egg development, cold temperatures appear to have induced inactivity in *A. quadriannulatus*. In this way, there was not much variation in metabolic rates as temperatures were reduced from 15 °C to 10 °C.

Results of the current study indicate that an additional effect other than temperature was induced by mating. The percentage decrease in metabolic rates for mated sugar fed mosquitoes were relatively higher than those for unmated mosquitoes measured at both cold and warm temperatures intervals. This observed difference in response to temperature changes between mated and unmated mosquitoes could have resulted from the effects of molecular interaction on reproductive behaviour and physiology of females during copulation (Baldini *et al*., 2013). For

monoandrous mosquitoes such as *A. gambiae*, multiple copulations are prevented by restricting the movement of female mosquitoes using substances secreted by male mosquitoes during copulation Klowden (1999). Thus, mated mosquitoes had lowered metabolic rates due to reduced activity induced by copulation with male mosquitoes. After discovering that virgin female mosquitoes live longer than their inseminated counterparts, the sexual activity in mosquitoes could accelerate the process of senescence (Collatz, 2003).

The role of mating in mosquitoes involves merely the laying of eggs and not their development (Gillett, 1971; Yaro *et al*., 2012). This idea is supported by Klowden and Russell (2004) who found no evidence of significant impacts of mating on the size of unmated and mated female accessory glands in *A. gambiae* between emergence and 36 h post emergence. Gray and Bradley (2005) also found that at 25 °C, mating had no significant effects on the metabolic rates of *C. tarsalis* as mosquitoes aged from emergence to 12 days old. Based on these findings, the impacts of mating on metabolic rates observed in *A. quadriannulatus*, could have resulted from sources other than egg development.

5.3.2 Blood feeding only

Unexpectedly high metabolic rates were measured at 10 °C (Table 4.9) in unmated blood fed three days old mosquitoes. There are two possible processes which could have resulted in this outcome, namely blood digestion and egg development. Both blood digestion and egg development consume relatively large amounts of energy. Thus, to sustain these processes, the mosquitoes had to maintain relatively high metabolic rates at this temperature. Because unmated *Anopheles* mosquitoes are capable of resuming egg development without insemination, it follows that egg development was underway in unmated three days old mosquitoes at 10 °C. According to, Baldini *et al*. (2013) *A. gambiae* mosquitoes would choose to use the blood meal for oogenesis,

if the mosquito is inseminated, otherwise the meal would be digested and used as an energy source. Contrary Gillett, (1971) and Yaro *et a*l., (2012), indicates that oogenesis would commence in *Anopheles* mosquitoes regardless of mating.

Since this observation was made at relatively low temperatures, one would think that gonotrophic dissociation rather than egg development was underway during the outcome. Muirhead-Thomson (1951) suggested that in cold temperatures, mosquitoes are most likely to invest their blood meals towards gonotrophic dissociation rather than egg development. According to Gray and Bradley, (2003), it takes more energy to digest one molecule of a blood meal than it does for a sugar meal. It is however, difficult to differentiate whether the observed outcome was a result of blood digestion or egg development. This is because blood digestion would still take place whether eggs were formed or not. What is different and could not be detected in this study was the fate of the digested blood meal. If the mosquitoes underwent gonotrophic dissociation, the digested blood meal was used to store energy and the mosquitoes did not develop eggs. But if egg development commenced then the mosquitoes became gravid thereafter.

The drop in metabolic rates when temperature decreased from 30 °C to 25 °C was higher than when temperature decreased from 15 °C to 10 °C for both unmated and mated blood fed mosquitoes. This was different in sugar fed mosquitoes (both mated and unmated) whereby the percentage decreases were relatively similar at those changes in temperature. Because *A. quadriannulatus* is non-autogenous, this explains why sugar fed mosquitoes did not indicate any differences in reducing their metabolic rates when temperatures were decreased from 30 °C to 25 °C and when they were reduced from 15 °C. Thus, a blood meal initiated blood digestion and egg development resulting in observed outcome. In blood fed mosquitoes (both unmated and mated), the mean VCO_2 were relatively higher at hot temperature intervals than at the lower temperature

intervals. This is because at higher temperatures, the mean VCO_2 were always significantly different and higher whereas at lower temperatures the mosquitoes retained constant and lower metabolic rates. Gonotrophic processes and duration are dependent on ambient temperatures and are quicker at higher temperatures (Gillett, 1971). This is one of the reasons why metabolic rates of blood fed *A*. *quadriannulatus* were higher at high temperatures. Blood digestion and egg development in mosquitoes are positively correlated to temperature (Mala *et al*., 2014).

5.3.3 Mating and blood feeding

Two and three day old mated and blood fed mosquitoes followed the general temperature-metabolic rate trend observed in other treatments. Surprising results obtained from four days old mosquitoes were different as these mosquitoes maintained relatively steady metabolic rates across all temperatures. Since blood digestion commences 20-24 h after a blood meal (Houk *et al*., 1979, Gray and Bradley, 2003), it follows that *A*. *quadriannulatus* mosquitoes commenced blood digestion on day two as they were blood fed on their day of emergence. At the end of day two, it means the mosquitoes were half gravid and gravid (ready to lay eggs) on day three (egg development and maturation was completed). Eggs should have been laid on the night of day three. Therefore, metabolic rates were lower on day four to mark the end of egg production. This explains why at 30 °C, metabolic rates increased to a peak on three days old but subsequently decreased on four days old (Table 4.5, Table 4.9).

Speculated egg production in this treatment could have been confirmed by checking for eggs in the cages on day four but this activity was out of the scope of this study. However, because *A*. *gambiae* mosquitoes can only oviposition their eggs after finding a suitable site (Kaiser *et al*., 2014), we assume mosquitoes from this treatment were unable to lay eggs as the experiment did not make provision for oviposition site. According to Muirhead-Thomson (1951), under optimal

conditions, a female mosquito which receives a blood meal on Monday night will be half gravid on Tuesday night and fully gravid by Wednesday night before it disappears to find a suitable oviposition site where it can lay its eggs the following morning at dusk. Based on the findings of the present study, *A. quadriannulatus* most probably underwent similar procedure at time frames similar to those stipulated by Muirhead-Thomson (1951). However, this could only be true at comparable temperatures such as 30 °C and 25 °C. A similar process could have commenced at low temperatures but at much slower rates resulting in the extension of the gonotrophic cycle. Also, due to egg development the mosquitoes needed to maintain high metabolic rates as described in section 5.3.2. Thus, metabolic rates remained relatively higher at 10 °C for four days old mosquitoes.

Four day old mated and blood fed mosquitoes maintained similar metabolic rates at temperature range of 30 °C to 10 °C. This is because at 30 °C, metabolic rates were lower than expected most probably due to completion of egg development. At the same time, metabolic rates at 10 °C were also higher than expected because egg development was still underway hence more energy was required to maintain the process at that temperature. Because the rates of blood digestion and egg development strongly depend on ambient temperature (Baldini *et al*., 2013; Mala *et al*., 2014), it is possible that duration of gonotrophic cycle was less at 30 °C than was at 10 °C. But because the test mosquitoes were only exposed to these temperatures for twenty minutes. this effect could not be accounted for in this experiment.

Another reason for steady metabolic rates at different temperatures could be gravidity of the female mosquitoes. Female *A. gambiae* mosquitoes become gravid only when mated and blood fed (Klowden, 1999). Gravid *A. gambiae* mosquitoes are sedentary during the day and become more active at dawn or dusk when they seek oviposition sites (Muirhead-Thomson, 1951). Since

metabolic rates of all mosquitoes were measured during day time when the gravid-semi gravid mosquitoes were all sedentary, it logically follows that their metabolic rates were similar at all temperatures.

Four day old mated and blood fed mosquitoes decreased their metabolic rates more at the colder temperature interval than at the warmer temperature interval (Table 4.9). This shows the strong dependence of blood digestion and egg production on temperature. Both processes could be retarded at cold temperatures resulting in steadier rates of reaction than when the temperatures are higher. This is also demonstrated by one of the outcomes of this study, whereby only at 10 °C, the metabolic rates for four days old mosquitoes with different mating and feeding state were significantly different from each other.

5.5   Future research / recommendations

My experiment has been able to answer some questions regarding factors that may affect the dynamics of mosquito populations across season. However, there are some aspects that can be improved from this experiment as well as some grey areas with regards to population dynamics and ecology of *A. quadriannulatus* that still needs to be researched. The first improvement I would recommend from the current experimental setup is increasing the incubation time of respective mosquitoes to assimilate the exposure time experienced by wild caught mosquitoes. Instead of using different individual mosquitoes to measure metabolic rates at different temperatures, metabolic rates can be monitored continually on a sampled group of mosquitoes as temperatures are decreased. This will allow each mosquito to be exposed to a variety of temperatures and indicate a better trend of change in metabolism with decrease in temperature (Lyons *et al*., 2012). If we are to infer on overwintering strategies such as diapause, it would be necessary to incorporate

all the aspect of day length in the experimental set up. The experiment can be redesigned in a way that mimics natural light regime in both length of exposure and quality.

Future research should incorporate oviposition sites to allow gravid mosquitoes to lay eggs. This will help differentiate if mosquitoes underwent gonotrophic dissociation or egg development. Presence of eggs in the cages will confirm egg development. Again, monitoring the presence of black faeces caused by blood digestion can help to differentiate the fate of blood meal. Therefore, presence of black faeces and lack of eggs in the cage will confirm gonotrophic dissociation. If both methods are incorporated into the whole system, mosquitoes which only undertook gonotrophic dissociation at low temperatures, could be differentiated from those that developed eggs. Because the duration of gonotrophic cycle is dependent on temperature, it will be imperative for future studies to use older mosquitoes to accommodate delays in egg production due to temperature changes.

Another possible source of mosquitoes at the beginning of spring could be overwintered pupae. It takes 2-3 days for pupae to develop into an adult. If the pupae which emerge at the end of summer, overwinter as pupal stage throughout winter it is possible that young adults will be produced within the first 2-3 days in spring. Thus, mosquitoes will be able to re-establish their populations earlier than expected. This is therefore an important research area whose findings can even help in crafting of more effective malaria control measures.

5.6   Conclusions

The main finding of this study was that despite being inactive at low temperatures, *A. quadriannulatus* mosquitoes failed to significantly lower their metabolic rates. Instead, they maintained constant metabolism as required for blood digestion and egg development regardless

of the cooler temperatures. This indicates that they are unable to overwinter using survival strategies such as diapause and quiescence. Therefore, if these mosquitoes are to survive low temperatures and still maintain high metabolic rates, they can only do so over short cold periods. This appears to be possible especially in sub-Saharan regions where winter seasons do not reach temperatures below freezing and are usually interceded by warm days. Thus, the mosquitoes will maintain constant metabolisms when temperatures are unbearably low but will be able to replenish their energy resources on warm days. The results of the present study could have implications on the future distribution of most mosquitoes due to climate change. The predicted rise in temperature and increased rainfall might prolong and intensify breeding in mosquitoes resulting in increased abundance and invasion of new habitats.

References

Adamou, A., Dao, A., Timbine, S., Kassogué, Y., Diallo, M., Traoré, S.F., Huestis, D.L., Lehmann, T., 2011. The contribution of aestivating mosquitoes to the persistence of Anopheles gambiae in the Sahel. Malar. J. 10, 151.

Afrane, Y.A., Githeko, A.K., Yan, G., 2012. The ecology of Anopheles mosquitoes under climate change: Case studies from the effects of deforestation in East African highlands. Ann. N. Y. Acad. Sci. 1249, 204–210. doi:10.1111/j.1749-6632.2011.06432.x

Baber, I., Keita, M., Sogoba, N., Konate, M., Diallo, M., Doumbia, S., Traoré, S.F., Ribeiro, J.M.C., Manoukis, N.C., 2010. Population size and migration of anopheles gambiae in the Bancoumana region of Mali and their significance for efficient vector control. PLoS One 5. doi:10.1371/journal.pone.0010270

Baldini, F., Gabrieli, P., South, A., Valim, C., Mancini, F., Catteruccia, F., 2013. The Interaction between a Sexually Transferred Steroid Hormone and a Female Protein Regulates Oogenesis in the Malaria Mosquito Anopheles gambiae. PLoS Biol. 11. doi:10.1371/journal.pbio.1001695

Bayoh, M.N., 2001. Studies on the development and survival of Anopheles gambiae sensu stricto at various temperatures and relative humidities. PhD thesis 134.

Beck-Johnson, L.M., Nelson, W.A., Paaijmans, K.P., Read, A.F., Thomas, M.B., Bjørnstad, O.N., 2013. The effect of temperature on Anopheles mosquito population dynamics and the potential for malaria transmission. PLoS One 8. doi:10.1371/journal.pone.0079276

Beier, J.C., Copeland, R., Oyaro, C., Masinya, a, Odago, W.O., Oduor, S., Koech, D.K., Roberts, C.R., 1990. Anopheles gambiae complex egg-stage survival in dry soil from larval

development sites in western Kenya. J. Am. Mosq. Control Assoc. 6, 105–109.

Bowen, M.F., 1992. Terpene-sensitive receptors in female Culex pipiens mosquitoes: Electrophysiology and behaviour. J. Insect Physiol. 38, 759–764. doi:10.1016/0022-1910(92)90028-C

Canzano, A.A., Krockenberger, A.A., Jones, R.E., Seymour, J.E., 2006. Rates of metabolism in diapausing and reproductively active tropical butterflies, Euploea core and Euploea sylvester (Lepidoptera: Nymphalidae). Physiol. Entomol. 31, 184–189. doi:10.1111/j.1365-3032.2006.00506.x

Capinera, J.L., 2008. Encyclopedia of Entomology, Igarss 2014. doi:10.1007/s13398-014-0173-7.2

Coetzee, M., Craig, M., Le Seur, D., 2000. Distribution of Africa Malaria Mosquitoes Belonging to the Anopheles gambiae complex. Parasitol. Today 16, 149–151.

Coetzee, M., Kruger, P., Hunt, R.H., Durrheim, D.N., Urbach, J., Hansford, C.F., 2013. Malaria in South Africa: 110 years of learning to control the disease. South African Med. J. 103, 770–778. doi:10.7196/SAMJ.7446

Collatz, K.-G., 2003. Aging and environmental conditions in insects, in: Aging of Organisms. Springer, pp. 99–123.

Craig, M., Le Sueur, D., Snow, B., 1999. A climate-based distribution model of malaria transmission in sub-Saharan Africa. Parasitol. Today 15, 105–111. doi:10.1016/S0169-4758(99)01396-4

Craig, M.H., Kleinschmidt, I., Nawn, J.B., Le Sueur, D., Sharp, B.L., 2004. Exploring 30 years

of malaria case data in KwaZulu-Natal, South Africa: Part I. The impact of climatic factors. Trop. Med. Int. Heal. 9, 1247–1257. doi:10.1111/j.1365-3156.2004.01340.x

Dao, A., Yaro, A.S., Diallo, M., Timbiné, S., Huestis, D.L., Kassogué, Y., Traoré, A.I., Sanogo, Z.L., Samaké, D., Lehmann, T., 2014. Signatures of aestivation and migration in Sahelian malaria mosquito populations. Nature 516, 387–90. doi:10.1038/nature13987

De Meillon, B., 1951. Malaria survey of South-West Africa. Bull. World Health Organ. 4, 333–417.

Denlinger, D.L., 2002. Regulation of diapause. Annu. Rev. Entomol. 47, 93–122. doi:10.1146/annurev.ento.47.091201.145137

Denlinger, D.L., 1974. Diapause Potential in Tropical Flesh Flies. Nature 252, 223–224. doi:10.1038/252223a0

Denlinger, D.L., Armbruster, P.A., 2014. Mosquito Diapause. Annu. Rev. Entomol 59, 73–93. doi:10.1146/annurev-ento-011613-162023

Diseases, V.P., Diseases, S., For, S., Resistance, A., Borders, B.O.U.R., 2017. Communicable Diseases Communiqué 16, 1–9.

Eldridge, B.F., 1987. Diapause and Related Phenomena in Culex Mosquitoes: Their Relation to Arbovirus Disease Ecology, in: Current Topics in Vector Research. pp. 1–28. doi:10.1007/978-1-4612-4712-8

Eldridge, B.F., Bailey, C.L., 1979. Experimental hibernation studies in Culex pipiens (Diptera: Culicidae): reactivation of ovarian development and blood-feeding in prehibernating females. J. Med. Entomol. 15, 462–467.

Gillett, J.D., 1971. Mosquitos. Weiden-feld & Nicolson Ltd., 5 Winsley Street, London, W.1.

Gillies, M., Meillon, D., 1968. The Anophelinae of Africa south of the Sahara (Ethiopian zoogeographical region). Sahara Ethiop. Zoogeographical 343 pp.

Gillies, M.T., Coetzee, M., 1987. A Supplement to the Anophelinae of the South of the Sahara (Afrotropical Region). Publ. South African Inst. Med. Res.

Gray, E.M., 2013. Thermal acclimation in a complex life cycle: The effects of larval and adult thermal conditions on metabolic rate and heat resistance in Culex pipiens (Diptera: Culicidae). J. Insect Physiol. 59, 1001–1007. doi:10.1016/j.jinsphys.2013.08.001

Gray, E.M., Bradley, T.J., 2005. Physiology of desiccation resistance in Anopheles gambiae and Anopheles arabiensis. Am. J. Trop. Med. Hyg. 73, 553–559.

Gray, E.M., Bradley, T.J., 2003. Metabolic rate in female Culex tarsalis (Diptera: Culicidae): age, size, activity, and feeding effects. J. Med. Entomol. 40, 903–911. doi:10.1603/0022-2585-40.6.903

Gubler, D.J., 1998. Resurgent Vector-Borne Diseases as a Global Health Problem 4, 442–450.

Gubler, D.J., Casta-Valez, A., 1992. A program for prevention and control of epidemic dengue hemorrhagic fever in puetro rico and the US Virgin Islands. Bol. la Of. Sanit. Panam. 113, 109–120.

Gullan, P., Cranston, P., 2010. Insects in populare culture and commerce, in: The Insects: An Outline of Entomology. p. 10.

Hahn, D. a., Denlinger, D.L., 2011. Energetics of Insect Diapause. Annu. Rev. Entomol. 56, 103–121. doi:10.1146/annurev-ento-112408-085436

Houk, E.J., Obie, F., Hardy, J.L., 1979. Peritrophic membrane formation and the midgut barrier to arboviral infection in the mosquito, Culex tarsalis Coquillett (Insecta, Diptera). Acta Trop. 36, 39–45.

Huestis, D.L., Yaro, A.S., Traoré, A.I., Adamou, A., Kassogué, Y., Diallo, M., Timbiné, S., Dao, A., Lehmann, T., 2011. Variation in metabolic rate of Anopheles gambiae and A. arabiensis in a Sahelian village. J. Exp. Biol. 214, 2345–2353. doi:10.1242/jeb.054668

Huestis, D.L., Yaro, A.S., Traoré, A.I., Dieter, K.L., Nwagbara, J.I., Bowie, A.C., Adamou, A., Kassogué, Y., Diallo, M., Timbiné, S., Dao, A., Lehmann, T., 2012. Seasonal variation in metabolic rate, flight activity and body size of Anopheles gambiae in the Sahel. J. Exp. Biol. 215, 2013–21. doi:10.1242/jeb.069468

Hunt, R.H., Coetzee, M., Fettene, M., 2000. The Anopheles gambiae complex: a new species from Ethiopia. Trans. R. Soc. Trop. Med. Hyg. 92, 231–5.

Kaiser, M.L., Duncan, F.D., Brooke, B.D., 2014. Embryonic development and rates of metabolic activity in early and late hatching eggs of the major malaria vector Anopheles gambiae. PLoS One 9, 1–15. doi:10.1371/journal.pone.0114381

Kambule, I.N., Hanrahan, S.A., Duncan, F.D., 2011. Metabolic rate in diapause and nondiapause brown locust eggs correlated with embryonic development. Physiol. Entomol. 36, 299–308. doi:10.1111/j.1365-3032.2011.00792.x

Kao, L.S., Green, C.E., 2008. Analysis of Variance: Is There a Difference in Means and What Does It Mean? J. Surg. Res. 144, 158–170. doi:10.1016/j.jss.2007.02.053

Klowden, J., 1999. The check is in the male: Male mosquitoes affect female physiology and

behaviour. J Am Mosq Control Assoc 15, 213-220

Klowden, M.J., Russell, R.C., 2004. Mating affects egg maturation in Anopheles gambiae Giles (Diptera : Culicidae) 135–139.

Koenraadt, C.J., Paaijmans, K.P., Githeko, A.K., Knols, B.G., Takken, W., 2003. Egg hatching, larval movement and larval survival of the malaria vector Anopheles gambiae in dessicating habitats. Malar J 2, 1–9. doi:10.1186/1475-2875-2-20

Lanzaro, G.C., Lee, Y., 2013. Speciation in Anopheles gambiae — The Distribution of Genetic Polymorphism and Patterns of Reproductive Isolation Among Natural Populations. Anopheles mosquitoes - New insights into Malar. vectors 813. doi:10.5772/3392

Leeson, H.S., 1931. Anopheline Mosquitos in Southern Rhodesia 1926-1928, in: Mem. Lond. Sch. Hyg. Trop. Med. p. ix + 55 pp.

Lehmann, T., Dao, A., Yaro, A.S., Adamou, A., Kassogue, Y., Diallo, M., Sékou, T., Coscaron-Arias, C., 2010. Aestivation of the African malaria mosquito, Anopheles gambiae in the Sahel. Am. J. Trop. Med. Hyg. 83, 601–6. doi:10.4269/ajtmh.2010.09-0779

Lehmann, T., Dao, A., Yaro, A.S., Diallo, M., Timbiné, S., Huestis, D.L., Adamou, A., Kassogué, Y., Traoré, A.I., 2014. Seasonal Variation in Spatial Distributions of Anopheles gambiae in a Sahelian Village: Evidence for Aestivation. J. Med. Entomol. 51, 27–38. doi:10.1603/ME13094

Lehmann, T., Diabate, A., 2008. The molecular forms of Anopheles gambiae: A phenotypic perspective. Infect. Genet. Evol. 8, 737–746. doi:10.1016/j.meegid.2008.06.003

Lyons, C.L., Coetzee, M., Terblanche, J.S., Chown, S.L., 2014. Desiccation tolerance as a

function of age, sex, humidity and temperature in adults of the African malaria vectors Anopheles arabiensis and Anopheles funestus. J. Exp. Biol. 217, 3823–33. doi:10.1242/jeb.104638

Lyons, C.L., Coetzee, M., Terblanche, J.S., Chown, S.L., 2012. Thermal limits of wild and laboratory strains of two African malaria vector species, Anopheles arabiensis and Anopheles funestus. Malar. J. 11, 226. doi:10.1186/1475-2875-11-226

Mala, A.O., Irungu, L.W., Mitaki, E.K., Shililu, J.I., Charles, M., 2014. Gonotrophic cycle duration , fecundity and parity of Anopheles gambiae complex mosquitoes during an extended period of dry weather in a semi arid area in Baringo County , Kenya 1, 28–34.

Maxwell, C.A., Chambo, W., Mwaimu, M., Magogo, F., Carneiro, I.A., Curtis, C.F., 2003. Variation of malaria transmission and morbidity with altitude in Tanzania and with introduction of alphacypermethrin treated nets. Malar. J. 2, 28. doi:10.1186/1475-2875-2-28

Miller, R.L., Ikram, S., Armelagoss, G.J., Walker, R., Harers, B., Shiffi, C.J., Baggett, D., Carrigan, M., Maret, S.M., 1994. Diagnosis of Plasmodium falciparum infections in mummies using the rapid manual ParaSightTM-F test. Trans. R. Soc. Trop. Med. Hyg. 88, 31–32. doi:10.1016/0035-9203(94)90484-7

Mitchell, C.J., Briegel, H., 1989. Inability of Diapausing Culex pipiens (Diptera: Culicidae) to Use Blood for Producing Lipid Reserves for Overwinter Survival. J. Med. Entomol. 26, 318–326. doi:10.1093/jmedent/26.4.318

Muirhead-Thomson, R.C., 1951. Mosquito Behaviour in relation to Malaria Transmission and Control in the Tropics. Mosq. Behav. Relat. to Malar. Transm. Control Trop.

Nelms, B.M., Macedo, P.A., Kothera, L., Savage, H.M., Reisen, W.K., 2013. Overwintering biology of Culex (Diptera: Culicidae) mosquitoes in the Sacramento Valley of California. J. Med. Entomol. 50, 773–90. doi:10.1055/s-0029-1237430.Imprinting

Nunes, C., Capistrano, G., Ferreira, D., Ferreira, S.S., Mexia, J.T., 2015. One-way fixed effects ANOVA with missing observations, in: AIP Conference Proceedings. doi:10.1063/1.4912415

Philip, C.B., Rozeboom, L.E., 1973. Medico-veterinary entomology: a generation of progress. Hist. Entomol.

Reisen, W., Brault, A.C., 2007. West Nile virus in North America: Perspectives on epidemiology and intervention. Pest Manag. Sci. doi:10.1002/ps.1325

Reisen, W.K., 1995. Effect of temperature on Culex tarsalis (Diptera: Culicidae) from the Coachella and San Joaquin Valleys of California. J. Med. Entomol. Entomol. 32, 636–45. doi:10.1093/jmedent/32.5.636

Reisen, W.K., Fang, Y., Lothrop, H.D., Martinez, V.M., Wilson, J., O'Connor, P., Carney, R., Cahoon-Young, B., Shafii, M., Brault, A.C., 2006. Overwintering of West Nile Virus in Southern California. J. Med. Entomol. 43, 344–355. doi:10.1093/jmedent/43.2.344

Reisen, W.K., Fang, Y., Martinez, V.M., 2006. Effects of temperature on the transmission of west nile virus by Culex tarsalis (Diptera: Culicidae). J. Med. Entomol. 43, 309–317. doi:10.1603/0022-2585(2006)043[0309:eotott]2.0.co;2

Reisen, W.K., Reeves, W.C., 1990. Bionomics and ecology of Culex tarsalis and other potential mosquito vector species. Bionomics Ecol. Culex tarsalis other potential Mosq. vector

species. 254–329.

Reisen, W.K., Smith, P.T., Lothrop, H.D., 1995. Short-term reproductive diapause by Culex
tarsalis (Diptera: Culicidae) in the Coachella Valley of California. J. Med. Entomol. 32,
654–62.

Robert, V., Rocamora, G., Julienne, S., Goodman, S.M., 2011. Why are anopheline mosquitoes
not present in the Seychelles? Malar. J. 10, 31. doi:10.1186/1475-2875-10-31

Roberts, R.M., 1978. Seasonal Strategies in Insects. New Zeal. Entomol. 6, 350–356.
doi:10.1080/00779962.1978.9722286

Robich, R.M., Denlinger, D.L., 2005. Diapause in the mosquito Culex pipiens evokes a
metabolic switch from blood feeding to sugar gluttony. Proc. Natl. Acad. Sci. 102, 15912–
15917. doi:10.1073/pnas.0507958102

Roca-Feltrer, A., Schellenberg, J., Smith, L., Carneiro, I., 2009. A simple method for defining
malaria seasonality. Malar. J. 8, 276. doi:10.1186/1475-2875-8-276

Russell, R.C., Otranto, D., Wall, R.L., 2013. The Encyclopedia of medical and veterinary
Entomology. CABI.

Sanburg, L.L., Larsen, J.R., 1973. Effect of photoperiod and temperature on ovarian
development in Culex pipiens pipiens. J. Insect Physiol. 19. doi:10.1016/0022-
1910(73)90202-3

Stuckenberg, B.R., 1969. Effective temperature as an ecological factor in Southern Africa. Zool.
Africana 4, 145–197. doi:10.1080/00445096.1969.11447371

Thomson, A.J., 2010. Climate indices, rainfall onset and retreat, and malaria in Nigeria. J. Vector

Borne Dis. 47, 193–203.

Wallace, J.R., Grimstad, P.R., 2002. A preliminary characterization of the physiological ecology of overwintering Anopheles mosquitoes in the midwestern USA. J Am Mosq Control Assoc 18, 126–127.

Wallace, J.R., Merritt, R.W., 1999. Influence of microclimate, food, and predation on Anopheles quadrimaculatus (Diptera : Culicidae) growth and development rates, survivorship, and adult size in a Michigan pond. Environ. Entomol. 28, 233–239.

World Health Organization, 2016. World Malaria Report 2016, World Health Organization. doi:10.1071/EC12504

Yang, C.F., Chen, C.F., Su, C.L., Teng, H.J., Lu, L.C., Lin, C., Wang, C.Y., Shu, P.Y., Huang, J.H., Wu, H.S., 2010. Screening of mosquitoes using SYBR Green I-based real-time RT-PCR with group-specific primers for detection of Flaviviruses and Alphaviruses in Taiwan. J. Virol. Methods 168, 147–151. doi:10.1016/j.jviromet.2010.05.006

Yaro, A.S., Traoré, A.I., Huestis, D.L., Adamou, A., Timbiné, S., Kassogué, Y., Diallo, M., Dao, A., Traoré, S.F., Lehmann, T., 2012. Dry season reproductive depression of Anopheles gambiae in the Sahel. J. Insect Physiol. 58, 1050–1059. doi:10.1016/j.jinsphys.2012.04.002

Zhou, G., Miesfeld, R.L., 2009. Energy metabolism during diapause in Culex pipiens mosquitoes. J. Insect Physiol. 55, 40–46. doi:10.1016/j.jinsphys.2008.10.002