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UNIVERSITY OF THE WITWATERSRAND

Evaluating alternative insecticides for use in malaria vector control

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MSc(Med)
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Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2017.

DECLARATION

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I dedicate this work to my beloved Aunt Rachel, whose love and encouragement throughout my life will never be forgotten; my parents, Chris and Sue Samuel, whose continued support is a gift for which I'm grateful beyond words, and my brother Matthew whose persistent nagging pushed me to finally get done.



Rachel Samuel

21 August 1959 - 16 February 2017

PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS STUDY

ORAL PRESENTATIONS

Title: Evaluation of the toxicity and repellence of an organic fatty acids mixture (C8910) against insecticide susceptible and resistant strains of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae).

Presented at: The 19th Congress of the Entomological Society of South Africa, held at Rhodes University, Grahamstown, from the 12-17th of July, 2015

POSTER PRESENTATIONS

Title: Samuel, M., Oliver, S.V., Coetzee, M. and Brooke, B.D., 2016. The larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of *Anopheles* malaria vector mosquitoes.

Presented at: The 2nd Annual Malaria Research Meeting, held at the University of the Pretoria, Pretoria, from the 31st of July - 2nd of October, 2016

Title: Samuel, M., Oliver, S.V., Wood, O.R., Coetzee, M. and Brooke, B.D., 2015. Evaluation of the toxicity and repellence of an organic fatty acids mixture (C8910) against insecticide susceptible and resistant strains of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae).

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PUBLICATIONS

Samuel, M., Oliver, S.V., Coetzee, M. and Brooke, B.D., 2016. The larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of *Anopheles* malaria vector mosquitoes. *Parasites and Vectors*, ***9***:238.

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and resistant strains of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae). *Parasites and Vectors*, ***8***:321.

ABSTRACT

Malaria is a vector-borne disease responsible for morbidity and mortality on a global scale, with particular severity in sub-Saharan Africa. Interventions targeting *Anopheles* malaria vectors rely heavily on synthetic insecticides. This may select for insecticide resistance in the target population. Exacerbating the resistance problem is the excessive use of the pyrethroid class of insecticide and the limited number of alternatives available for public health use. This raises an urgent need for new alternatives, such as naturally occurring biochemicals, to be investigated.

In this study, the larvicidal capabilities of the food spice, black pepper (*Piper nigrum*), and its principle alkaloid, piperine when administered as a food source to anopheline malaria vector species were evaluated. Additionally, a straight-chain fatty acid mixture patented as "C8910" was also investigated as a larvicide and adulticide against several *Anopheles* species, with the adulticidal efficacy of C8910 being specifically evaluated against *An. arabiensis* and a single strain of *An. funestus* derived from a wild population.

The results indicate that black pepper is an effective larvicide when administered as a food source, whereas piperine is not. This suggests that piperine is not the primary larvicidal constituent in black pepper. C8910 demonstrated larvicidal properties, inducing mortality in both *An. arabiensis* and *An. funestus*. C8910 was also adulticidal against *An. arabiensis* and a wild sample of *An. funestus*.

It is concluded that, pending further development and assessment of compliance with WHO standards, black pepper and C8910 could be considered as potential tools for future use in vector control.

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Finally, and most importantly, I'd like to thank the Lord God Almighty, through whom I am more than a conqueror.

Philippians 4:13

I can do all things through Christ who gives me strength

LIST OF ABBREVIATIONS

a.i.	Active ingredient
AChE	Acetylcholinesterase
ACT	Artemisinin-combination therapy
ANOVA	Analysis of variance
BDMI	Botha De Meillon Insectary
CDC	Centres for Disease Control and Prevention
DDT	Dichlorodiphenyltrichloroethane
DVS	Dominant vector species
GABA	γ-aminobutyric acid
GPIRM	Global Plan for Insecticide Resistance Management
GST	Glutathione S-transferase
IGS	Intergenic space
IRAC	Insecticide Resistance Action Committee
IRS	Indoor residual spraying
ITN	Insecticide-treated bednet
IVCC	Innovative Vector Control Consortium
IVM	Integrated Vector Management
kdr	Knockdown resistance
KZN	KwaZulu-Natal
LLIN	Long-lasting insecticide-treated bednet
LSM	Larval source management
NICD	National Institute for Communicable Diseases
SADC	Southern African Development Community
SNP	Single nucleotide polymorphisms in the
RBM	Roll Back Malaria
USAID	United States Agency for International Development
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 MALARIA

Malaria is an infectious tropical disease caused by infected *Anopheles* mosquitoes injecting *Plasmodium* parasites into the human cardiovascular system. Adult female mosquitoes acquire the parasite during an initial bloodmeal taken from an infected individual. The ingested gametocytes then undergo a sporogonic cycle within the mosquito and during subsequent bloodmeals, the resultant sporozoites (which are present in the saliva of the infected mosquito) are injected into the host (Garnham, 1966). There are four *Plasmodium* species commonly responsible for human malaria: *P. falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. A fifth species, *P. knowlesi*, which was previously associated with simian malaria and considered a zoonotic form of the disease, has also been increasingly occurrent in humans in Malaysia, (Knowles and Gupta, 1932; Coatney *et al.*, 1971; Singh *et al.*, 2004; William *et al*., 2013, 2014).

Malaria is preventable when the appropriate interventions are correctly and consistently applied. Once contracted, however, it can lead to fatality if not appropriately and timeously treated. Cerebral malaria, caused specifically by *P. falciparum*, is considered the most severe complication resulting from malaria, and in cases where it does not prove fatal, long-term neurocognitive impairments may occur due to brain injury (Idro, 2010).

The World Health Organization (WHO) estimates that in 2015 there were 212 million cases of malaria globally – 429,000 of which proved fatal (WHO, 2016a). This was a significant decrease from the year 2000 (262 million cases, 839,000 fatalities). The WHO African region, which accounts for 88% of the 2015 case estimate and 90% of the fatalities, has consistently carried the bulk of the disease burden and while substantial progress has been made, weak health systems continue to impede progress toward the ultimate goal of malaria elimination (WHO, 2015a).

In 2015, South African health facilities confirmed 555 malaria cases, while 602 cases were confirmed at community level. In the same year, 110 malaria-related deaths were reported (WHO, 2016a). The country is considered to have low disease transmission and malaria endemicity has been restricted to the Lowveld areas of Mpumalanga, KwaZulu-Natal and Limpopo provinces with occasional cases of Odyssean (imported) malaria being recorded in more inland parts of the country (Maharaj *et al*., 2013; Frean *et al.*, 2014). The ongoing

transmission does not however bode well for the national aim of malaria elimination (that is, of locally transmitted malaria) by 2018.

1.2 COMBATING MALARIA

Recent advances in the development of a malaria vaccine have shown some promise, with the recombinant protein candidate, RTS,S/AS01, drawing particular interest. In 2015, trials of the RTS,S/AS01 vaccine were reported to have prevented a "substantial" number of cases of clinical malaria in young infants and older children over its 3-4 year period (RTS,S Clinical Trials Partnership, 2015). However, several concerning safety signals warrant caution by the WHO, who have recommended additional research into the efficacy of the vaccine, the feasibility of consistent, widespread deployment and the possible correlation with the observed safety signals (WHO, 2016b).

For travellers visiting malaria-endemic areas, the United States Centres for Disease Control and Prevention (CDC, Atlanta, Georgia) prescribes several measures for personal protection, including: covering exposed skin by wearing long-sleeved shirts, long pants and socks, using appropriate insect repellents and using prescription chemoprophylaxis. The main disadvantage among these measures is that they must be consistently maintained and may be considered inconvenient to some extent – leading to neglected application.

In terms of treating malaria-diagnosed patients, artemisinin-combination therapy (ACT) is the current recommendation of the WHO for treating uncomplicated malaria (WHO, 2015b). Complicated (severe) malaria is treated as a medical emergency and the primary objectives of treatment are to prevent the patient from dying as well as to prevent disability and recrudescent infection (Trampuz *et al.*, 2003).

With the rollout of an effective vaccine currently stifled by uncertainty and the use of ACT as a reactive solution rather than a proactive one, controlling malaria vectors to reduce disease transmission remains a primary approach. In Africa, several countries have collaborated, identifying vector control strategies, insecticide resistance and vector genetics as key areas requiring research and enhancement in order to eradicate the disease in the future (Hougard *et al.*, 2002).

1.2.1 Vector control: programs, interventions and strategies

The WHO makes recommendations to its participant countries regarding acceptable vector control approaches. In 1998, the Roll Back Malaria campaign (RBM) was introduced and adopted by the WHO (Nabarro and Tayler, 1998; Nabarro, 1999; WHO, 1999). The RBM campaign sought to approach malaria control in a different way: by strengthening public health services and increasing availability of anti-malaria interventions. The ultimate goals of RBM were to halve malaria by 2010, and again by 2015 (Nabarro and Tayler, 1998). The WHO has since issued several guidance manuals, position statements and progress reports to assist with surveillance and decision making in order to achieve these goals. The WHO later took a stance to support integrated vector management (IVM), which is a more holistic approach aimed at achieving optimal use of resources for vector control (Beier *et al*., 2008). One of the major problem areas addressed by IVM was the increasing seriousness of insecticide resistance among vector species. This led to the conception and implementation of The Global Plan for Insecticide Resistance Management (GPRIM) (WHO, 2012a).

There are also more localized initiatives aimed at malaria elimination. In Africa, 8 SADC countries (including South Africa) have established the Elimination 8 (E8) coordination which aims to interrupt malaria transmission to the point of elimination in Botswana, Namibia, South Africa and Swaziland by 2020, and in Angola, Mozambique, Zambia and Zimbabwe by 2030 (SADC, 2016). The emphasis of this collaboration is on identifying malaria-prevalent foci and targeting them specifically with interventions to control malaria transmission and, where possible (in areas where there are residual levels of malaria), to eliminate it.

According to the WHO, the goals of vector control are two-fold (WHO, 2013a). They are to "reduce human-vector contact and protect individuals from mosquitoes that carry malaria-causing parasites" and to "lower the intensity of malaria transmission at community level by reducing the average lifespan of the local mosquito population". Effective achievement of these goals requires the mosquito life cycle to be taken into consideration and to target multiple stages.

The mosquito life cycle is divided into four stages: egg, larva, pupa and adult (Figure 1.1). The longevity of the various life stages is dependent on species-specific traits, temperature and nutritional factors amongst others (Gillies and De Meillon, 1968; Oliver and Brooke, 2013; Davies *et al.*, 2016).

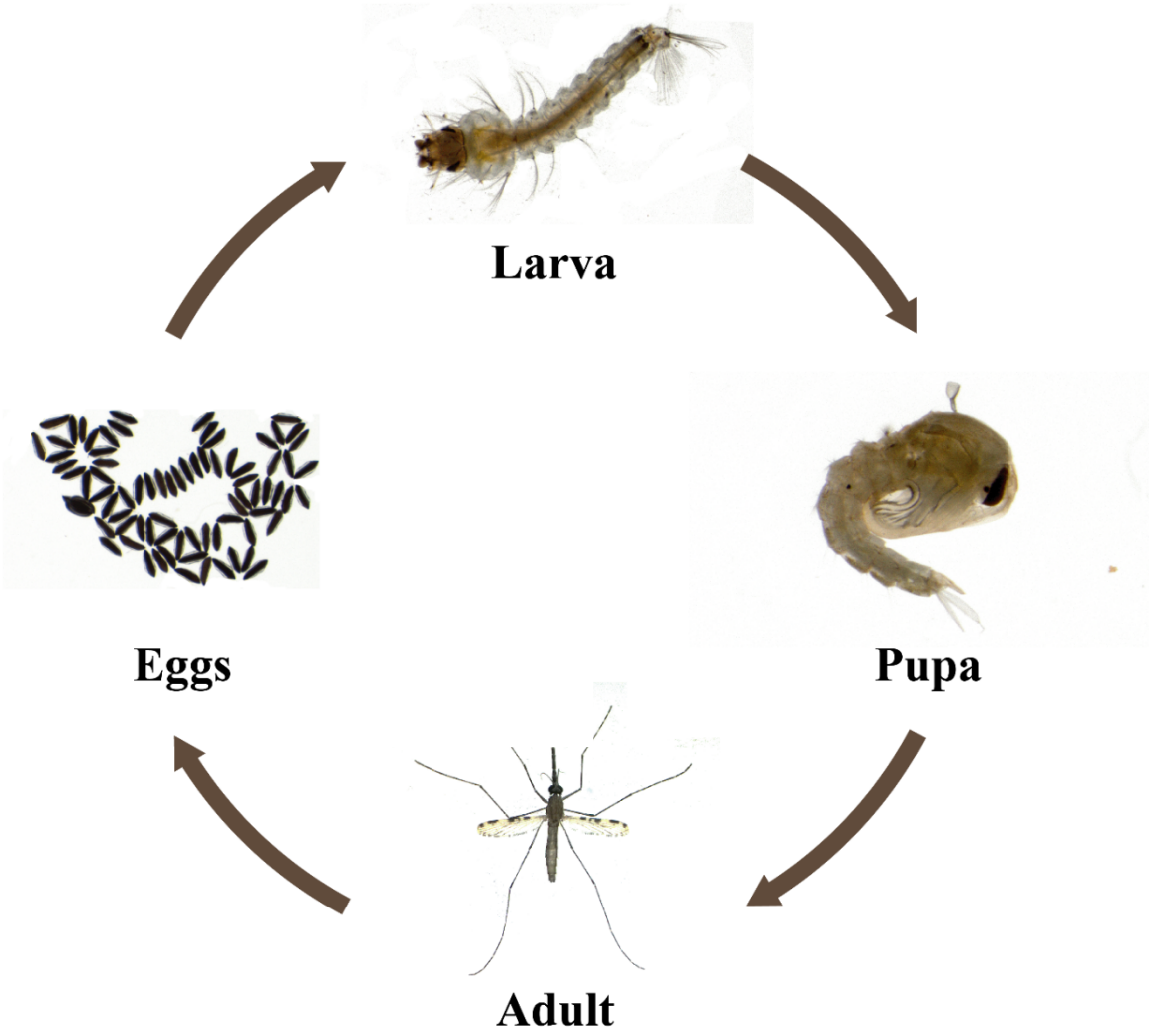


Figure 1.1 Life cycle of an *Anopheles* mosquito. The egg, larval and pupal stages are each aquatic. It is during the adult stage that a female mosquito may acquire and transmit the malaria parasite. The larval and adult life stages are the common and recommended targets of vector control interventions.

There are two strategies currently recommended by the WHO to target the adult life stage: Indoor residual spraying (IRS) and the use of long-lasting insecticidal bednets (LLINs). These preventative measures are heavily funded (more so than national health systems and malaria treatment) by the Global Fund and United States Agency for International Development (USAID) (WHO, 2016a). Larval source management (LSM), which is a secondary vector control approach, targets the mosquito larvae to control the number of larvae which may reach adulthood and serve as potential vectors. These strategies can be further defined as follows:

1.2.1.1 *Indoor residual spraying (IRS)*

IRS, as the name suggests, involves the application of long-lasting insecticides to interior surfaces such as walls, ceilings and eaves which mosquitoes may potentially rest on (WHO, 2013b). It targets endophilic (indoor resting) and endophagic (indoor feeding) mosquito species by repelling them from entering treated households and killing resting females. Toxicity is dependent on the mosquito making contact with the surface that is treated with a lethal dose of insecticide, and while this may occasionally occur before a bloodmeal is taken, it most often occurs afterward (WHO, 2013b). In this manner, the transmission of parasites by infected mosquitoes is curbed. It must, however, be implemented on a large scale for a significant decrease in transmission to be achieved. In 2015, 106 million people were protected by IRS – 3.1% of the global population at risk of acquiring malaria (WHO, 2016a). This figure has however declined from 2010 (5.7% IRS protection), most likely due to the use of more expensive insecticide alternatives to pyrethroids.

1.2.1.2 *Long-lasting insecticidal bednets (LLINs)*

LLINs, which are systematically being distributed to replace regular insecticide-treated bednets (ITNs), serve as both a physical barrier between host-seeking mosquitoes and humans and as a contact insecticide. LLINs are cheaper to produce than the regular treated nets and can be used with greater efficacy than IRS to protect children, who are at higher risk of being infected than adults (WHO, 2007). Like IRS, they target endophilic and endophagic species. Unlike IRS, they target mosquitoes seeking a blood meal rather than those which have had a blood meal and have proceeded to rest on treated surfaces. In sub-Saharan Africa, an estimated 53% of the population at risk slept under ITN in 2015 – 23% more than was the case in 2010. A problem

that been noted though, is that there are often too few nets distributed per household. So, increasing access to these nets remains imperative (WHO, 2016a).

1.2.1.3 *Larval source management (LSM)*

Anopheles mosquitoes spend three of their four life stages in an aquatic environment and can thus be targeted during these stages while they are limited to a water body and are unable to transmit malaria. LSM involves the management of such aquatic habitats which may serve as potential breeding sites for malaria vector species. Although quantitative evidence of the impact of larval source management on malaria transmission is lacking and difficult to acquire (WHO, 2012b), it is considered by the WHO as a useful supplementary vector control strategy where there are clearly delineated habitats which can be described as "few, fixed, and findable" (WHO, 1997, 2013c). As such, by 2013, it is only employed in 38 of the 97 countries with ongoing malaria transmission (WHO, 2014).

Larviciding is one of four types of LSM, the others being habitat modification, habitat manipulation, and biological control (WHO, 2012b). Larviciding utilizes insecticides (biological and chemical) and oils, to target mosquitoes during the larval stage of their life cycle either by ingestion or blocking the air passages. It was a prominent form of vector control prior to the development of synthetic insecticides for IRS, specifically dichlorodiphenyltrichloroethane (DDT), which could be used with great efficiency to target adult mosquitoes (Killeen *et al.*, 2002).

During its period of prominence, successful larviciding and other LSM initiatives were carried out in Europe, Asia and the Americas and were responsible for the decrease and elimination of malaria transmission in substantial continental areas (Killeen *et al.*, 2002). Conversely, there have also been numerous cases reporting the failure of LSM to control vector populations, but these are usually attributed to the incorrect application of interventions resulting in the waste of valuable resources (Tusting *et al*., 2013).

There are several groups of insecticides that are approved of by the WHO Pesticide Evaluation Scheme (WHOPES) as larvicides. These include bacterial larvicides, benzoylureas, juvenile hormone mimics, organophosphates and spinosyns (WHO, 2013c). Each has been rigorously tested and comply with several strict criteria which assess how hazardous they are to humans and the environment, their storage requirements and shelf life, the susceptibility of local vectors and the costs associated of utilizing them (WHO, 2012b)

1.2.2 Vector control in South Africa

In South Africa, vector control strategies have been successfully implemented owing to the effective use of DDT for IRS. The use of DDT began in 1946 when it replaced pyrethrum as the insecticide of choice (Coetzee *et al*., 2013a). Its use was halted in 1996 as a result of pressure from the government to protect the environment from hazardous chemicals, communities' opposition to residual spraying, and the development of a seemingly suitable alternative, the pyrethroid deltamethrin, with which it was substituted (Maharaj *et al*., 2005; Coetzee *et al*., 2013a). In the period that followed DDT exclusion, however, a coinciding relaxation of border security allowing rural Mozambicans into the country (including individuals who were infected with malaria and seeking medical treatment) and high rainfalls (increasing potential mosquito breeding sites) led to increased malaria incidence - peaking in 2000 when 64 622 cases of the disease were recorded (Maharaj *et al*., 2005).

Entomological investigations into the epidemic found that *Anopheles funestus,* which was previously eradicated from the Kwa-Zulu Natal (KZN) province in the 1940's (Sharp and le Sueur, 1996), had re-emerged in the northern part of the province displaying resistance to the pyrethroids used in IRS (Hargreaves *et al*., 2000; Brooke *et al*., 2001). Subsequent efforts made by national government at the Stockholm Convention for Persistent Organic Pollutants led to an exemption allowing the reversion to DDT in 2000. By 2005, malaria incidence had dropped 91% (Maharaj *et al*., 2005) and the endemic areas were progressively confined to the north-eastern areas of the Kwa-Zulu Natal, Mpumalanga and Limpopo provinces (Maharaj *et al*., 2013).

1.2.3 Difficulties faced in vector control

The South African malaria epidemic highlighted the impact of prevalent insecticide resistance on malaria incidence. Insecticide resistance, which may be defined as "the selection of a heritable trait in an insect population that results in an insect control product no longer performing as intended" (McCaffery and Nauen, 2006), has since been included as a vital facet of vector control research. This research has also had to take into account several factors compounding the severity of insecticide resistance.

There are currently only four classes of insecticides recommended by the WHOPES for used in vector control: carbamates, organochlorines, organophosphates and pyrethroids (Zaim and Guillet, 2002). All of these insecticide classes are recommended for IRS (Najera and Zaim, 2001), but only the pyrethroid class is currently recommended for the treatment of bednets

(Zaim *et al*., 2000). Pyrethroids are ideal for this due to their rapid knockdown effect, high insecticidal potency at low dosages and relative safety for human contact and handling (Zaim *et al*., 2000). As such, pyrethroids have been deemed the main tool for decreasing malaria incidence in Africa by the RBM initiative, and are commended as such (Zaim and Guillet, 2002). This commendation is however tarnished by the fact that the widespread use of pyrethroids exacerbates selection pressure on mosquito populations and thus, while succeeding in preventing malaria transmission in the present, also builds insecticide resistance for the future - and resistance to a single pyrethroid insecticide generally confers cross-resistance to others (Zaim and Guillet, 2002). A map of known resistance is available online and can be accessed at http://www.irmapper.com (Knox *et al*., 2014).

Between the four classes of insecticides, there are only two modes of action (Nauen, 2007). Organophosphates and carbamates both inhibit acetylcholinesterase (AChE) (Fukuto, 1990) whereas pyrethroids and the organochlorine DDT cause sustained activation of voltage-gated sodium channels (Soderlund and Bloomquist, 1989; Hemingway and Ranson, 2000). In addition to cross-resistance, multiple resistance (resistance to insecticides acting on different, unrelated target-sites) may also occur (McCaffery and Nauen, 2006). There are two physiological mechanisms underlying insecticide resistance (Hemingway and Ranson, 2000), both of which, along with their roles in insecticide-specific resistance, will be discussed further on in this chapter.

Agricultural use of insecticides also plays a major role in selecting for resistance (Chandre *et al*., 1999; Nkya *et al*., 2013). In 2006, it was estimated that insecticides constituted 44% of the pesticides used in agriculture and that many of these were of identical insecticidal class to those used in public health (McCaffery and Nauen, 2006). The often-unrestricted use of these insecticidal classes in agriculture at multiple points during the mosquito's life cycle intensifies selection pressure and elicits resistance to the same insecticides (and sometimes others where cross resistance or multiple resistance occur) which are used for public health protection. This situation is however, from an agricultural perspective, being monitored and progressively addressed by the Insecticide Resistance Action Committee (IRAC) (Sparks and Nauen, 2015).

Whereas the public health insecticides are limited to the two previously mentioned modes of action and target-sites, insecticides used in the agricultural sector have many modes of action and improving chemical development allows them to take advantage of target-sites which are not utilized by public health insecticides (Nauen, 2007). In fact, there have been no new mosquito adulticides approved for use in public health use since the pyrethroid Etofenprox in

1990 (Nauen, 2007), which has led to increasing disparity in the list of insecticidal options available to the agricultural sector in comparison to the public health sector.

Another problem is that insecticides used for public health may be persistent in the environment. DDT in particular is one of 12 chemicals which the Stockholm Convention on Persistent Organics Pollutants, an international agreement recognized by the WHO, considers as a persistent pollutant (van den Berg, 2009; WHO, 2011). Its chemical stability allows it to accumulate in food webs and in animal tissue which has led to suspicion of it having long-term toxic effects. Although it is non-toxic to mammals, DDT has been the subject of much investigation for long-term toxicity, though the evidence is consistently inadequate to support ideas such as its implications in clinical carcinogenicity (Turusov *et al*., 2002).

Finally, since the introduction of the pyrethroids during the 1980's, development of mosquito adulticides has stalled. This is partially attributed to the process being laborious and expensive, with the cost ranging around US$70 million for a single product (Hemingway *et al*., 2006). Following insecticide synthesis, a new challenge arises in attempting to gain WHO approval. This requires compliance with the WHOPES stringent 4-phase evaluation and testing programme which may be time consuming - potentially taking up to two years (Marris, 2007). Such difficulties have prompted the foundation of the Innovative Vector Control Consortium (IVCC), funded by the Bill and Melinda Gates Foundation, which aims to see through the complete development-to-production of three selected active ingredients with novel modes of action (Hemingway *et al*., 2006).

1.2.3.1 *Mechanisms of insecticide resistance*

There has been development of resistance to all four classes of public health insecticides as well as microbial agents and insect growth regulators (Brogdon and McAllister, 1998). Resistance may manifest either in physiological and/or behavioural changes in the mosquito which may nullify insecticidal effects (David *et al*., 2013). Three mechanisms may lead to resistance. The first involves physical adaptation, such as cuticular thickening, which decreases the availability of a toxicant (Wood *et al*., 2010). The other two mechanisms are metabolic resistance and target-site resistance respectively (Feyereisen, 1995; Hemingway *et al*., 2004), the relative impacts of which in insecticide-specific resistances is described in Table 1.1.

Table 1.1 The major biochemical mechanisms responsible for resistance to the four classes of insecticides approved for use in malaria vector control. The number of plus signs indicate the relative impact of each mechanism on the resistance to the corresponding insecticide class. Adapted from McCaffery and Nauen (2006)

	Biochemical mechanisms of resistance				
	Metabolic			**Target-site**	
	Esterases	**Monooxygenases**	**Glutathione S-Transferases**	***Kdr***	**Altered AChE**
Pyrethroids	+	++		++	
DDT		+	++	++	
Carbamates	+				++
Organophosphates	++	+			++

Metabolic resistance mechanisms involve the enhanced detoxification capacities of enzymes from three superfamilies: cytochrome P450s, non-specific esterases and glutathione S-transferases (GST). Of these, elevated levels of cytochrome P450 activity are implicated in resistance to most insecticides including pyrethroids (David *et al*., 2013). Likewise, hydrolysis of pyrethroids by esterases and over-expressed GST are often evident in pyrethroid resistance. The mechanism of action of these enzymes involves the excretion of insecticides before they can exert a toxic effect, though the causative biochemical relationships (those preceding pyrethroid resistance in particular) have remained unclear (Hemingway and Ranson, 2000; David *et al*., 2013).

Target-site resistance is the second most common resistance mechanism, after metabolic resistance (McCaffery and Nauen, 2006). Since insecticides usually act as neurotoxins, they require specific binding sites to elicit a response. In target site resistance, these sites are mutated and disallow effective binding by the insecticide – resulting in reduced sensitivity. Insensitive AChE as well as mutations to receptors of the γ-aminobutyric acid (GABA) neurotransmitter and voltage gated-sodium ion channels (Dong *et al*., 2014) are each implicated as resistance mechanisms in various arthropods including vector mosquitoes (Hemingway *et al*., 2004).

1.3 *ANOPHELES* MOSQUITOES

1.3.1 Malaria vector species in sub-Saharan Africa

The first step in any malaria vector control programme is the accurate identification of local mosquito species (Brooke *et al*., 2013). This is also of great significance as mosquito complexes (where species are morphologically identical) may include vector and non-vector species possessing differing behavioural traits and resistance profiles and mechanisms, as is the case in sub-Saharan Africa. Incorrect identification may thus lead to the waste of important control-related resources. Although there are 400 *Anopheles* species, only 30 are considered to be malaria vectors of importance (WHO, 2013d) and Africa is inhabited by the most effective and efficient of them: in the *Anopheles gambiae* complex there are *An. gambiae, An. arabiensis, An. coluzzii*, *An. merus* and *An. melas*; in the *An. funestus* group only *An. funestsus s. s.* is considered important with other species, such as *An. vaneedeni* (Burke *et al*., 2017), playing minor roles; and finally, *An. nili, An. moucheti* and *An. rivulorum* (the latter from the *An. rivulorum* subgroup) are important but with restricted distribution (Coetzee *et al*., 2013b; Sinka *et al*., 2010; 2012). In South Africa, the elimination of *Anopheles funestus* has left *An. arabiensis* to be considered the primary malaria vector (Gillies and De Meillon, 1968; Coetzee *et al*., 2013a; Maharaj *et al*., 2013). The distributions of vector species of importance are illustrated in Figures 1.2 and 1.3.

Figure 1.2 The predicted distribution of *Anopheles gambiae* complex species in sub-Saharan Africa. Black dots indicate recorded occurrence of a species and the respective colours in each map indicate the probability (p = 1.0) of the labelled species occurring within a region. **A.** *An. arabiensis* (red) **B.** *An. gambiae* (green) **C.** *An. bwambae* (cyan), *An. melas* (blue) and *An. merus* (orange) and **D.** *An. quadriannulatus* species (yellow and pink). The maps included in this figure have been adapted by Lanzaro and Lee (2013) from Ayala and Coluzzi (2005) and Sinka *et al*. (2010) and are available as open access content at http://www.intechopen.com/books/anopheles-mosquitoes-new-insights-into-malaria-vectors/.

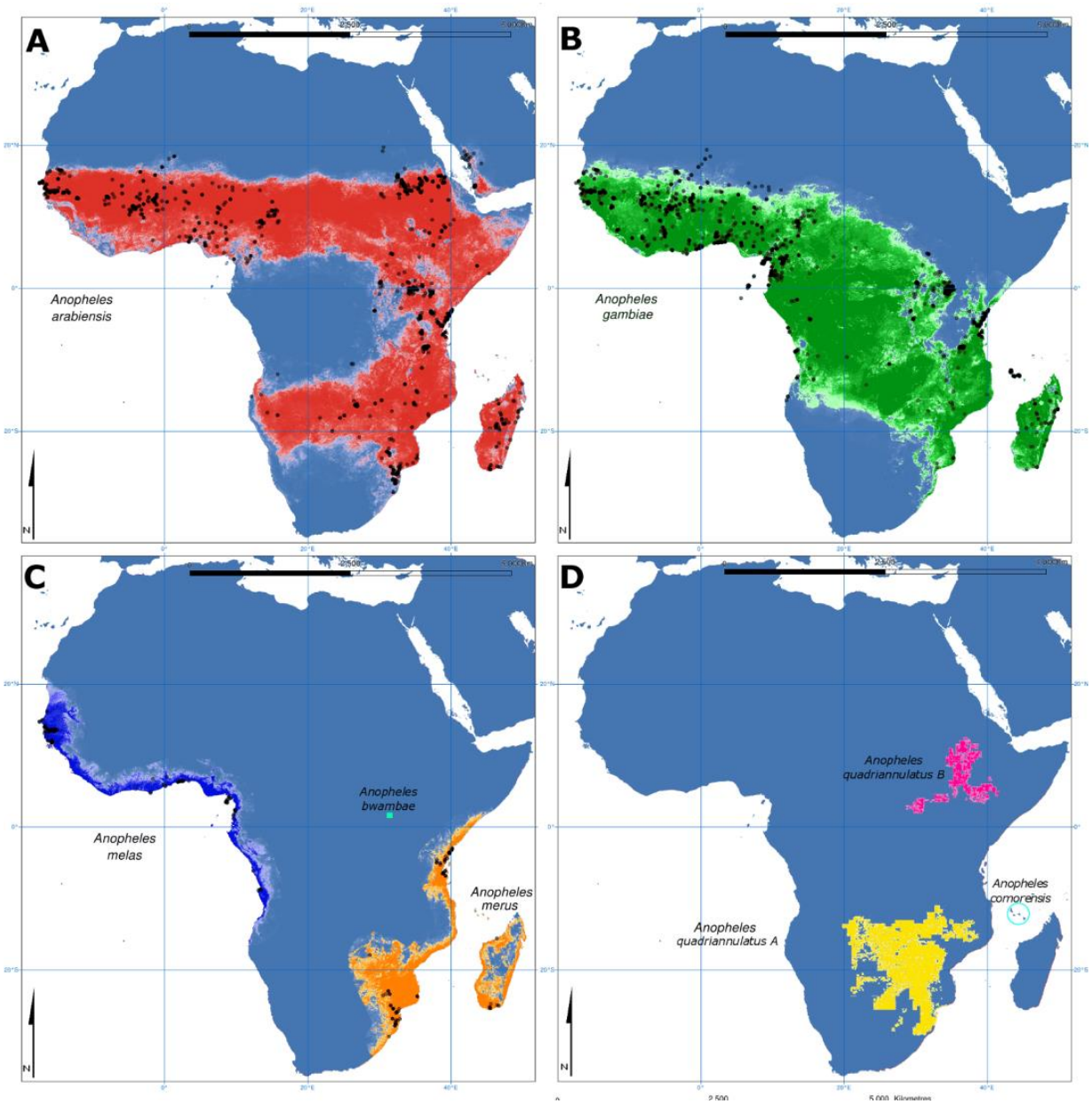
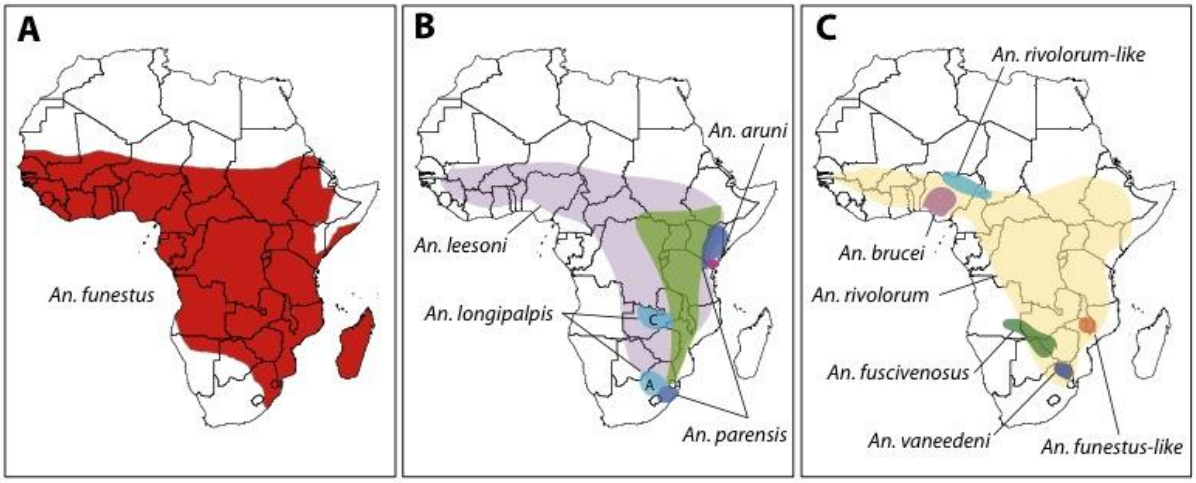


Figure 1.3 The predicted distribution of *Anopheles funestus* group species in sub-Saharan Africa. A. *An. funestus* (red) **B.** *An. aruni* (pink), An. leesoni (grey), *An. longipalpus* (light blue) and *An. parensis* (purple) **C.** *An. brucei* (purple), *An. funestus*-like (orange), *An. fuscivenosus* (green), *An. rivulorum* (pale yellow) and *An. rivulorum*-like (light blue). The maps included in this figure have been adapted by Dia *et al*. (2013) from Sinka *et al*. (2010) and are available as open access content at http://www.intechopen.com/books/anopheles-mosquitoes-new-insights-into-malaria-vectors/.



Anopheles gambiae complex

Anopheles (Cellia) gambiae Giles has been historically recognized as a major vector of malaria in Africa (Gillies and De Meillon, 1968). Of the species within the *Anopheles gambiae* complex (described below), *An. gambiae*, *An. arabiensis*, *An. coluzzii*, *An. merus* and *An. melas* are considered dominant vector species (DVS) in Africa, with the former three species being particularly efficient malaria vectors (Sinka *et al*., 2010).

Anopheles gambiae complex: species, bionomics and distribution

Initially, two salt-water breeding forms were identified in the 1940's and 1950's in East and West Africa. These were named *An. merus* Dönitz and *An. melas* Theobald respectively. Later, in the early-to-mid 1960's, genetic evidence supporting the concept of *An. gambiae* being a species complex of freshwater-breeding species arose. Towards the end of the 1960's the complex was broadened, consisting of the two salt-water breeders, three freshwater breeders (termed species A, B and C) and a mineral water breeder (designated species D). The species were distinguishable by inter-species hybridization producing sterile male progeny. The nomenclature proposed by Mattingly (1977) and White (1985) regarding these species has since been upheld: species A was named *Anopheles gambiae sensu stricto* (*s. s.*) Giles, species B named *An. arabiensis* Patton, species C named *An. quadriannulatus* Theobald and species D named *An. bwambae* White. Two sympatric populations within *An. gambiae s. s.*, labelled "M-form" and "S-form", were later characterized by form-specific single nucleotide polymorphisms (SNPs) in the rDNA intergenic space (IGS) region (della Torre *et al*., 2001). Of these, the reproductively isolated molecular "M-form" (della Torre *et al*., 2001) has recently been deemed an independent species and named *Anopheles coluzzii* Coetzee & Wilkerson (Coetzee *et al*., 2013b), while the "S-form" has retained the nominal name *Anopheles gambiae*. Likewise, the population of "*An. quadriannulatus*" from Ethiopia was shown to be a separate species and named *An. amharicus* Hunt, Wilkerson & Coetzee while the southern African populations retained the name *An. quadriannulatus* (Coetzee *et al.*, 2013b). Due to the lengthy period over which the species within the complex were differentiated, species belonging to the complex are often referred to in text as *An. gambiae sensu lato* (*s. l.*) and the species *An. gambiae* in its strictest sense as *An. gambiae s. s.* Hereafter, *An. gambiae* shall be retained to describe the nominal species.

The freshwater breeding members of *An. gambiae s. l.* show similarity in breeding site preference. These range from temporary rain pools to seasonal swamps to more permanent

water bodies such rice fields. Co-inhabitation of these water sources by *An. gambiae*, *An.coluzzii* and *An. arabiensis* has also frequently been recorded (Minakawa *et al*., 1999; Mutuku *et al*., 2006; Sinka *et al*., 2010). The salt water breeding species *An. melas* and *An. merus* lay their eggs in saline water bodies such as ponds (though *An. merus* has also been shown to survive in freshwater and in water concentrations not exceeding 60% sea-water). The distribution of these species, while mainly coastal, is not limited to the West and East coasts respectively and includes inland areas, usually associated with salt pans (Gillies and Coetzee, 1987). The distribution of several species of the *An. gambiae* complex is illustrated in Figure 1.2. *An. coluzzii* shares much of its distribution with *An. gambiae*, but does not extend past east-central Africa toward Madagascar as *An. gambiae* does (della Torre *et al*. (2005).

Anopheles gambiae complex: vectorial capacity and resistance

Female mosquitoes feed and rest either indoors or outdoors and during the night. Although *An. gambiae*, *An. coluzzii* and *An. arabiensis* are highly anthropophilic, *An. arabiensis* shows a relatively greater tendency toward zoophily. Since domestic animals are usually kept outdoors, they are susceptible to host-seeking *An. arabiensis* which resultantly show a greater tendency to rest and feed outdoors. This behavioural plasticity has gained *An. arabiensis* notoriety as it makes this species difficult to control by the more conventional means of ITNs and IRS (Gillies and Coetzee, 1987). It also means that the species has much lower sporozoite infection rates than *An. gambiae* or *An. coluzzii* which correlates with its lower human blood index (HBI).

Several mechanisms have been observed in *An. gambiae* insecticide resistance. Single point mutations in *An. gambiae*, referred to as knockdown resistance (*kdr*), are responsible for decreased binding of pyrethroids and DDT to their respective target-sites. There are two such *kdr* mutations, evident in West (Martinez-Torres *et al*., 1998), Central (Pinto *et al*., 2006) and East Africa (Ranson *et al*., 2000). Samples from western Kenya displayed elevated expression of the adult-specific cytochrome P450 CYP6Z1 in permethrin-resistant compared to permethrin-susceptible *An. gambiae* (Nikou *et al*., 2003). Additionally, *An. gambiae* from Nigeria have exhibited multiple pyrethroid resistance mechanisms including both elevated P450 enzymes of known association and over-expression of two cuticular genes (Awolola *et al*., 2009). *Anopheles arabiensis* resistance to DDT, permethrin and deltamethrin has been mapped in Ethiopia, with cross-resistance between DDT and permethrin also evident (Balkew *et al*., 2010).

Anopheles funestus group

In the past decade, research into the major malaria vector *Anopheles funestus* Giles has dramatically increased. Previously, difficulty with rearing F_1 progeny from wild *An. funestus* females and colonizing these, as well as the perception in some areas of it being a less important vector than *An. gambiae*, had hindered field and laboratory studies from being conducted on the species (Coetzee and Fontenille, 2004; Coetzee and Koekemoer, 2013).

Anopheles funestus group: species, bionomics and distribution

There are eleven species comprising the *An. funestus* group, including major and minor vectors. These, although maintaining some morphological differentiation in their egg and larval stages, are remarkably similar in their adult stage (Gillies and De Meillon, 1968). Gillies and De Meillon (1968) termed three members of the group, *An. funestus* Giles, *An. aruni* Sobti and *An. parensis* Gillies, as the "*funestus* sub-group" due to them being morphologically inseparable during their early life stages. The sub-group was later extended to include *An. vaneedeni* Gillies & Coetzee (1987), and an independent species in Malawi provisionally called *An. funestus*-like (Spillings *et al*., 2009), characterized by hybrid female chromosome asynapis that is typically observed in inter-species crosses. Furthermore, *An. funestus* can be split into two subdivisions referred to as Clade I and Clade II according to mitochondrial DNA (Choi *et al*., 2012). The epidemiological implications of this difference for malaria transmission, however, remain unclear. The "*rivulorum* subgroup" comprises *An. rivulorum* Leeson, *An. rivulorum-like*, *An. brucei* Service and *An. fuscivenosus* Leeson. Of this subgroup, only *An. rivulorum* is implicated as a minor malaria vector based on a single locality in Tanzania (Wilkes *et al*., 1996). Finally, *An. leesoni* Evans is a member of the "*An. minimus* subgroup" and is not considered a vector of importance.

Anopheles funestus group species breed in clear, large bodies of water with emergent vegetation. These are usually semi-permanent or permanent water bodies, such as swamps, or near the banks of lakes or ponds which may be shaded (though not necessarily so). Unusual breeding sites such as wells or in domestic water containers have also been identified on occasion. The larvae are often difficult to find where there are low densities, as they tend to remain submerged for extended period of time (Gillies and De Meillon, 1968). The distribution of important *An. funestus* group species is illustrated in Figure 1.3.

Anopheles funestus group: vectorial capacity and resistance

Anopheles funestus, like *An. gambiae*, was first shown by Ross (1910) to be a malaria vector in Sierra Leone. It is the nominal member of the *An. funestus* group and, along with *An. rivulorum* and *An. vaneedeni*, is currently implicated in malaria transmission (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Adult *An. funestus* are highly anthropophilic (Gillies and Coetzee, 1987) and it is thought to be one of the first species to have adapted to a human host because of its breeding habitat's likely close proximity to early civilizations (Charlwood *et al*., 1995). Its habit of late night biting decreases the prospect of host-avoidance, while its considerable longevity, endophilic resting and endophagic behaviour each contribute significantly to its efficiency as a parasitic vector (Sinka *et al*., 2010). The latter two characteristics do, however, also make it susceptible to ITN and IRS control interventions. *Anopheles funestus* also has the potential for higher *P. falciparum* sporozoite infection rates than *An. gambiae* (Shililu *et al*., 1998).

Resistance to all four classes of insecticide has been recorded in *An. funestus* (Coetzee and Koekemoer, 2013). Current characterizations of resistance mechanisms in this species is cause for concern as the levels of pyrethroid resistance can be extremely high, to the extent that wild populations from Zambia and Zimbabwe could withstand up to 8 hours exposure to a standard WHO discriminating dose for deltamethrin, giving only ~80% mortality (Choi *et al*., 2014).

In localities where the species is prevalent, the possibility of a malaria epidemic such as the one experienced in KwaZulu-Natal (KZN) remains increasingly likely (Coetzee and Koekemoer, 2013; Coetzee *et al*., 2013a). In the KZN case, Brooke *et al.* (2001) found that elevated levels of mixed function oxidases in *An. funestus* were responsible for detoxifying pyrethroids as well as conferring cross-resistance to the carbamate propoxur. Further work by Amenya *et al.* (2008) assessing two laboratory-reared colonies of *An. funestus* established by Hunt *et al.* (2005) identified over-expression of the cytochrome P450 CYP6P9 (a gene of the monooxygenase CYP6 family) in the egg and adult stages of the pyrethroid-resistant phenotype when compared to an insecticide susceptible phenotype. Using a positional cloning technique, Wondji *et al*. (2009) found that CYP6P4 (in addition to CYP6P9) was overexpressed in resistant *An. funestus* females. Both CYP6P9 and CYP6P4 were found and confirmed to be duplicate genes and were initially termed CYP6P9a and CYP6P9b and, CYP6P4a and CYP6P4b (Wondji *et al*., 2009; Matambo *et al*., 2010).

1.4 THE ROLE OF NATURALLY OCCURRING BIOCHEMICALS IN VECTOR CONTROL

A tendency towards naturally occurring insecticides, particularly those of botanical origin, has gained increasing interest in recent years. Plants have co-evolved with insects over millenia, as have their defence mechanisms in response to insect predation. Plant allelochemicals can be very advantageous as insecticidal actives compared to their synthetic counterparts as they are biodegradable and, in many instances, show reduced or no adverse effects on non-target organisms, including mammals (Tehri and Singh, 2015).

The success in using botanical-based insecticides is none more evident than in the case of pyrethrum, an extract from the pyrethrum daisy (*Tanacetum cinerariaefolium* Trev.), which is the basis of the most widespread insecticide class: pyrethroids (Casida, 1980). Initially, extracts of the dried pyrethrum flower were used for insect pest control in households, barns, farms and plantations. They were also directly applied to humans and livestock. One feature of early natural and synthetic pyrethroids was instability in light and air, which meant that persistent residues of the insecticide would be diminished in comparison to synthetic organic insecticides (such as DDT and organophosphates) which were later introduced. Synthesis of modern pyrethroids has, however, allowed for improvement in stability of pyrethrins (the esters which comprise pyrethrum), thereby inducing long-term potency (among other favourable characteristics of earlier pyrethroids) while retaining low environmental persistence (Elliot *et al*., 1978).

A plethora of botanical insecticidal actives have since been identified, many of which have shown toxicity to mosquito vectors from several genera at different stages of the mosquito life cycle. A prominent example is piperine, an extract of *Piper nigrum* Linn. (black pepper) which has shown distinct larvicidal capabilities (Park *et al*., 2002; Simas *et al*., 2007). In addition to these strictly botanical actives, mixtures of naturally occurring compounds with known insecticidal properties have also shown promise. For instance: a combination of straight-chain fatty acids (C8910), initially designed as an insect repellent for use on cattle, and later, personnel in the US Navy assigned to the CDC. has recently been shown to have a toxic effect on mosquito vectors (Reifenrath, 2015; Dunford *et al.*, 2014). Such extracts and mixtures may yet have a significant role to play in the control of malaria vectors on the backdrop of an ever-intensifying resistance situation, though this depends largely on their efficacy relative to the current alternatives.

1.5 AIMS AND OBJECTIVES

The aims of this study were to evaluate the toxicity of black pepper, piperine and C8910 to larvae and C8910 to adults of several *Anopheles* mosquito strains. The specific objectives and the methods of approach were as follows:

1. To investigate the toxicity of powdered black pepper and piperine when ingested by late instar larvae drawn from several insecticide resistant and susceptible laboratory-reared strains of several *Anopheles* species.
2. To investigate whether C8910 is larvicidal to late instar larvae drawn from insecticide resistant and susceptible laboratory strains of *An. funestus* and *An. arabiensis*.
3. To determine the adulticidal effects of C8910 on laboratory-reared *An. arabiensis* strains with differing resistance profiles and a single wild-caught strain of *An. funestus* from Zambia.

CHAPTER 2

COMMON METHODS AND MATERIALS

2.1 INSECTARY CONDITIONS

All of the *Anopheles* mosquito strains/samples used in this study are housed in the Botha De Meillon Insectary (BDMI) at the National Institute for Communicable Diseases (NICD) in Johannesburg. All larvae were reared and bioassays conducted under the standard insectary conditions described by Hunt *et al*. (2005): an ambient temperature of 25°C, with 80% relative humidity and a 12-hour day/night lighting regime with 45-minute dusk/dawn cycles. Thrice during the day cycle, larvae were fed a 3:1 mixture of finely crushed dog biscuits and brewer's yeast (hereafter referred to as standard larval food).

2.2 MOSQUITO STRAINS

2.2.1 Laboratory strains

The laboratory-reared *Anopheles* mosquito strains used for this study include four members of the *An. gambiae* complex: *An. arabiensis*¸ *An. coluzzii*, *An. gambiae* and *An. quadriannulatus*, and a single species of the *An. funestus* group, *An. funestus s. s*. Pertinent details regarding the utilized strains of these species is given inable 2.1.

Table 2.1 *Anopheles* species by laboratory strain and insecticide susceptibility profile used in various larvicidal and adulticidal bioassays.

Species	Strain (Country of origin)	Resistance profile/ additional information	Resistance Mechanisms
Anopheles arabiensis	KGB (Zimbabwe)	Insecticide susceptible	N/A
	SENN (Sudan)	Mostly susceptible. Low-level resistance to permethrin	N/A (Oliver and Brooke, 2014)
	SENN-DDT (Sudan)	Selected for resistance to DDT from SENN base colony. It is also resistant to permethrin, deltamethrin and malathion (Oliver and Brooke, 2014; Nardini *et al*., 2012)	Elevated cytochrome P450, glutathione S-transferase (GST) and general esterase activity (Oliver and Brooke, 2014; Nardini *et al*., 2012)
Anopheles coluzzii	SILC* (Sierra Leone)	Resistant to deltamethrin and DDT	No data
	SUA (Liberia)	Insecticide susceptible	N/A
Anopheles gambiae	GAH (Ghana)	Resistant to pyrethroids, DDT, carbamates and organophosphates (Kaiser *et al*., 2010)	Monooxygenase and esterase mediated detoxification coupled with the L1014F *kdr* mutation is implicated in pyrethroid resistance. An assortment of L1014F *kdr* is also implacted in DDT resistance. Mutations of the Alanine296-glycine (*Rdl*) GABA receptor and acetylcholinesterase receptor (*ace-1*^{R}) are associated with dieldrin and bediocarb resistance respectively (Kaiser *et al.*, 2010).
	TONGS* (Cote d'Ivoire)	Resistant to pyrethroids, DDT, carbamates and organophosphates	No data
Anopheles quadriannulatus	SANGWE (Zimbabwe)	Insecticide susceptible. *Anopheles quadriannulatus* is zoophilic and not considered to be a vector species.	N/A
Anopheles funestus	FANG (Angola)	Insecticide susceptible	N/A
	FUMOZ-R (Mozambique)	Resistant to pyrethroids and carbamates (Amenya *et al*., 2008)	Overexpression of the cytochrome P450 CYP6P9 (Amenya *et al*., 2008). Thickened cuticles also contribute to adult insecticide resistance (Wood *et al*., 2010).

*SILC was used for black pepper bioassays but not for piperine bioassays as the insecticide resistance levels in the strain had significantly dropped during the period between the conducting of the two bioassays and it would not have been an accurate proxy by which to compare insecticide susceptible and resistant strains. SILC was replaced with the *An. gambiae* strain TONGS for the piperine bioassays.

2.2.2 Collection of wild samples

Samples of wild, indoor-resting *Anopheles* adult females were collected from the Nchelenge District of northern Zambia (Figure 2.1) during February 2014. This was done during daytime with the use of aspirator tubes. This was done with permission to enter households granted by each household owner. Samples were separated according to morphology as either *Anopheles gambiae* complex or *Anopheles funestus* group (See section 2.2.2.1) and kept in gauze-covered plastic cups which had their interior surfaces roughened with sand paper (Figure 2.2A). Roughening the interior walls of the cups enables the mosquitoes to grip the sides, causing them less stress. The mosquitoes were given access to 10% sucrose solution as a food source and the cups were covered with a plastic sheet and a warm, moist towel to maintain humidity. The live adult mosquitoes were transported to the BDMI in Johannesburg where their F_1 progeny were collected and reared to adulthood.

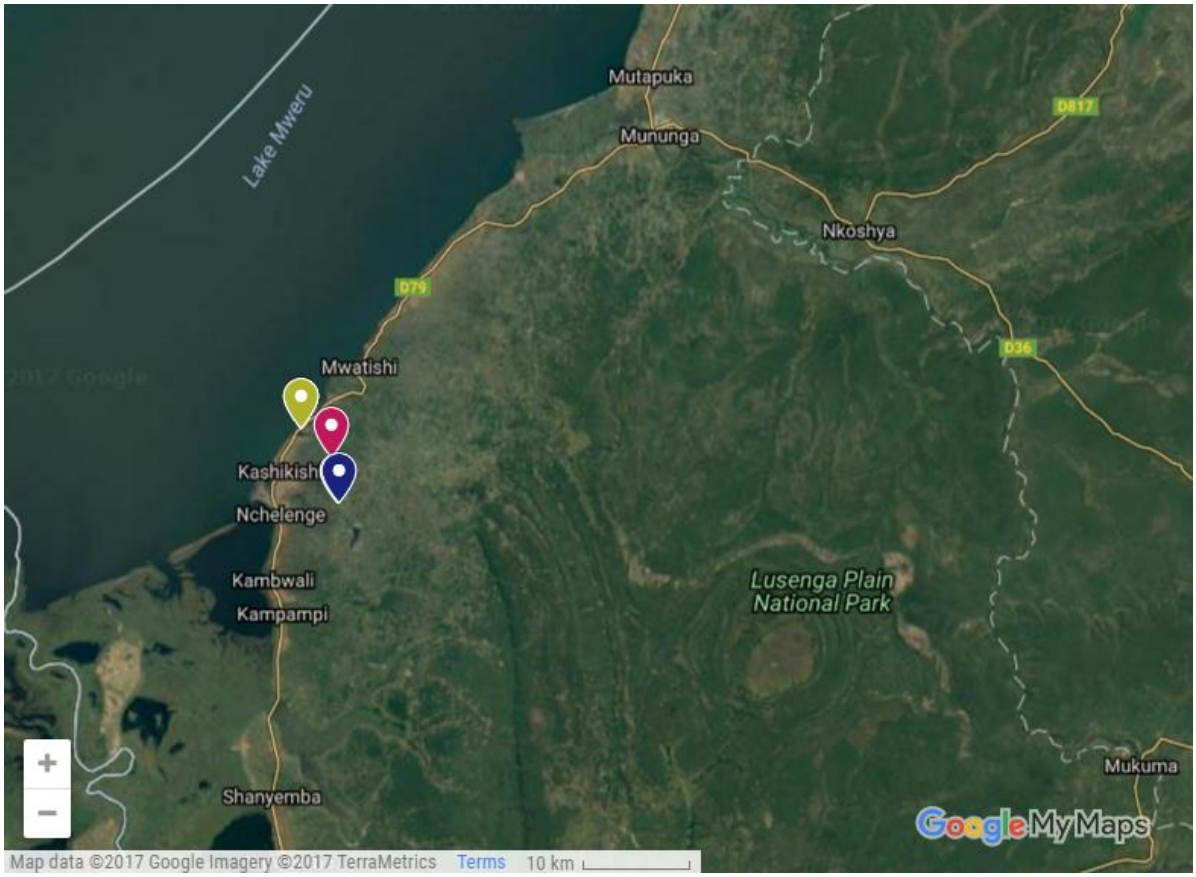


Figure 2.1 Mosquito collection sites in the Nchelenge district of northern Zambia. Nchelenge is a malaria endemic area which borders the southern shore of Lake Mweru**.**

Once at the BDMI, each wild adult female was individually inserted into a glass vial, each containing a damp section of filter paper to promote egg-laying (Figure 2.2B). These females were bloodfed and given access to 10% sucrose solution. Following oviposition, egg batches from each adult female were initially placed in separate containers of distilled water and the hatchlings were reared using the standard protocol of Hunt *et al*. (2005). Once identified to clade (see section 2.2.2.1), the respective larvae were pooled accordingly and Clade I larvae were designated "ZamF". Samples of the ZamF F1 adult progeny were used as representative of the wild population and, in experiments conducted concurrently by Choi *et al*. (2014), were found to be highly resistant to pyrethroids and carbamates yet susceptible to DDT and organophosphates.

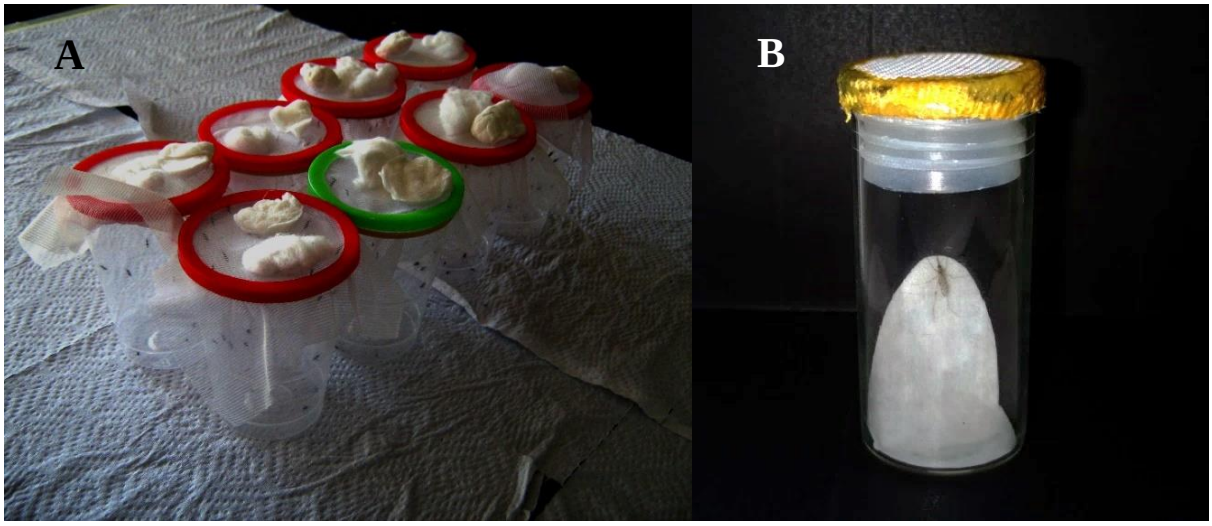


Figure 2.2 Holding containers for wild-caught *Anopheles* samples. A. In the field, plastic containers were used for holding *Anopheles* females. These were covered with a plastic sheet and warm, moist towel (not shown) to maintain humidity. **B.** Once transported to the Botha De Meillon Insectary in Johannesburg, adult female mosquitoes were individually inserted into glass vials lined with a section of dampened filter paper to promote egg-laying.

2.2.2.1 *Identification of wild samples*

Morphological identification of adult females was conducted in accordance with the keys developed by Gillies and De Meillon (1968) and Gillies and Coetzee (1987)**.** These keys pay particular attention to abdominal scaling and segmentation, leg and tarsal colours, palpal banding and wing veination.

Characteristics of *An. gambiae* complex species were determined as follows (Figure 2.3A-D): laterally projecting tufts of scales were absent from abdominal segments II-VII. The legs were speckled (sometimes sparsely), rather than having pale hind tarsi or a combination of pale hind tarsi and tarsus 5 being dark. If the palps had three pale bands, the 3rd main dark area (preapical dark spot) of vein 1 was assessed for a pale interruption, sometimes fused with a preceding pale spot; and the scaling on abdomen was observed to determine if it was very scanty and confined to 8th (or rarely 7th) terga. If this was so, the species could be confirmed as *An. gambiae* complex. If the palps, however, had 4 pale bands, tarsi 1-4 were assessed for conspicuous pale bands on at least the apices and the wings were assessed for pale fringe spots up to vein 5.2 or 6.

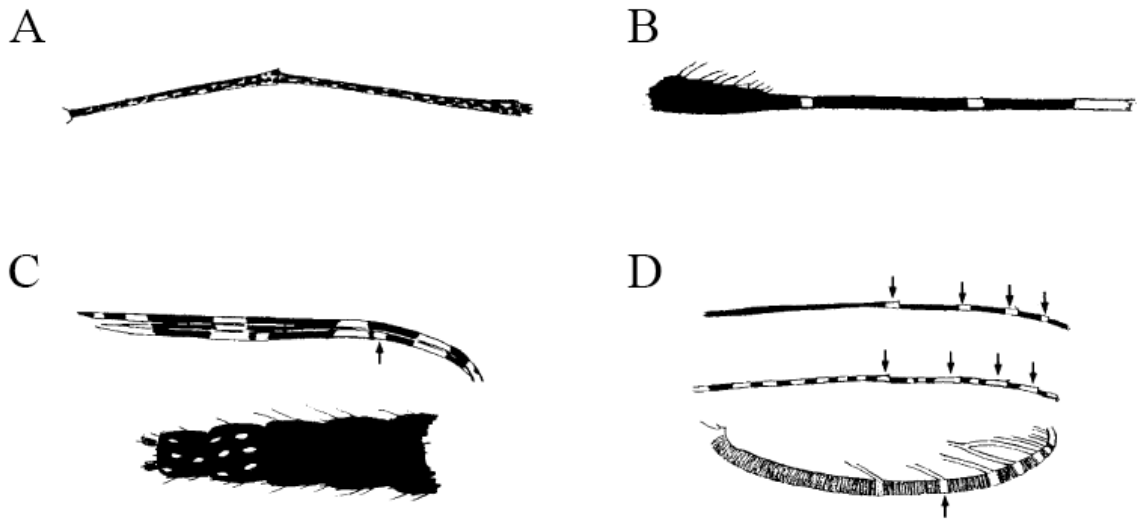


Figure 2.3 Morphologically identifiable diagnostic points of *Anopheles gambiae* complex species (from Gillies and Coetzee, 1987). A. Speckled legs **B.** Three pale palpal bands **C.** Pale interruption on the 3rd main dark area of wing vein 1 (above) and scanty scaling present on the 8th or rarely 7th terga (below) **D.** Conspicuous pale bands on tarsi 1-4 (above) and pale fringe spots on wing veins up to 5.2 or 6 (below)

Characteristics of *An. funestus* subgroup species were determined as follows (Figure 2.4A-D): Mosquitoes without laterally projecting tufts of scales on their abdomens. They possessed a pale spot on vein 5.1, with no pale interruption on 3rd main dark area (preapical dark spot) of vein 1. The costa had at least a pale spot on basal half. The palps had less than 3 bands, and were pale at apex. The subapical pale band on the palps was either much narrower than the subapical dark band or the 3rd main dark area was broader than the subcostal pale spot. The legs were not speckled and the hind tarsus 4 and 5 were not entirely pale, but did possess pale, narrow and apical banding. Preaccessory dark spots were either absent or if present, were narrower or only slightly broader than adjoining pale spots. On the wing, the basal area of vein 1 was entirely pale and the tip of vein 6 was dark, with no 6th fringe spot present. The wing length was 3.3mm or less, belonging to a small species.

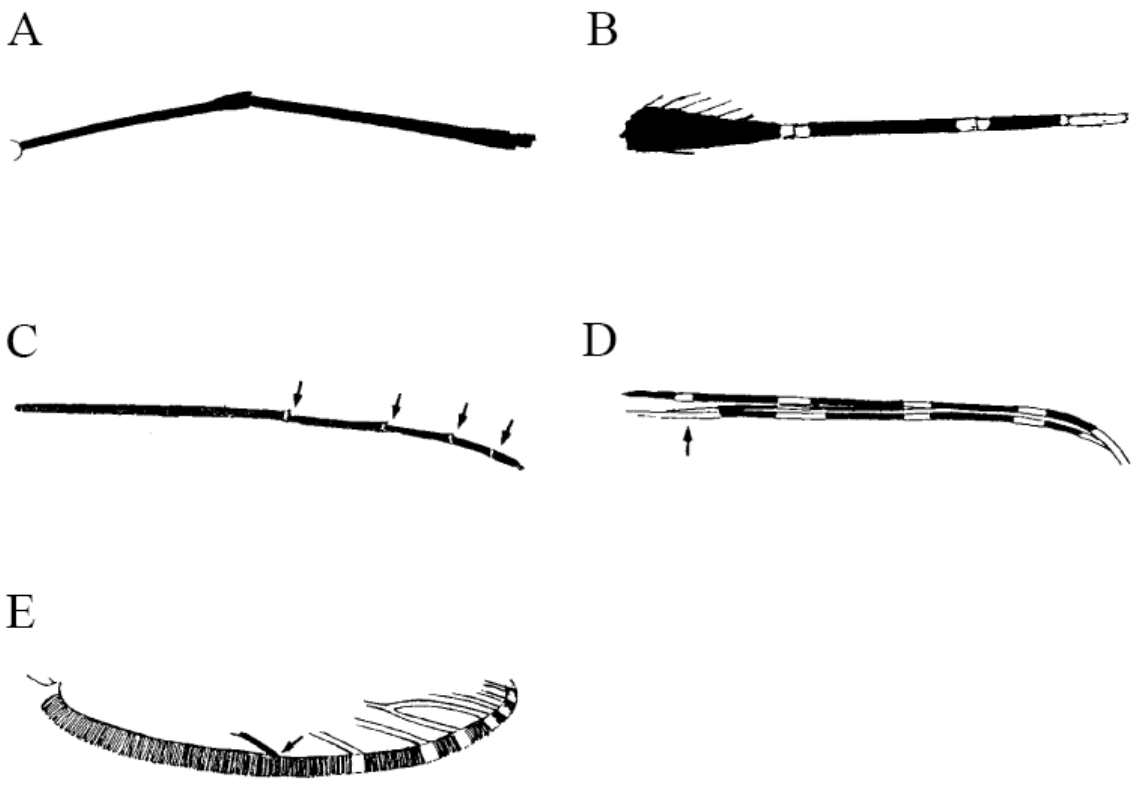


Figure 2.4 Morphologically identifiable diagnostic points of *Anopheles funestus* group species (from Gillies and Coetzee, 1987). A. No speckling on legs **B.** Three pale palpal bands **C.** Pale, narrow banding on apices of the hind tarsi **D.** Basal area of vein 1 is entirely pale **E.** Tip of vein 6 is dark and there is no fringe spot present

After laying eggs, the wild *An. funestus* group females were removed from the vials and killed by exposure to diethyl ether. Morphological identification was carried out as described above and *An. funestus* group individuals were identified to species using the polymerase chain reaction method described by Koekemoer *et al*. (2002). They were then further identified to clade using hydrolysis probe analysis (Taqman assay) as described by Choi *et al*. (2013). All specimens were identified as *An*. *funestus s. s.* and of these, 265 were identified as Clade I and 61 as Clade II.

2.3 SAMPLE SELECTION FOR BIOASSAYS

2.3.1 Larval sampling

Anopheles larvae were selected from their respective laboratory colonies according to age for each species. For strains of the *Anopheles gambiae* complex, $3^{rd}/4^{th}$ instar larvae were sampled 8-10 days following egg collection. Likewise, $3^{rd}/4^{th}$ instar *Anopheles funestus* larvae were sampled 12-15 days following egg collection. Larvae were not fed on the day of the toxicity bioassays.

2.3.2 Adult sampling

Adult *Anopheles* were collected from their respective colonies at 3-5 days post-emergence. Of these, the non-bloodfed females were separated from their male counterparts according to the sexual dimorphisms illustrated in Figure 2.5. The key distinguishing features of *Anopheles* females are the straight-tipped palps and lack of bushy antennae. The samples were given access to 10% sucrose solution up until the point of insecticide exposure.

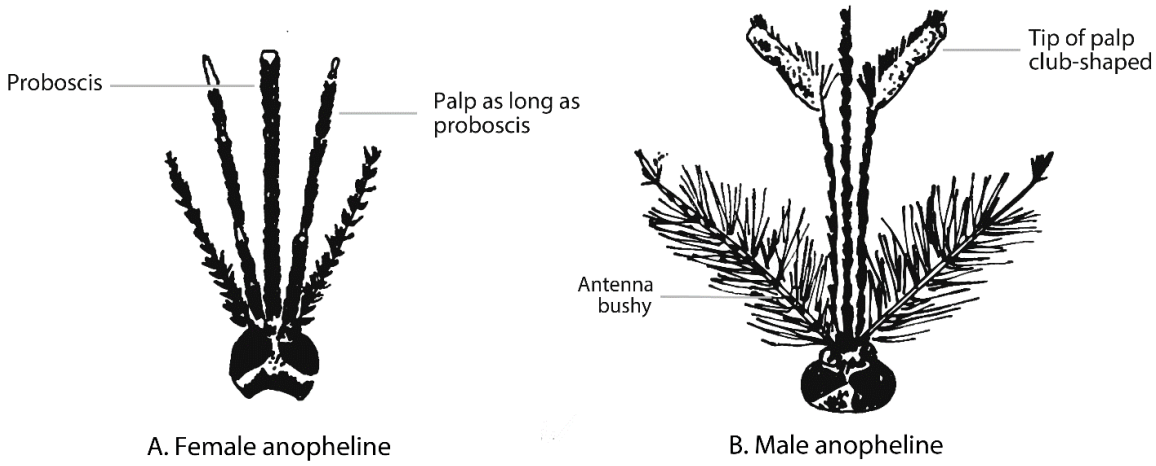


Figure 2.5 Head appendages of female and male *Anopheles* mosquitoes (from WHO 2013d). Distinguishing features were used to separate sexes for use in adult toxicity bioassays

2.4 Statistical analysis

Statistical analyses were conducted in IBM SPSS Statistics (Armonk, NY) v22 with significance set at 95% confidence. Abbott's formula was used to correct mortality in replicates where control mortality <20% occurred. Further information is detailed in the relevant chapters.

CHAPTER 3

THE LARVICIDAL EFFECTS OF BLACK PEPPER (*PIPER NIGRUM* LINN.) AND PIPERINE AGAINST INSECTICIDE RESISTANT AND SUSCEPTIBLE STRAINS OF *ANOPHELES* MALARIA VECTOR SPECIES

3.1 INTRODUCTION

Black pepper is considered as a food grade spice by the U.S. Food and Drug Administration (FDA) and is categorised as "generally recognised as safe". The uses of pepper are extensive and diverse, ranging from being a food spice, to having numerous medical benefits and effects, to functioning as an insecticide. A comprehensive list of its traditional and modern uses is available in Meghwal and Goswami's (2013) review of *P. nigrum* and piperine. White pepper and black pepper are both derived from *P. nigrum*, but are produced from different parts of the fruit being subjected to different processes. White pepper is from the dried, ripe seeds whereas black pepper production involves cooking and drying the unripened fruit. The principle bioactive component of both derivatives (as well as that of the sister species *Piper longum* L. – commonly known as long pepper), is the alkaloid piperine (Meghwal and Goswami, 2013).

Piperine (together with its stereoisomer chavicine) is the constituent responsible for the pungency of *P. nigrum* and *P. longum*. The isolation of piperine from *P. nigrum* was first reported in 1820 (Oerstedt, 1820). In rats, piperine was shown to be a potent unspecific inhibitor of drug metabolism, showing little discrimination between various forms of P450s (Atal *et al*., 1985). This is believed to be the basis of enhanced drug bioavailability as a result of piperine ingestion.

In an attempt to utilize powdered white pepper as a dry organic carrier for spores of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, Bukhari *et al*. (2011) found that the pepper caused 100% mortality of *An. gambiae* and *An. stephensi* larvae - even in the absence of the fungal spores. While this apparent toxicity eliminated pepper as a potential carrier for these fungal larvicides, it highlighted the potential for pepper itself to be used as a larvicide against *Anopheles* species. In addition, isolated piperine has demonstrated toxicity against fourth-instar larvae of the dengue and yellow fever vector *Aedes aegypti* (Siddiqui *et al*., 2002; Gulzar *et al*., 2013). Reports regarding the efficacy of this toxicity are, however, conflicting.

Park *et al*. (2002) compared several isobutylamide alkaloids derived from *Piper nigrum* fruits, including pellitorine, guineensine, pipercide and refractomide A to reagent grade piperine in larval bioassays against third-instar *Culex pipiens pallens* ($LC50_{piperine}$ = 3.21ppm), *Ae. aegypti* ($LC50_{piperine}$ = 5.10ppm) and *Ae. togoi* ($LC50_{piperine}$ = 4.60ppm). They concluded that of the analysed compounds, piperine was the least toxic and ultimately declared it nontoxic by comparison. More recently, Simas *et al*. (2007) found that piperine achieved an LC50 of 1.53ppm when tested on third-instar *Ae. aegypti* larvae with an established resistance to pyrethroids. Gulzar *et al*. (2013) conducted WHO larval susceptibility tests (WHO, 1970) using six *P. nigrum* derivatives, including piperine, on fourth-instar larvae of *Ae. aegypti*. Piperine achieved an LC50 of 10.00ppm, the lowest among the tested compounds. However, a lack of a discussion or a conclusion in their publication makes it difficult to rationalise the considerably higher LC50 against *Ae. aegypti* than that which was achieved by both Park *et al*. (2002) and Simas *et al*. (2007). Possible key differences may lie in extraction method and larval age: both Simas *et al*. (2007) and Gulzar *et al*. (2013) extracted and isolated their own piperine from *P. nigrum* as opposed to purchasing it as Park *et al*. (2002) did, and Gulzar *et al.* (2013) experimented on older larvae than the other two.

The aim of this project was to investigate the toxicity of powdered *P. nigrum* (black pepper) and piperine respectively, when administered as a food source, to larvae of several insecticide resistant and susceptible strains of *Anopheles* species including *An. arabiensis*, *An. coluzzii*, *An. gambiae*, *An. quadriannulatus* and *An. funestus*.

3.2 METHODS AND MATERIALS

3.2.1 Mosquito strains

The laboratory-reared mosquito strains used included the insecticide susceptible and insecticide resistant laboratory strains of *An. arabiensis*, KGB and SENN-DDT respectively as well as the SENN strain from which SENN-DDT is selected for resistance. The *An. coluzzii* strains SILC and SUA, the *An. gambiae* strains GAH and TONGS, the *An. quadriannulatus* strain SANGWE and the insecticide susceptible and insecticide resistant *An. funestus* strains FANG and FUMOZ-R respectively, were also included.

3.2.2 Technical material and feeding mixtures

Commercial ground black pepper was further homogenized in a Qiagen Tissue Lyser II until a fine powdery consistency was achieved. Analytical grade piperine in powder form (expiry date: December 2016) was purchased from Sigma Aldrich (Saint Louis, MO). The larval toxicities of black pepper and piperine ingestion were analysed separately, using a similar protocol. A range of feeding mixtures were created by mixing a standard larval food (Hunt *et al*. 2005) with powdered black pepper and piperine respectively. These mixtures ranged from 10% to 100% of either technical material (Table 3.1).

Table 3.1 Proportions of either black pepper (*Piper nigrum*) or piperine to standard larval food used to create feeding mixtures for *Anopheles* larval toxicity bioassays

Black pepper/ Piperine (mg)	Standard larval food (mg)	Black pepper/ Piperine dose (%)
0	50*	-
5	45	10
10	40	20
20	30	40
40	10	80
50	0	100

* 50mg of standard food only was used as a control

3.2.3 Bioassays

Fifty 3rd/4th instar larvae of an *Anopheles* strain were gently introduced into each of 6 plastic containers containing 500ml distilled water (treatable surface area = 150mm×215mm). Each of the feeding mixtures (Table 3.1) was applied respectively to the water surface in the containers. A control with only standard larval food was also conducted concurrently. All food in both the control and treatments was dispensed once at the beginning of the bioassay. Mortality was recorded in each of the containers 24 hours after exposure in the black pepper bioassays and 48 hours after exposure in the piperine bioassays (little or no mortality was observed following 24 hours larval exposure to piperine). The mortality status of each larva was determined as per the WHO larviciding protocol (WHO, 2005), by assessing

responsiveness to being probed at the siphon or cervical region. The full range of treatments for both black pepper and piperine was replicated three times per *Anopheles* strain.

3.2.4 Statistical analysis

One-way ANOVA and Tukey HSD post-hoc tests were used to determine a) if the mortality in treated bioassays significantly differed from that of the controls and at which doses in particular, b) if there were significant differences in response between insecticide susceptible and resistant strains by species where pertinent, and c) if there were significant differences in response between species. A Student t-test was used to determine if there were significant differences in response between intoxication with black pepper and piperine.

3.3 RESULTS

For this study "dose", which is represented by a percentage, refers to the proportion (Table 3.1) of black pepper or piperine constituting 50mg of treatment mixture administered to larvae.

3.3.1 Black pepper

Black pepper, when applied to the water, tended to spread over the surface evenly and effectively. Mortality was induced in all species and strains by all treatments containing pepper (Figure 3.1). In general, mortality increased with increasing proportions of pepper in the treatment mixture. Furthermore, the presence of pepper (even in low quantities) consistently induced significantly higher mortalities in the *An. gambiae* complex larvae than in *An. funestus* (Table 3.2). Dead *An. gambiae* complex larvae tended to cluster, pivoting around their tracheal gills (Figure 3.2)

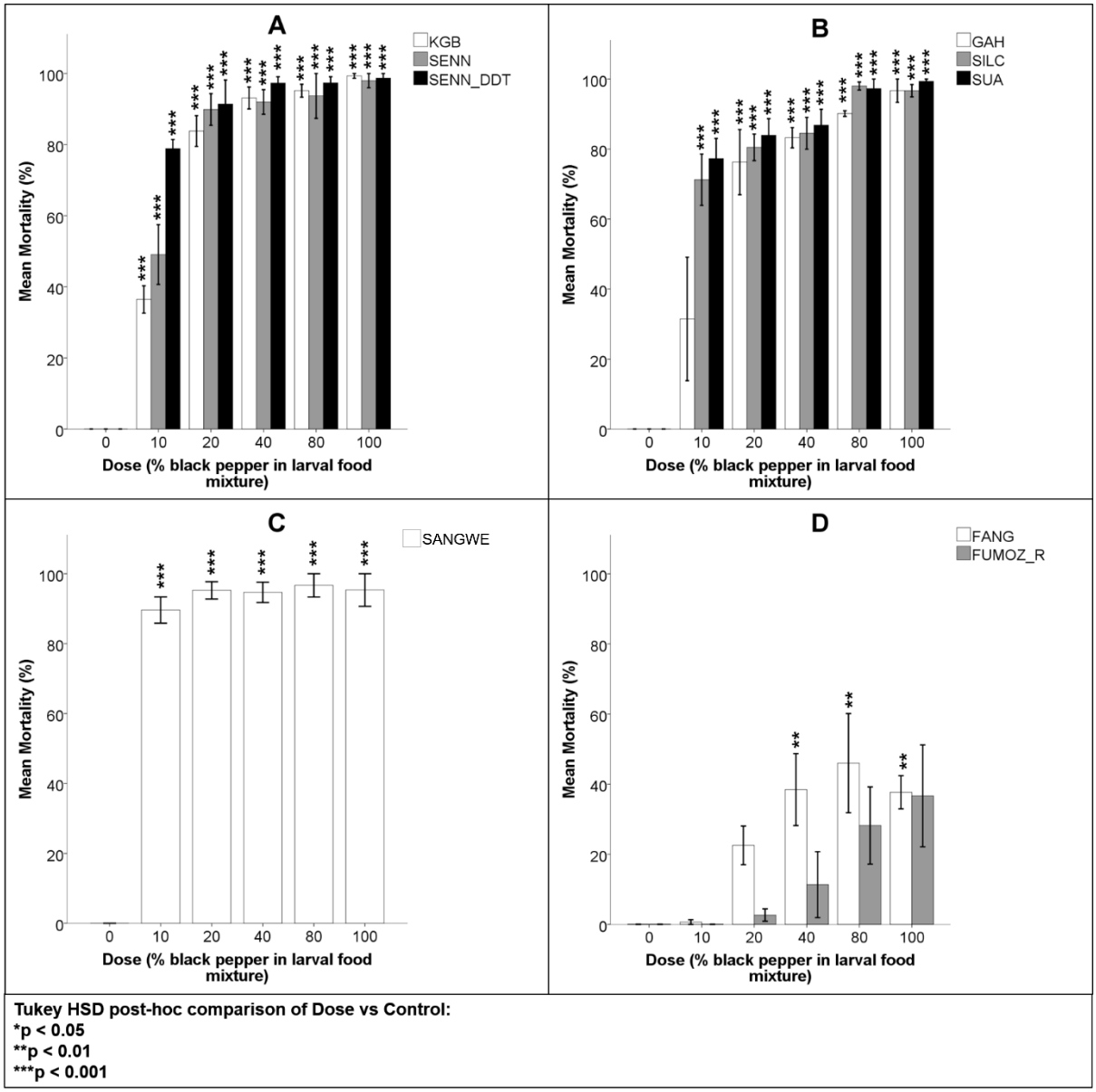


Figure 3.1 Mean mortalities of laboratory-reared *Anopheles* larvae 24 hours after being fed powdered black pepper (*Piper nigrum*) at various concentrations. The dose value represents the percentage of black pepper in a 50mg treatment mixture. 0% represents the control group which was fed standard larval food only and 100% represents a treatment comprised of black pepper only. A One-way ANOVA and Tukey HSD post-hoc comparison was used for each strain to determine significant differences in mortality at each dose compared to that of the relative control at 95% confidence. **A.** *Anopheles arabiensis* strains. **B.** *Anopheles gambiae* (GAH) and *An. coluzzii* (SILC and SUA) strains. **C.** *Anopheles quadriannulatus* (SANGWE) and **D.** *Anopheles funestus* strains.

Table 3.2 Tukey HSD post-hoc analysis of *Anopheles* larval mean mortalities 24 hours post-exposure to black pepper (*Piper nigrum*). A one-way ANOVA ($p < 0.01$; $F = 38.11$ at $df = 134$), supplemented with a Tukey HSD post-hoc, was used to determine significant differences in the overall mean mortalities induced in each *Anopheles* strain at 95% confidence.

	Strain	Mean Mortality
		Mean ± SD (95%CI)
1	KGB	$81.59 \pm 24.35^{8\text{-}9***}$
2	SENN	$84.54 \pm 20.17^{8\text{-}9***}$
3	SENN-DDT	$92.75 \pm 9.170^{8\text{-}9***}$
4	GAH	$75.55 \pm 27.38^{8\text{-}9***}$
5	SILC	$86.18 \pm 12.20^{8\text{-}9***}$
6	SUA	$88.93 \pm 10.47^{8\text{-}9***}$
7	SANGWE	$94.30 \pm 5.723^{8\text{-}9***}$
8	FANG	$29.07 \pm 20.78^{1\text{-}7***}$
9	FUMOZ-R	$15.77 \pm 20.07^{1\text{-}7***}$

Tukey HSD post-hoc multiple comparisons of strains:

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$



Figure 3.2 Clustering of dead *Anopheles quadriannulatus* larvae 24 hours after being fed a powdered black pepper treatment mixture. Similar clustering was observed among all strains of all *An. gambiae* complex species tested.

Anopheles arabiensis

The treatment dose of 100% black pepper achieved upward of 98% mortality in all three of the *An. arabiensis* strains and all of the treatment doses induced significantly higher mortality than the control for each KGB (One-way ANOVA: $p < 0.01$; $F = 211.51$ at $df = 17$), SENN (One-way ANOVA: $p < 0.01$; $F = 62.05$ at $df = 17$) and SENN-DDT (One-way ANOVA: $p < 0.01$; $F = 148.30$ at $df = 17$) (Figure 3.1A). There were no significant differences in mean mortality between SENN-DDT and either KGB or SENN (Table 3.2).

Anopheles gambiae and *An. coluzzii*

With the exception of GAH exposed to 10% black pepper (One-way ANOVA: $p < 0.01$; $F = 21.31$ at $df = 17$), all of the treatment mixtures achieved significantly higher mortality against larvae of both GAH (*An. gambiae*) and the *An. coluzzii* strains SILC (One-way ANOVA: $p < 0.01$; $F = 86.25$ at $df = 17$) and SUA (One-way ANOVA: $p < 0.01$; $F = 98.61$ at $df = 17$) (Figure 3.1B). When exposed to just the black pepper treatment (100%), the mean mortalities of the respective strains each exceeded 95%; even from the lowest treatment dose (10%), again with the exception of GAH at this dose, upward of 70% mean mortality was achieved in all of the strains. There were no significant differences in the mean mortalities induced in GAH versus those of either SILC or SUA (Table 3.2).

Anopheles quadriannulatus

The SANGWE strain was particularly susceptible to black pepper. Significantly higher mean mortalities were achieved in all pepper-inclusive treatments compared to the control, with approximately 90% mortality observed even at the lowest pepper dose (One-way ANOVA: $p < 0.01$; $F = 145.06$ at $df = 17$) (Figure 3.1C).

Anopheles funestus

Other than the mortality of FANG exposed to the 100% dose, which was less than in the 40% and 80% doses, mortalities among *An. funestus* larvae generally increased with increasing proportions of black pepper. Even with the proportional increase, ingestion of black pepper did not cause more than 50% mortality in either *An. funestus* strain. There was, however, significantly higher mortality among FANG larvae in the 40%-100% treatment doses than in the control (One-way ANOVA: $p < 0.01$; $F = 6.70$ at $df = 17$) (Figure 3.1D). For FUMOZ-R,

although one-way ANOVA indicated a significant difference in mortality between the doses ($p < 0.05$; $F = 3.51$ at $df = 17$), none of the treatment mortalities differed significantly from the control. This is likely because of the wide variation in response to pepper ingestion (Figure 3.1D). While this suggests that FUMOZ-R is less susceptible to black pepper than FANG, this was found to be statistically untrue (Table 3.2).

3.3.2 Piperine

Unlike black pepper, the powdered piperine did not spread effectively and if not scattered carefully over the surface area of water, it tended to clump. The larval clustering observed in the black pepper experiments did not occur in the piperine trials. All mortality results were recorded 48 hours post-feeding with piperine (Figure 3.3). SILC was used for black pepper bioassays but not for piperine bioassays as the insecticide resistance levels in the strain had significantly dropped during the period between the conducting of the two bioassays and it would not have been an accurate proxy by which to compare insecticide susceptible and resistant strains. The strain was subsequently replaced with *An. gambiae* TONGS for the piperine bioassays. The mortality induced in SENN, SENN-DDT and GAH did not significantly differ from the mortality in the *An. funestus* larvae, whereas the mortality in the other *An. gambiae* complex strains was significantly higher (Table 3.3).

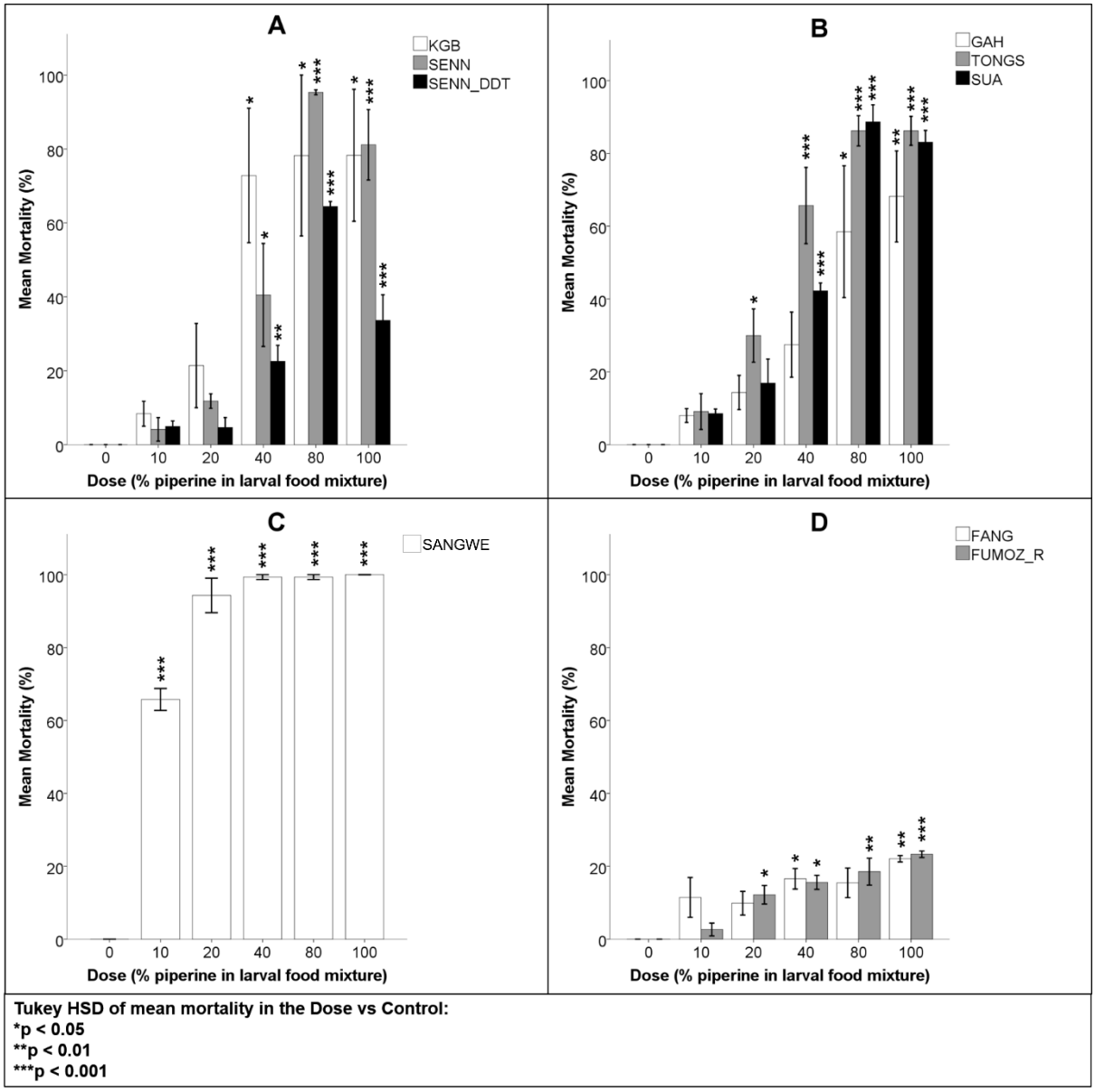


Figure 3.3 Mean mortalities of laboratory-reared *Anopheles* larvae 48 hours after being fed powdered piperine. The dose value represents the percentage of piperine in a 50mg treatment mixture. 0% represents the control group which was fed standard larval food only and 100% represents a treatment comprised of piperine only. A One-way ANOVA and Tukey HSD post-hoc comparison was used for each strain to determine significant differences in mortality at each dose compared to that of the relative control at 95% confidence. **A.** *Anopheles arabiensis* strains. **B.** *Anopheles gambiae* (GAH and TONGS) and *An. coluzzii* (SUA) strains. **C.** *Anopheles quadriannulatus* (SANGWE) and **D.** *Anopheles funestus* strains.

Table 3.3 Tukey HSD post-hoc analysis of *Anopheles* larval mean mortalities 48 hours post-exposure to piperine. A one-way ANOVA ($p < 0.01$; $F = 10.83$ at $df = 134$), supplemented with a Tukey HSD post-hoc, was used to determine significant differences in the overall mean mortalities induced in each *Anopheles* strain at 95% confidence.

	Strain	Mortality (%)
		Mean ± SD (95%CI)
1	KGB	$51.83 \pm 39.23^{7**,8\text{-}9*}$
2	SENN	$46.58 \pm 39.33^{7***}$
3	SENN-DDT	$26.05 \pm 23.61^{7***}$
4	GAH	$35.29 \pm 29.44^{7***}$
5	TONGS	$55.43 \pm 33.49^{7*,8\text{-}9**}$
6	SUA	$47.89 \pm 34.65^{7**,8\text{-}9*}$
7	SANGWE	$91.75 \pm 14.11^{1,6**,2\text{-}4,8\text{-}9***,5*}$
8	FANG	$15.09 \pm 6.91^{1,6*,5**,7***}$
9	FUMOZ-R	$14.46 \pm 7.96^{1,6*,5**,7***}$

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Anopheles arabiensis

All of the treatment mixtures including and exceeding 40% piperine induced significantly higher mortality than the control in KGB (One-way ANOVA: $p < 0.01$; $F = 6.54$ at $df = 17$), SENN (One-way ANOVA: $p < 0.01$; $F = 33.77$ at $df = 17$) and SENN-DDT larvae (One-way ANOVA: $p < 0.01$; $F = 46.63$ at $df = 17$) (Figure 3.3A). However, the significantly higher mortalities achieved range from approximately 20%-95% among the strains; and while this was seemingly indicative of the strains having significantly different responses from each other, ANOVA showed otherwise for both KGB versus SENN-DDT and SENN versus SENN-DDT (Table 3.3).

Anopheles gambiae and *An. coluzzii*

In GAH, only the 80% and 100% doses induced significantly higher larval mortality than the control (One-way ANOVA: $p < 0.01$; $F = 7.95$ at $df = 17$) and in both TONGS (One-way ANOVA: $p < 0.01$; $F = 40.05$ at $df = 17$) and SUA (One-way ANOVA: $p < 0.01$; $F = 108.22$ at $df = 17$), all dosages greater than and equal to 40% piperine induced significantly higher mortalities than the control (Figure 3.3B). There were no significant differences in mortality induced by piperine between either GAH and TONGS or GAH and SUA (Table 3.3).

Anopheles quadriannulatus

SANGWE larvae were more susceptible to the piperine than other *An. gambiae* complex strains (Table 3). Up to 100% mortality was induced in larvae exposed to 100% piperine and even 10% piperine induced more than 60% mortality. All of the piperine-inclusive doses induced significantly higher mortality than the control (One-way ANOVA: $p < 0.01$; $F = 292.01$ at $df = 17$) (Figure 3.3C).

Anopheles funestus

FANG larvae only showed significantly higher mortalities than that of the control in the 40% and 100% piperine dosages (One-way ANOVA: $p < 0.01$; $F = 5.14$ at $df = 17$). However, the highest mean mortality, which was induced by 100% piperine, was only 22%. The mortalities induced by piperine in FUMOZ-R larvae were significantly higher than that of the control in all dosages exceeding 10% piperine (One-way ANOVA: $p < 0.01$; $F = 17.88$ at $df = 17$) though the highest mean mortality achieved was again quite low at of only 24% using 100% piperine

(Figure 3.3D). There was no significant difference in the mortality induced by piperine between FANG and FUMOZ-R (Table 3.3).

3.3.3 Black pepper versus Piperine

The SILC and TONGS strains were excluded from the statistical analyses comparing mortalities induced by black pepper versus piperine as neither were exposed to both. Mortality among the *An. gambiae* complex strains 24 hours after being fed black pepper was significantly higher than that induced by piperine 48 hours following feeding (Student's t-test: $p < 0.01$; $t = 8.34$ at $df = 178$). Although it appeared that black pepper induced higher mortality than piperine in the *An. funestus* strains as well, the difference is not statistically significant at 95% confidence (Student's t-test: $p = 0.07$; $t = 1.87$ at $df = 58$).

3.4. DISCUSSION

Prior to this study, *Anopheles* larval toxicity caused by the ingestion of either black pepper or piperine had not been explicitly investigated. To date, the evaluations of the insecticidal activity of *P. nigrum* has been focussed mainly on the constituent alkaloids and their effects on a variety of insect species ranging from those of economic importance (Scott *et al*., 2003; de Paula *et al*., 2000) to disease vectors such as *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (Amer and Mehlhorn, 2006; Park *et al*., 2002). Of the compounds comprising *P. nigrum*, piperine is the most recognized and is largely abundant in fully differentiated shoots of the plant (Scott *et al*., 2008; Semler and Gross, 1998).

Commercial, ground black pepper and piperine showed marked toxic effects against 3/4th instar larvae of all the *An. gambiae* complex strains tested. Piperine ingestion was evidently less toxic to *Anopheles* larvae than black pepper, but did elicit larval mortality over 48 hours and may serve as a synergist amongst the various amides constituting black pepper. Black pepper and piperine induced mortality in insecticide susceptible and resistant *An. gambiae* complex strains with no statistically significant differences between them. This suggests that insecticide resistance mechanisms play no role in the detoxification of black pepper and piperine in these species.

The *An. funestus* strains, however, showed markedly lower sensitivities to black pepper and piperine. This may reflect differences in their feeding habits and metabolism. Alternatively, their larval rearing conditions, which partially reflect the characteristics of their natural

breeding sites, may have influenced their relative sensitivities. This is because the FANG and FUMOZ-R *An. funestus* strains are reared in water containing algae obtained from a nearby fish pond whereas the *An. gambiae* complex larvae are reared in water containing no additives other than larval food. However, it is not clear as to how or why this may have affected their responses to black pepper and piperine.

There were no differences in response between insecticide resistant and their corresponding insecticide susceptible strains by species to intoxication by either black pepper or piperine, suggesting that insecticide resistance mechanisms have no effect on their relative susceptibilities to these compounds/derivatives.

A potential advantage of utilizing piperamides for larval control is that they act as neurotoxins, but in a manner distinct from pyrethroids, and so represent a novel mode of action (Scott *et al*., 2008) - something urgently required for alternative methods of vector control (Zaim and Guillet, 2002). They also have an inhibitory effect on enzymes and have shown synergistic effects when used in conjunction with pyrethrum (Jensen *et al*., 2006). They have low mammalian toxicity and are not environmentally persistent, degrading quickly under full sunlight (Scott *et al*., 2003; Scott *et al*., 2004).

3.5 CONCLUSION

An appropriately formulated black pepper or derivative product may have potential as a larvicide for malaria vector control in settings where larval source management adds benefit under the auspices of integrated vector control. Assessments of the other piperamides present in black pepper may provide further insights into the larvicidal effects of this plant and its derivatives.

CHAPTER 4

EVALUATING THE TOXICITY OF A MIXTURE OF ORGANIC FATTY ACIDS (C8910) AGAINST *ANOPHELES* MOSQUITO LARVAE AND ADULTS

4.1 INTRODUCTION

Skinner *et al*. (1965) first reported the potential of lipids derived from the human skin surface to repel the host-seeking yellow fever vector *Aedes aegypti*. The isolation of the free fatty acid fraction, along with an assessment of its repellent properties, was conducted in 1970 - confirming that it was responsible for repellence in human skin lipids (Skinner *et al*., 1970). Such knowledge of repellent constituents of human skin lipids is of importance because specific groups of carboxylic acids may augment attractiveness rather than cause repellence, as was later demonstrated by Bosch *et al*. (2000), also testing repellence on *Ae. aegypti*. In addition to repelling female mosquitoes attempting to blood-feed, straight-chain fatty acids (particularly nonanoic acid) were also shown to repel *Ae. aegypti* and *Culex* species attempting to oviposit (Hwang *et al*., 1982).

More recently, a blend of octanoic ($CH_3(CH_2)6COOH$), nonanoic ($CH_3(CH_2)7COOH$) and decanoic (CH_3 $(CH_2)8COOH$) acids in equal proportions has shown promise both as a repellent and as an insecticide when combined with particular carriers (such as kaolin clay or silicone oil). Developed by Stratacor Inc. (Richmond, CA), the mixture has been patented as 'C8910', (Reifenrath, 2001). All of the constituents are considered to be inexpensive and are naturally occurring. For example, octanoic and decanoic acids are derived from palm kernel and coconut oils whereas nonanoic acid is from cattle tallow.

A 15% mixture of C8910 in a silicone carrier was highly repellent against houseflies (*Musca domestica*), horn flies (*Haematobia irritans*) and stable flies (*Stomoxys calcitrans*) (Mullens *et al*., 2009). The mixture also repelled *Culicoides* midges (Diptera: Ceratopogonidae) from suction light traps (Venter *et al*., 2011). Insecticidal effects against colony-reared, insecticide susceptible mosquito vector species including *An. gambiae, An. dirus, An. farauti, An. freeborni, An. minimus, An. stephensi, Ae. aegypti* and *Ae. albopictus* have also been recorded in CDC bottle bioassays conducted by Dunford *et al*. (2014, 2015). The mode of action of C8910 has yet to be fully elucidated, although fatty acids are known to cause respiratory inhibition (Scholefield, 1963). This has been the suggested mode of action in preliminary

studies (Dunford *et al*., 2014). If this were to be confirmed, such a novel mode of action would be well received by vector control strategists.

To date, insect larval exposure to C8910 has not been investigated, as most research has been geared toward its efficacy as either an insect repellent or adulticide.

The aim of this project was to evaluate the toxicity of the fatty acid mixture C8910 against the larvae and adults of insecticide resistant and susceptible strains of *An. arabiensis* and *An. funestus* as well as a single insecticide susceptible strain of the non-vector species, *An. quadriannulatus*. Evaluation of the toxicity of C8910 against adults of a wild-caught strain of *An. funestus* was conducted to supplement initial experiments on laboratory strains of this species. These data were published together in Samuel *et al*. (2015).

4.2 METHODS AND MATERIALS

4.2.1 Mosquito strains

The laboratory-reared *Anopheles arabiensis* strains KGB, SENN and SENN-DDT were used. KGB is an insecticide susceptible strain whereas SENN-DDT is a DDT-resistant strain selected from the susceptible SENN strain. An *Anopheles funestus* strain was reared from the progeny of a wild-caught Zambian sample and designated the name ZamF.

4.2.2 Technical material

A pure C8910 fatty acid blend (dated July 8, 2013) developed by Emery Oleochemicals (Cincinnati, OH) was supplied in liquid form.

4.2.3 Larval toxicity

4.2.3.1 *WHO larval bioassay*

The larval bioassay protocol recommended by the WHO was used to determine the biological activity of C8910 (WHO, 2005). Only the WHO-categorized "Phase I" stage of evaluation was conducted. This involved laboratory studies to determine the biopotency and activity of a potential larvicide as well as the diagnostic concentration and assessment of possible cross-resistance. This was done by exposing larvae to a range of concentrations of the technical material, C8910, and determining lethal concentrations from the mortality data.

200mg of liquid C8910 was diluted in 20ml of acetone to establish a stock solution which was stored at room temperature in a sealed and foil-covered glass bottle. 2ml of the stock solution was further dissolved in 18ml acetone to create the final solution from which test concentrations were derived. Aliquots of this solution were added to distilled water to establish 100ml treatment solutions to which 20-25 third and fourth instar *Anopheles* larvae were exposed. Test concentrations were refined from a range of 0.0001ppm-100.00ppm on insecticide susceptible *An. quadriannulatus* (SANGWE) and *An. arabiensis* (KGB) larvae. The final range concentrations decided upon were 0.50, 0.75, 1.00, 1.50, 2.00, 4.00 and 8.00ppm. Following 24 hours of exposure, larval mortality was recorded using the WHO standard for larval mortality (WHO, 2005). Bioassays were replicated 3 times for each concentration.

4.2.3.2 *Statistical Analysis*

Mean percentage mortalities at each concentration were compared between strains using one-way ANOVA. The corrected mortality data (using Abbott's formula) were also subjected to probit analysis, plotting the probit of the mortality percentage against log dose (Finney, 1952). From the resultant dose-response curves, lethal concentrations inducing 50% mortality (LC50) were determined for each replicate. The LC50s for the three replicates were then averaged to determine a final LC50 for each strain. LC50s of insecticide susceptible and resistant strains of the same species were compared using a Student t-test.

4.2.4 Adult toxicity

4.2.4.1 *CDC Bottle Bioassay*

The CDC bottle bioassay protocol of Brogdon and McAllister (1998) was used to assess the insecticidal effects of C8910 on the *An. arabiensis* strains and a wild-caught sample of *An. funestus*. Pure C8910 fatty acid blend was diluted in acetone to obtain a series of dilutions at a concentration range of 50-400µg active ingredient per litre (a.i /l) of acetone (Table 4.1). As *An. arabiensis* adults were observably more susceptible to C8910, the range of concentrations utilized was refined to 50-250µg a.i./l. The 250ml glass bottles were thoroughly washed, rinsed and dried and the interior of each was treated with 1ml of C8910 dilution. Controls included bottles treated with 1ml of acetone only.

Adult female mosquitoes (1-5 days old, 20-25 per bottle) were gently transferred into each bottle using an aspirator tube. Knockdown, which was defined as a mosquito on its back and unable to right itself to a standing position, was recorded at 15 minute intervals over the 2 hour exposure period for *An. funestus* and 1 hour period for *An. arabiensis* (again, due to the species' higher susceptibility). Three replicates of each concentration per colony/wild-caught sample were conducted. Following exposure, mosquitoes were removed from the bottles and placed into holding cups with access to 10% sucrose solution. Environmental controls were conducted concurrently with unexposed mosquitoes directly inserted into a holding cup with access to sucrose solution. Final mortalities were recorded at 24 hours post-exposure.

4.2.4.2 *Statistical Analysis*

The mean lethal concentrations inducing 50% mortality (LC50s) were determined for each colony/wild-caught sample using regression lines of log-transformed mortality data (Finney, 1952). One-way analysis of variance (ANOVA) was used to compare LC50s between the data sets belonging to insecticide susceptible and resistant strains of the same species.

Table 4.1 Volume of pure C8910 diluted in 50ml of acetone to achieve treatment concentrations. 1.00µg = 1.07µl of C8910.

C8910 (µl)	Treatment concentration (µg a.i./l)
2.68	50
4.01	75*
5.35	100
6.69	125*
8.03	150
10.7	200
13.38	250
16.05	300**
18.73	350**
21.40	400**

*Treatment concentrations only used for *An. arabiensis* bioassays.

**Treatment concentrations only used for *An. funestus* bioassays.

4.3 RESULTS

4.3.1 Larval toxicity

C8910 induced mortality in larvae of both the insecticide susceptible and resistant strains of *An. arabiensis* and *An. funestus*, as well as in the *An. quadriannulatus* strain, following 24 hours of exposure (Figure 4.1). The percent mortality generally increased as the concentration of C8910 increased and up to 100% mortality was induced in each of the strains at the highest C8910 concentration. Control mortality did not exceed 10% for any of the strains. For the One-way ANOVA and Tukey HSD post-hoc results, refer to Table 4.2 and 4.3 respectively.

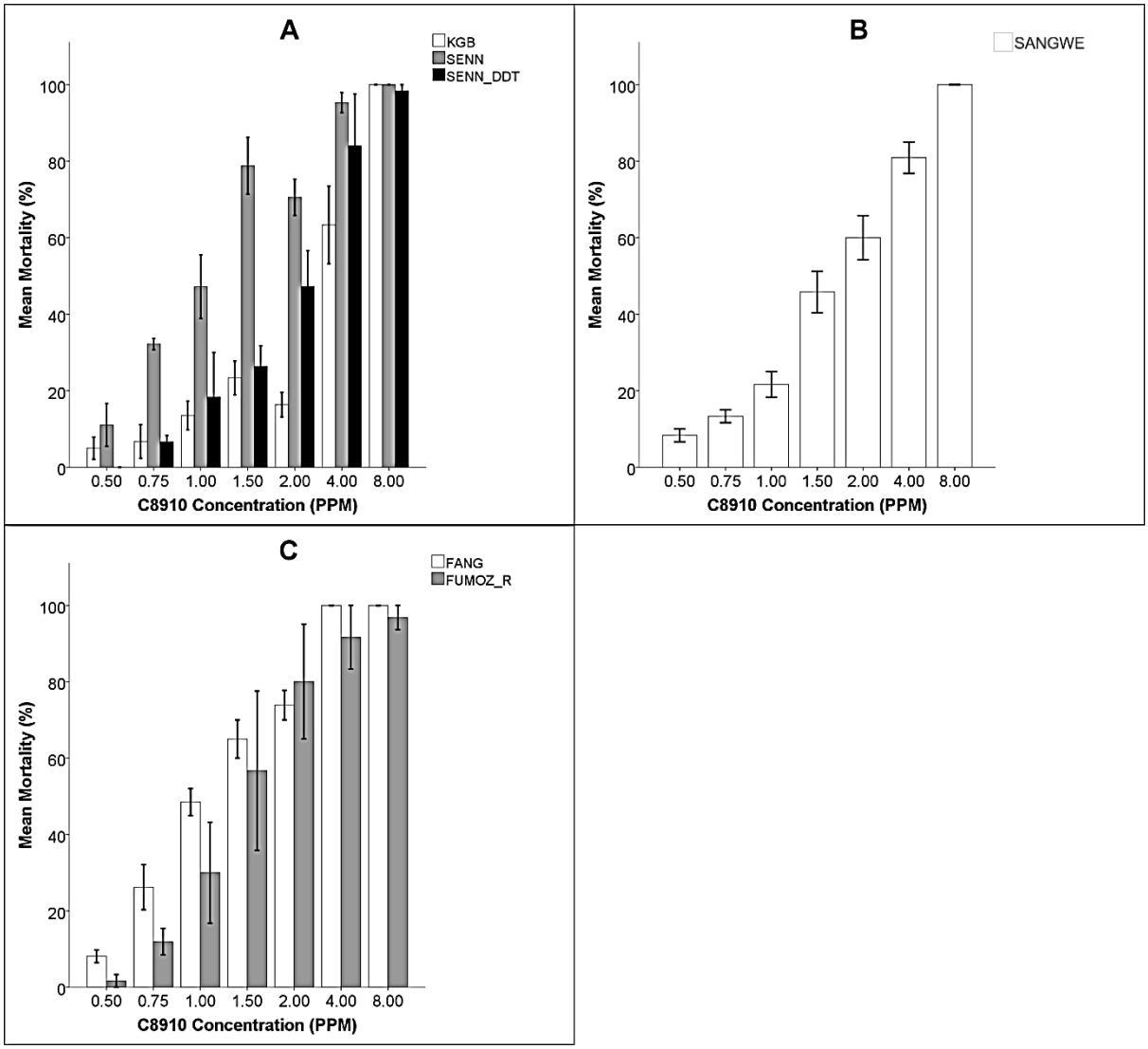


Figure 4.1 Mean percentage mortalities of laboratory-reared *Anopheles* larvae after 24 hour exposures to C8910. A. *Anopheles arabiensis* strains with differing insecticide resistance profiles: KGB (insecticide susceptible), SENN (baseline DDT resistance) and SENN-DDT (selected for DDT resistance). **B.** *Anopheles quadriannulatus* strain, SANGWE (insecticide susceptible) **D.** *Anopheles funestus* strains FANG (insecticide susceptible) and FUMOZ-R (pyrethroid and carbamate resistant).

Table 4.2 One-way ANOVA results of C8910 larvicide experiments conducted on *Anopheles* larvae. Late instar larvae of laboratory-reared strains of *Anopheles arabiensis*, *An. quadriannulatus* and *An. funestus* were exposed to a concentration gradient of the fatty acid mixture C8910. 24 hour mortality was recorded and statistically analysed using IBM SPSS v22.0.0.0.

C8910 Concentration (PPM)		df	F	p
0.05	Between Groups	5	2.261	0.115
	Within Groups	12		
	Total	17		
0.75	Between Groups	5	9.135	*0.001
	Within Groups	12		
	Total	17		
1.0	Between Groups	5	3.198	*0.046
	Within Groups	12		
	Total	17		
1.5	Between Groups	5	4.818	*0.012
	Within Groups	12		
	Total	17		
2.0	Between Groups	5	8.379	*0.001
	Within Groups	12		
	Total	17		
4.0	Between Groups	5	2.717	0.072
	Within Groups	12		
	Total	17		
8.0	Between Groups	5	0.840	0.546
	Within Groups	12		
	Total	17		

* Significant at 95% confidence

Table 4.3 Tukey HSD post-hoc results of C8910 larvicide experiments conducted on *Anopheles* larvae. Late instar larvae of laboratory-reared strains of *Anopheles arabiensis*, *An. quadriannulatus* and *An. funestus* were exposed to a concentration gradient of the fatty acid mixture C8910. 24 hour mortality was recorded and a one-way ANOVA was conducted in IBM SPSS v22.0.0.0. This was supplemented with a Tukey HSD post-hoc to identify significant differences between strains (Strain A and Strain B) at each concentration.

		p						
		C8910 Concentration (PPM)						
Strain A	**Strain B**	**0.05**	**0.75**	**1.0**	**1.5**	**2.0**	**4.0**	**8.0**
KGB	SENN	0.663	*0.003	0.115	*0.019	*0.005	0.116	1.000
	SENN-DDT	0.806	1.000	0.998	1.000	0.146	0.477	0.967
	SANGWE	0.955	0.766	0.980	0.616	*0.023	0.634	1.000
	FANG	0.967	*0.019	0.096	0.096	0.003	0.059	1.000
	FUMOZ-R	0.955	0.894	0.729	0.239	*0.001	0.192	0.643
SENN	KGB	0.663	*0.003	0.115	*0.019	*0.005	0.116	1.000
	SENN-DDT	0.133	*0.003	0.216	*0.027	0.381	0.909	0.967
	SANGWE	0.981	*0.024	0.321	0.248	0.933	0.790	1.000
	FANG	0.972	0.824	1.000	0.915	1.000	0.998	1.000
	FUMOZ-R	0.249	*0.015	0.695	0.630	0.956	0.999	0.643
SENN-DDT	KGB	0.806	1.000	0.998	1.000	0.146	0.477	0.967
	SENN	0.133	*0.003	0.216	*0.027	0.381	0.909	0.967
	SANGWE	0.357	0.757	1.000	0.737	0.869	1.000	0.967
	FANG	0.385	*0.019	0.183	0.136	0.258	0.716	0.967
	FUMOZ-R	0.998	0.888	0.913	0.323	0.113	0.981	0.967
SANGWE	KGB	0.955	0.766	0.980	0.616	*0.023	0.634	1.000
	SENN	0.981	*0.024	0.321	0.248	0.933	0.790	1.000
	SENN-DDT	0.357	0.757	1.000	0.737	0.869	1.000	0.967
	FANG	1.000	0.173	0.276	0.746	0.823	0.557	1.000
	FUMOZ-R	0.576	1.000	0.978	0.967	0.527	0.923	0.643
FANG	KGB	0.967	*0.019	0.096	0.096	*0.003	0.059	1.000
	SENN	0.972	0.824	1.000	0.915	1.000	0.998	1.000
	SENN-DDT	0.385	0.019	0.183	0.136	0.258	0.716	0.967
	SANGWE	1.000	0.173	0.276	0.746	0.823	0.557	1.000
	FUMOZ-R	0.611	0.111	0.633	0.990	0.993	0.972	0.643
FUMOZ-R	KGB	0.955	0.894	0.729	0.239	*0.001	0.192	0.643
	SENN	0.249	*0.015	0.695	0.630	0.956	0.999	0.643
	SENN-DDT	0.998	0.888	0.913	0.323	0.113	0.981	0.967
	SANGWE	0.576	1.000	0.978	0.967	0.527	0.923	0.643
	FANG	0.611	0.111	0.633	0.990	0.993	0.972	0.643

* Significant at 95% confidence

Anopheles arabiensis

Apart from a deviation from the mortality trend observed in KGB and SENN at the 2.0PPM concentration, C8910 induced consistently increasing proportional mortality with increasing concentration in the *An. arabiensis* strains (Figure 4.1A). The mean mortality of the SENN strain was significantly higher than that of KGB in the 0.75PPM, 1.5PPM and 2.0PPM concentrations. The mortality in the SENN strain was also significantly higher than SENN-DDT in the 0.75PPM and 1.5PPM concentration.

Anopheles quadriannulatus

C8910 consistently induced mortality in SANGWE and proportionately increased with increasing concentration (Figure 4.1B).

Anopheles funestus

There were no significant differences between the insecticide susceptible FANG and the insecticide resistant FUMOZ-R strains at any concentration (Figure 4.1C).

Lethal concentrations

The KGB strain of *An. arabiensis* was found to have the highest LC50 of all the exposed strains (Table 4.4). SENN and FANG had lower LC50s than the more resistant strains of their respective species. These differences were however not significant. With the exception of KGB, the SANGWE strain had a higher LC50 than the other insecticide susceptible strains.

Table 4.4 Lethal concentrations (LC50) of C8910 against laboratory-reared *Anopheles* larvae after 24 hour exposures to C8910. Third and fourth instar *Anopheles* larvae were exposed for 24 hours to C8910 in accordance with the WHO larvicide assessment protocol. Probit analysis of Abbott-corrected mortality results was used to determine the lethal concentrations, the means of which were compared using a One-way ANOVA ($P = 0.015$; $F = 4.51$ at $df = 12$) supplemented with a Tukey HSD post-hoc comparison.

	Strain	LC50 (PPM)
		Mean ± SD (95%CI)
1	KGB	2.77 ± 0.37 [2**,5*]
2	SENN	1.09 ± 0.12
3	SENN-DDT	2.16 ± 0.81
4	SANGWE	1.72 ± 0.30
5	FANG	1.15 ± 0.06
6	FUMOZ-R	1.59 ± 0.84

Tukey HSD post-hoc multiple comparisons of strains:

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

4.3.2 Adult toxicity

Adult mortality generally increased as C8910 concentration increased in the *An. funestus* and *An. arabiensis* strains (Figure 4.2). Control mortality did not exceed 10% in any of the bioassays. An additional replicate of the KGB bioassay was done as two replicates produced linear mortality trends and the third showed a sudden spike in mortality from the 50-75µg/l concentrations. This fourth replicate, however, also showed a similar dramatic fluctuation in mortality between the 50-75µg a.i./l concentrations, so no clear linear relationship between mortality and C8910 concentration could be established. Subsequently, the KGB strain was excluded from LC50 calculations.

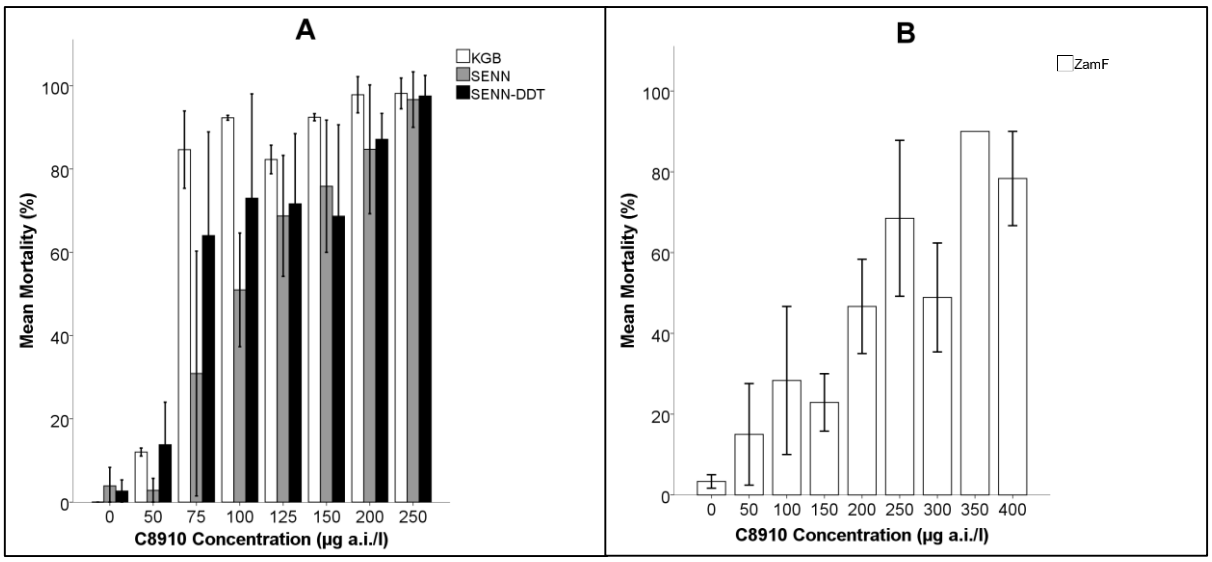


Figure 4.2 Mean percentage mortalities of *Anopheles* adults 24 hours after being exposed to C8910 at various concentrations. A. *Anopheles arabiensis* strains with differing insecticide resistance profiles: KGB (insecticide susceptible), SENN (baseline DDT resistance) and SENN DDT (selected for DDT resistance). **B.** The *Anopheles funestus* strain ZamF. Only one replicate was achieved at the 350µg a.i./l concentration.

Anopheles arabiensis

As previously mentioned, there was a sudden dramatic fluctuation in mortality observed in two of the four replicates of KGB exposure. These fluctuations were consistently followed by particularly high levels of mortality (exceeding 80%). Despite the observably high levels of variation, however, no significance difference was found between the mortalities of the four replicates for KGB (One-way ANOVA: $P = 0.170$; $F = 1.81$ at $df = 29$). For both the SENN and SENN-DDT strains, mortality generally increased as the C8910 concentration increased. Between the three *An. arabiensis* strains, there was no significant difference in response (One way ANOVA: $P = 6.13$; $F = 0.49$ at $df = 77$) (Figure 4.2A).

Anopheles funestus

The dose-mortality trend of the wild-caught ZamF sample was less obvious than in SENN and SENN-DDT (Figure 4.2B). A Tukey HSD post-hoc test of the ZamF showed that the significant trend indicated by one-way ANOVA does not reflect a significant difference in mean mortalities (One-way ANOVA: $P < 0.05$; $F = 3.76$ at $df = 22$).

Lethal concentrations

There was no significant difference in the mean lethal concentrations required to induce 50% mortality (LC50) in the *An. arabiensis* strains SENN and SENN-DDT (Table 4.5). Neither was there a difference between the mean LC50s of these respective strains and *An. funestus* of the wild-caught sample ZamF.

Table 4.5 Lethal concentrations (LC50) of C8910 against *Anopheles* mosquitoes following 24-hour exposure to C8910. Probit analysis of the mortality results were used to determine the lethal concentrations, the means of which were compared using a One-way ANOVA ($P > 0.05$; $F = 2.95$ AT $df = 12$) supplemented with a Tukey HSD post-hoc comparison. There were no significant differences between the LC50s of the three strains.

	Strain	LC50 (µg a.i/l)
		Mean ± SD (95%CI)
1	SENN	104.33 ± 13.43
2	SENN-DDT	79.33 ± 19.30
3	ZamF	202.67 ± 97.51

4.4 DISCUSSION

4.4.1 Larval toxicity

The *Anopheles* larvae do not possess a respiratory siphon, but rather have spiracles on their eighth abdominal segment through which they take in oxygen. As such, they must frequently position themselves parallel to the surface of the water in order to breathe. As C8910 is a mixture of fatty acids, its hydrophobic nature caused it to fall out of solution and clump at the surface of the water during the bioassays. The combination of these phenomena may well have resulted in suffocation of the larvae and hence contributed to the increased mortality where higher concentrations of C8910 were present.

It is not unusual for larvicidal agents to kill larvae in this manner, and oils and surface films are a WHO-recognized method of larviciding (WHO, 2013c). Other agents that work similarly include petroleum distillates and monomolecular surface films. There are several advantages to using oil-based larvicides based on the fact that mosquitoes cannot develop

resistance to oil. Among the currently employed options, there is also no toxicity against non-target organisms (including fish and mammals) when utilized according to their recommended doses. They are relatively cheap for targeting smaller water bodies such as borrow-pits, pools and latrines. However, they can become expensive for large scale treatment. The disadvantages of these oils are that they coat vegetation and their effectiveness can be negated by floating vegetation and debris. The longevity of their efficacy is also short, with treatment often having to be done on a weekly basis (WHO, 2013c).

The susceptibility levels of KGB and SANGWE was surprisingly lower than that of the more resistant *An. arabiensis* strains. This may be related to differences in detoxification gene expression between strains and species.

An. arabiensis is particularly adaptable in terms of breeding site selection and breeds in non-permanent water bodies such as temporary rain pools. This makes the larvae more difficult to target operationally with larvicides than *An. funestus* which prefers permanent swamp-like habitats. However, if C8910 were found not to hinder oviposition, its toxic effects against the larvae of DDT-resistant *An. arabiensis* may be particularly important for future vector control efforts.

C8910 was equally effective against the two strains of *An. funestus*, suggesting that the pyrethroid-resistant phenotype associated with adult FUMOZ-R mosquitoes (over-expression of the cytochrome P450 genes) did not decrease larval susceptibility to C8910. The breeding sites of *An. funestus* are more permanent water bodies and since this species is anthropophilic, potential breeding sites within close proximity to areas where people be consistently targeted. This is, however, subject to further investigation into the safety of C8910 for larviciding.

4.4.2 Adult toxicity

The variation in the mortality of KGB is difficult to explain considering that the same exposure conditions were maintained for all four replicates. Both KGB and SENN were observably less susceptible to C8910 than SENN-DDT and the mortality induced in SENN compared to SENN-DDT suggests that the DDT resistance mechanisms, related to elevated cytochrome P450, glutathione S-transferase (GST) and general esterase activity (Oliver and Brooke 2014), do not decrease susceptibility to C8910.

The experimentation on *An. funestus* adults that is reported on here is a continuation of initial work conducted by Samuel *et al*. (2015) on laboratory strains of this species. The

variation of responses recorded for the wild-caught *An. funestus* sample, ZamF, was notable in comparison to that observed by Samuel *et al*. (2015) in the laboratory-reared insecticide susceptible and pyrethroid resistant strains of the same species (both of which were susceptible to C8910). This likely reflects greater genetic heterogeneity in the wild-caught sample compared to the laboratory strains which are expected to show reduced genetic variation, and thereby reduced heterogeneity in response to insecticide intoxication, owing to laboratory colonisation and the effects of bottle-necking. The population from which the ZamF sample was derived also carries high levels of pyrethroid resistance (Choi *et al*. 2014). Although the resistance mechanisms in this population have not been fully characterised to date, preliminary data derived from synergist bioassays show that monooxygenases play a fundamentally important role.

The LD50 values recorded here for the *An. arabiensis* strains and ZamF are reasonably comparable to those recorded for other anophelines including *An. gambiae* (91.76 μg a.i./bottle), *An. dirus* (132.59 μg a.i./bottle), *An. farauti* (118.17 μg a.i./bottle), *An. freeborni* (119.87 μg a.i./bottle), *An. minimus* (55.44 μg a.i./bottle) and *An. stephensi* (112.58 μg a.i./bottle) (Dunford *et al.* 2014), allowing for the fact that the exposure times differed substantially (30 min for *An. gambiae, An. dirus, An. farauti, An. freeborni, An. minimus* and *An. stephensi* vs 2 hours for *An. funestus* and *An. arabiensis*).

The fatty acids comprising C8910 have been approved by the U.S. FDA and are categorized as “Generally recognized as safe”. They have low mammalian toxicity and are unlikely to pose significant environmental concerns (Dunford *et al.* 2014). As suggested by Samuel *et al*. (2015) however, further studies will be required to assess the residual activity of C8910 as an adulticide, though, a promising mechanism for prolonging residual activity is micro-encapsulation, and various strategies are currently underway to produce formulations with extended activity (Dunford *et al.* 2014).

4.5 CONCLUSION

C8910 proved to be an effective larvicide against laboratory-reared *Anopheles* larvae, and equally so against *An. arabiensis* and *An. funestus* strains. Further investigation into its effects on wild mosquito samples and non-target organisms will be necessary to establish the feasibility of C8910 as a larvicide for use in public health. These may be done through the Phase II and III WHO-recommended studies, small-scale and large-scale field testing, consecutively.

As an adulticide, it is concluded that C8910 is equally effective against DDT-resistant and insecticide susceptible *An. arabiensis*, though the mechanisms belying the decreased and variable mortality exhibited in the KGB strain requires further investigation. C8910 shows potential as an adulticide that can be used for malaria vector control, particularly in those instances where insecticide resistance management is required.

CHAPTER 5

DISCUSSION AND CONCLUDING REMARKS

Despite tremendous and somewhat successful efforts in reducing malaria incidence, particularly in Africa south of the Sahara, the disease continues to induce high levels of morbidity and mortality globally. Of the interventions employed, wide-scale vector control programmes remain a proactive and vitally important component. Among the interventions used for vector control, those that utilize synthetic insecticidal agents (i.e. LLINs and IRS) have achieved suppression of mosquito vector populations and thus reduction in disease incidence. This accomplishment is however jeopardized by several factors related to insecticide use. These include: insecticide resistance, insecticide persistence, and a limited number of alternatives for use in public health with little progress towards increasing the pool of WHO-approved insecticides.

Based on historical data, naturally occurring biochemicals may serve as a suitable and efficient (by WHOPES standards) solution for use in vector control under the current circumstances. In the present study, three potentially mosquitocidal and naturally-derived agents were assessed: black pepper (a powdery food spice derived from *P. nigrum*), piperine (the principle alkaloid of *Piper* species) and C8910 (a straight-chain fatty acid mixture). Each was assessed for their larvicidal capabilities and C8910 was also evaluated as an adulticide.

While larviciding is one of four types of larval source management (LSM) strategies recommended by the WHO (WHO, 2012b), it is not widely adopted, even as supplementary to IRS and LLINs. This is due to the logistical difficulties and costs of applying larvicides, particularly to temporary rainfall-dependent water bodies. However, the fact that it targets mosquitoes at a stage of their life cycle with limited mobility and bypasses the issues related to adulticide use, makes it an interesting prospect that should be seriously considered - even in an African context (Fillinger and Lindsay, 2011).

Although black pepper is larvicidal against *An. gambiae* complex species and may potentially be a useful larvicidal agent in localities where members of the complex are present, the affinity of these species to oviposit in temporary water bodies (such as rain puddles, etc.), makes it difficult to identify breeding sites that comply with the “few, fixed and findable” specifications of the WHO. The ingestion of pepper, even in the presence of a nontoxic food alternative also indicates that it acts as a competitive food source for the *Anopheles* species – which feed along the surface of the water. There are still certain caveats that must be

investigated though, such as the rate at which the pepper sinks from the surface (possibly decreasing efficacy) and the effect on non-target organisms, which is essential before it can be suggested as a possible wide-scale alternative for larviciding. Additionally, the black pepper would have to be appropriately formulated to achieve maximum impact.

Piperine, while toxic to various *Anopheles* species, was markedly less toxic than black pepper and took an additional 24 hours to produce mortality results comparable to that of the ground black pepper. This suggests that piperine may not be the only alkaloid responsible for the high mortality induced in the *Anopheles* species that were fed ground black pepper. Further research into additional amides present in pepper are required to determine which have insecticidal properties.

C8910 is both an efficient *Anopheles* larvicide and adulticide at high doses. A surprisingly low larval susceptibility to C8910 was evident in the insecticide susceptible KGB strain (lower than that of the DDT-resistant SENN-DDT strain). An important difference was also observed between the larval and adult susceptibility of this strain to C8910. This indicates that decreased larval susceptibility does not necessarily confer decreased adult susceptibility. Investigation into the mechanisms underying this difference in susceptibility is thus necessary.

As an adulticide, C8910 was effective against *An. arabiensis* but acted inconsistently against the *An. funestus* strain derived from a wild sample. The longevity of the toxic effect induced by pure C8910 is, however, low and suggestions of microencapsulation may be needed to increase the residual activity (Dunford *et al*., 2014). Should this be achieved, C8910 may have a potential use in LSM, IRS and LLINs especially in foci where malaria is at residual levels. This would be a much-welcomed addition to a waning arsenal of public health insecticides.

The most crucial property of these insecticides that remains to be investigated are their modes of action, which is paramount to them being considered for WHO-recommended vector control strategies. If these modes of action are confirmed as novel and are not nullified by cross-resistance to existing insecticides, with further research and development of applicable formulations, black pepper (and once identified, the constituents responsible for its toxicity) and C8910 may potentially serve as valuable alternatives/supplementaries for vector control and may contribute significantly to achieving the elimination of malaria transmission globally.

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EHICS WAIVER

Human Research Ethics Committee (Medical)

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

TO WHOM IT MAY CONCERN:

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

Investigator: **Prof Maureen Coetzee.**

Project title: **Research on mosquitos.**

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

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APPENDIX A

Samuel, M., Oliver, S.V., Coetzee, M. and Brooke, B.D., 2016. The larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of *Anopheles* malaria vector mosquitoes. *Parasites and vectors*, ***9***:238.

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The larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of *Anopheles* malaria vector mosquitoes

Michael Samuel[1,2], Shüné V. Oliver[1,2], Maureen Coetzee[1,2] and Basil D. Brooke[1,2*]

Abstract

Background: Insecticide resistance carries the potential to undermine the efficacy of insecticide based malaria vector control strategies. Therefore, there is an urgent need for new insecticidal compounds. Black pepper (dried fruit from the vine, *Piper nigrum*), used as a food additive and spice, and its principal alkaloid piperine, have previously been shown to have larvicidal properties. The aim of this study was to investigate the larvicidal effects of ground black pepper and piperine against third and fourth instar *Anopheles* larvae drawn from several laboratory-reared insecticide resistant and susceptible strains of *Anopheles arabiensis*, *An. coluzzii*, *An. gambiae*, *An. quadriannulatus* and *An. funestus*.

Methods: Larvae were fed with mixtures of standard larval food and either ground black pepper or piperine in different proportions. Mortality was recorded 24 h after black pepper and 48 h after piperine were applied to the larval bowls.

Results: Black pepper and piperine mixtures caused high mortality in the *An. gambiae* complex strains, with black pepper proving significantly more toxic than piperine. The *An. funestus* strains were substantially less sensitive to black pepper and piperine which may reflect a marked difference in the feeding habits of this species compared to that of the Gambiae complex or a difference in food metabolism as a consequence of differences in breeding habitat between species.

Conclusions: Insecticide resistant and susceptible strains by species proved equally susceptible to black pepper and piperine. It is concluded that black pepper shows potential as a larvicide for the control of certain malaria vector species.

Keywords: *An. arabiensis*, *An. coluzzii*, *An. funestus*, *An. gambiae*, *An. quadriannulatus*, Larvicides, Vector control

Background

Malaria is responsible for high levels of morbidity and mortality globally, with particular severity in sub-Saharan Africa [1]. This region is home to several of the most efficient malaria vectors, the principal species being *Anopheles gambiae*, *An. coluzzii* (formerly *An. gambiae* M form [2]), *An. arabiensis* and *An. funestus*. The former three species are members of the *Anopheles gambiae* species complex and the latter is the nominal member and only major vector of the *Anopheles funestus* species group [3, 4].

Insecticide based vector control strategies primarily directed against adult mosquitoes have significantly decreased malaria incidence over the past decade, with indoor residual spraying of insecticides (IRS) and distribution of long-lasting insecticide-treated bednets (LLINs) being pivotal [1]. Additionally, and especially in light of the increasing incidence of insecticide resistance in target vector populations [5], larval source management (LSM) is playing an increasingly important role in vector control [6].

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Larval source management as a vector control technique has dramatically decreased since the shift toward the use of synthetic mosquito adulticides, especially DDT and later pyrethroids – this despite distinct successes achieved by larviciding in the early-to-mid 1900's [7]. LSM is currently only recognized as supplementary to the core interventions of IRS and LLINs by the World Health Organization (WHO), being employed in only 38 of the 97 countries with ongoing malaria transmission [1]. Nevertheless, the WHO encourages the use of LSM where conditions are appropriate and it is feasible to do so [8] and recent evidence, though sparse, does suggest that larviciding may have a reinvigorated, valuable role to play in integrated vector management [7, 9, 10]. It is for these reasons that investigations into potential options for use in larviciding are ongoing.

A tendency towards naturally occurring insecticides, particularly those of botanical origin, has gained increasing interest in recent years. Plants have co-evolved with insects over many centuries, as have their defence mechanisms in response to insect predation. Plant allelochemicals can be very advantageous as insecticidal actives compared to their synthetic counterparts as they are biodegradable and in many instances show reduced or no adverse effects on non-target organisms [11].

Piper nigrum L. (the fruit of which are used to produce white and black pepper) has a plethora of traditional and modern day applications ranging from a US FDA recognized food additive to potential as an insecticide [12]. While assessing spreading agents for *Metarhizium anisopliae* and *Beauveria bassiana* entomopathogenic fungal spores, Bukhari et al. [13] observed 100 % mortality of *Anopheles* larvae exposed to white pepper, even in the absence of the fungal spores. This has prompted an interest in the use of pepper as a possible larvicide for use in malaria vector control. In addition, piperine, which is the principle alkaloid responsible for the pungency of pepper, has demonstrated toxicity against fourth-instar larvae of the dengue and yellow fever vector *Aedes aegypti* [14, 15].

The aim of this study was to investigate the toxicity of *P. nigrum* (black pepper) and piperine when administered as a food source to larvae of several insecticide resistant and susceptible strains of *Anopheles* species including *An. arabiensis*, *An. coluzzii*, *An. gambiae*, *An. quadriannulatus* and *An. funestus*.

Methods

Mosquito strains

All of the mosquito strains used in this study are housed in the Botha De Meillon Insectary (BMDI) at the National Institute for Communicable Diseases (NICD) in Johannesburg. All larvae were reared and bioassays conducted under the standard insectary conditions [16]. Table 1 gives detailed information on each of the strains used.

Technical materials

Commercial ground black pepper was further homogenized in a Qiagen Tissue Lyser II, for 5 min, until a fine powdery consistency was achieved. Analytical grade piperine in powder form (expiry date: December 2016) was purchased from Sigma Aldrich (Saint Louis, MO). The technical materials were each proportionately mixed with standard larval food [16] to obtain treatment mixtures ranging from 0 % pepper/piperine (control) to 100 % pepper/piperine (Table 2).

Larval toxicity bioassays

Fifty third to fourth instar larvae from each *Anopheles* strain were gently introduced into plastic containers containing 500 ml distilled water (surface area = 150 mm × 215 mm). Fifty milligram (1 mg treatment mixture/larva) of each treatment mixture, in addition to a control composed entirely of standard larval food, was tapped gently into each container to allow for an even spread over the surface of the water. Mortality was recorded 24 h after the application of the black pepper mixtures and 48 h after the piperine mixtures (little or no mortality was observed following 24 h larval exposure to piperine). At the end of the period, larvae were gently prodded and non-responsive larvae were recorded as dead [17]. Bioassays for each treatment mixture were replicated three times per strain.

Statistical analysis

Mortality was corrected using Abbott's formula in replicates where control mortality exceeded 10 %. One-way ANOVA and Tukey HSD *post-hoc* tests were used to determine (i) if the mortality in treated bioassays significantly differed from that of the controls and at which doses in particular; (ii) if there were significant differences in response between insecticide susceptible and resistant strains by species where pertinent; and (iii) if there were significant differences in response between species of the Gambiae complex and Funestus group. For the latter, analysis excluded control mortalities from the data. A Student's *t*-test was used to determine if there were significant differences in response between intoxication with black pepper and piperine. All statistics were conducted in IBM SPSS Statistics v22 with significance set at 95 % confidence.

Results

For this study "dose", which is represented by a percentage, refers to the proportion (Table 2) of black pepper or piperine constituting 50 mg of treatment mixture administered to larvae.

Table 1 *Anopheles* species by laboratory strain and insecticide susceptibility profile fed on powdered formulations of either *Piper nigrum* (black pepper) or piperine at the larval stage

Species	Strain (Country of origin)	Resistance profile/additional information	Resistance mechanisms
Anopheles arabiensis	KGB (Zimbabwe)	Insecticide susceptible	N/A
	SENN (Sudan)	Mostly susceptible. Low-level resistance to permethrin	N/A [27]
	SENN-DDT (Sudan)	Selected for resistance to DDT from SENN base colony. Also resistant to permethrin, deltamethrin and malathion [27, 28]	Elevated cytochrome P450, glutathione S-transferase (GST) and general esterase activity [27, 28]
Anopheles coluzzii	SILC[a] (Sierra Leone)	Resistant to pyrethroids and DDT	[a]No data
	SUA (Liberia)	Insecticide susceptible	N/A
Anopheles gambiae	GAH (Ghana)	Resistant to pyrethroids, DDT, carbamates and organophosphates [29]	Monooxygenase and esterase mediated detoxification coupled with the L1014F *kdr* mutation are implicated in pyrethroid resistance. An assortment of L1014F *kdr* is also implacted in DDT resistance. Mutations of the Alanine296-glycine (*Rdl*) GABA receptor and acetylcholinesterase receptor (*ace-1*^{R}) are associated with dieldrin and bediocarb resistance respectively [29].
	TONGS[a] (Cote d'Ivoire)	Resistant to pyrethroids, DDT, carbamates and organophosphates	[a]No data
Anopheles quadriannulatus	SANGWE (Zimbabwe)	Insecticide susceptible. *Anopheles quadriannulatus* is zoophilic and not considered to be a vector species.	N/A
Anopheles funestus	FANG (Angola)	Insecticide susceptible	N/A
	FUMOZ-R (Mozambique)	Resistant to pyrethroids and carbamates [30]	Overexpression of the cytochrome P450 CYP6P9 [30]. Thickened cuticles also contribute to adult insecticide-resistance [31].

[a]SILC was used for black pepper bioassays but not for piperine bioassays as the insecticide resistance levels in the strain had significantly dropped during the period between the conducting of the two bioassays and it would not have been an accurate proxy by which to compare insecticide susceptible and resistant strains. SILC was replaced with the *An. gambiae* strain TONGS for the piperine bioassays

Black pepper

Black pepper, when applied to the water, tended to spread over the surface quite evenly and effectively. Mortality was induced in all species and strains by all treatments containing pepper (Fig. 1a–d). In general, mortality increased with increasing proportions of pepper in the treatment mixture. Furthermore, the presence of pepper (even in low quantities) consistently induced significantly higher mortalities in the *An. gambiae* complex larvae than in *An. funestus* (Table 3). Dead *An. gambiae* complex larvae tended to cluster, pivoting around their tracheal gills (Fig. 2).

Table 2 Proportions of black pepper (*Piper nigrum*) or piperine to standard larval food used as treatment dosages for *Anopheles* larval toxicity bioassays

Black pepper/ piperine (mg)	Standard larval food (mg)	Dosage of black pepper/piperine (%)
0	50	0
5	45	10
10	40	20
20	30	40
40	10	80
50	0	100

Anopheles arabiensis

The treatment dose of 100 % black pepper achieved upwards of 98 % mortality in all three of the *An. arabiensis* strains and all of the treatment doses induced significantly higher mortality than the control (0 % black pepper) for KGB (ANOVA: $P < 0.01$; $F = 211.51$ at $df = 17$), SENN (ANOVA: $P < 0.01$; $F = 62.05$ at $df = 17$) and SENN-DDT (ANOVA: $P < 0.01$; $F = 148.30$ at $df = 17$) (Fig. 1a). There were no significant differences in mean mortality between SENN-DDT and either KGB or SENN (Table 3).

Anopheles gambiae and An. coluzzii

With the exception of GAH exposed to 10 % black pepper, all of the treatment mixtures achieved significantly higher mortality against larvae of both GAH (*An. gambiae*) (ANOVA: $P < 0.01$; $F = 21.31$ at $df = 17$) and the *An. coluzzii* strains SILC (ANOVA: $P < 0.01$; $F = 86.25$ at $df = 17$) and SUA (ANOVA: $P < 0.01$; $F = 98.61$ at

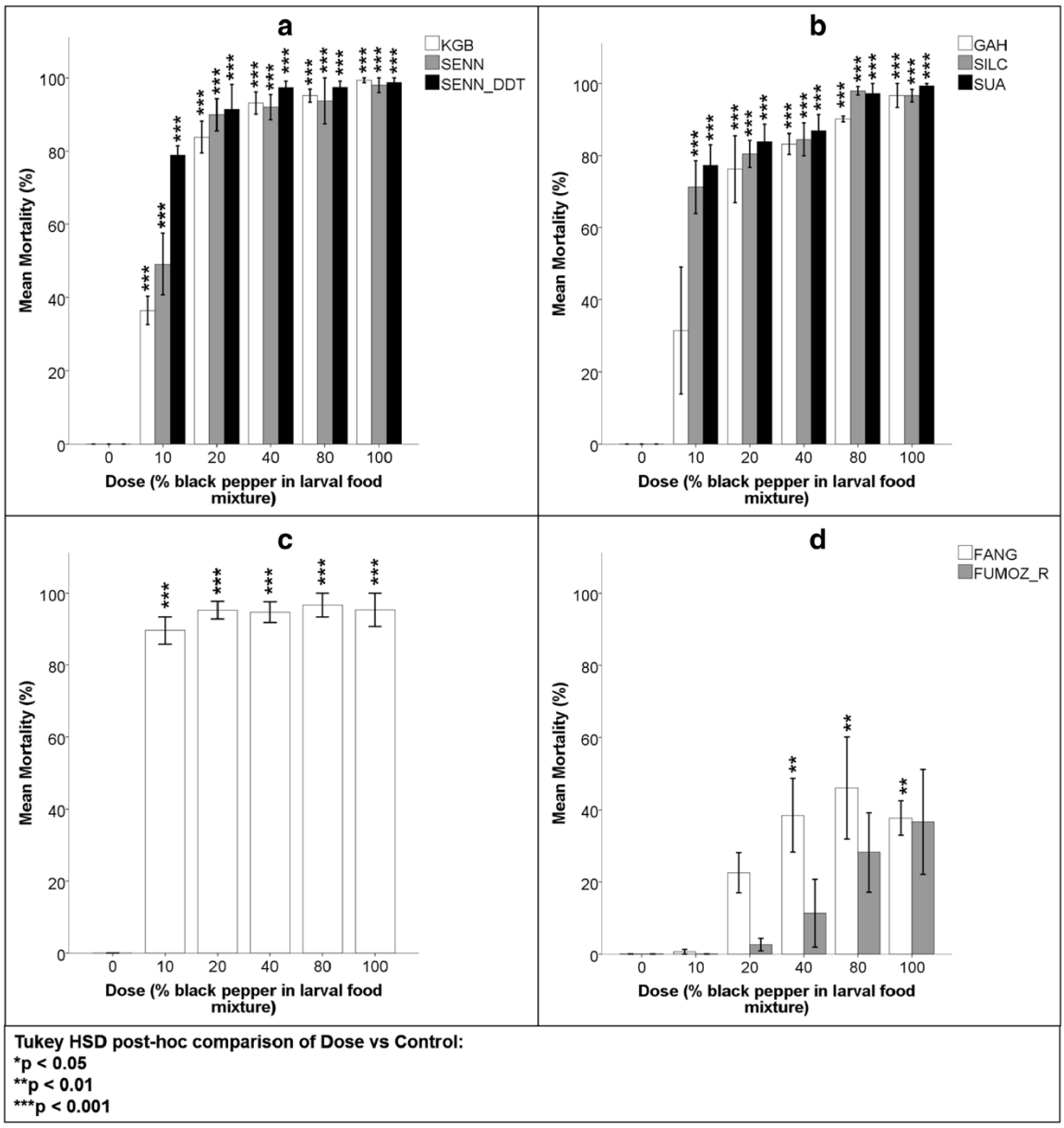


Fig. 1 Mean mortalities of laboratory-reared *Anopheles* larvae 24 h after being fed powdered black pepper (*Piper nigrum*) at various concentrations. The dose value represents the percentage of black pepper in a 50 mg treatment mixture. 0 % represents the control group which was fed standard larval food only and 100 % represents a treatment comprised of black pepper only. A One-way ANOVA and Tukey HSD *post-hoc* comparisons were used for each strain to determine significant differences in mortality at each dose compared to that of the relevant control at 95 % confidence. **a** *Anopheles arabiensis* strains. **b** *Anopheles gambiae* (GAH) and *An. coluzzii* (SILC & SUA) strains. **c** *Anopheles quadriannulatus*. **d** *Anopheles funestus* strains

df = 17) (Fig. 1b). When exposed to just the black pepper treatment (100 %), the mean mortalities of the respective strains each exceeded 95 %; even the lowest treatment dose (10 %), with the exception of GAH at this dose, showed upwards of 70 % mean mortality. There were no significant differences in the mean mortalities induced in GAH versus those of either SILC or SUA (Table 3).

Anopheles quadriannulatus

The SANGWE strain was particularly susceptible to black pepper. Significantly higher mean mortalities

Table 3 Tukey HSD *post-hoc* analysis of *Anopheles* larval mean mortalities 24 h post-exposure to black pepper (*P. nigrum*)

	Strain	Mean mortality	Standard deviation
1	KGB	81.59^{8-9***}	24.35
2	SENN	84.54^{8-9***}	20.17
3	SENN-DDT	92.75^{8-9***}	9.170
4	GAH	75.55^{8-9***}	27.38
5	SILC	86.18^{8-9***}	12.20
6	SUA	88.93^{8-9***}	10.47
7	SANGWE	94.30^{8-9***}	5.723
8	FANG	29.07^{1-7***}	20.78
9	FUMOZ-R	15.77^{1-7***}	20.07

A one-way ANOVA ($P < 0.01$; $F = 38.11$ at $df = 134$), supplemented with a Tukey HSD *post-hoc* test, was used to determine significant differences in the overall mean mortalities induced between *Anopheles* strains indicated by superscript numbering at 95, 99 and 99.9 % confidence
***$P < 0.001$

were achieved in all pepper-inclusive treatments compared to the control, with approximately 90 % mortality observed even at the lowest pepper dose (ANOVA: $P < 0.01$; $F = 145.06$ at $df = 17$) (Fig. 1c).

Anopheles funestus

Other than the mortality of FANG exposed to the 100 % dose, which was less than in the 40 and 80 % doses, mortalities among *An. funestus* larvae generally increased with increasing proportions of black pepper.



Fig. 2 Clustering of dead *Anopheles quadriannulatus* larvae 24 h after being fed a powdered black pepper treatment mixture. Similar clustering was observed among all strains of all *An. gambiae* complex species tested

Generally, ingestion of black pepper did not cause more than 50 % mortality in either of the *An. funestus* strains. There was, however, significantly higher mortality among FANG larvae in the 40–100 % treatment doses than in the control (ANOVA: $P < 0.01$; $F = 6.70$ at $df = 17$) (Fig. 1d). For FUMOZ-R, although one-way ANOVA indicated a significant difference in mortality between the doses ($P < 0.05$; $F = 3.51$ at $df = 17$), none of the treatment mortalities differed significantly from the control. This is likely due to the wide variation in response to pepper ingestion (Fig. 1d). While this suggests that FUMOZ-R is less susceptible to black pepper than FANG, this was found to be statistically insignificant (Table 3).

Piperine

Unlike black pepper, the powdered piperine did not spread effectively and if not scattered carefully over the surface area of water, it tended to clump. The larval clustering observed in the black pepper experiments did not occur in the piperine trials. All mortality results were recorded 48 h post-feeding with piperine (Fig. 3). The mortalities induced in SENN, SENN-DDT and GAH did not significantly differ from the mortalities in the *An. funestus* larvae, probably owing to high levels of variation around the means, whereas the mortalities in the other Gambiae complex strains were significantly higher than those recorded in the *An. funestus* strains (Table 4).

Anopheles arabiensis

All of the treatment mixtures including and exceeding 40 % piperine induced significantly higher mortality than the control in KGB (ANOVA: $P < 0.01$; $F = 6.54$ at $df = 17$), SENN (ANOVA: $P < 0.01$; $F = 33.77$ at $df = 17$) and SENN-DDT larvae (ANOVA: $P < 0.01$; $F = 46.63$ at $df = 17$) (Fig. 3a). However, the significantly higher mortalities achieved ranged from approximately 20–95 % among the strains; and while this was seemingly indicative of the strains having significantly different responses from each other, ANOVA showed otherwise for both KGB *versus* SENN-DDT and SENN *versus* SENN-DDT (Table 4).

Anopheles gambiae and An. coluzzii

In GAH, only the 80 and 100 % doses induced significantly higher larval mortality than the control (One-way ANOVA: $P < 0.01$; $F = 7.95$ at $df = 17$) and in both TONGS (ANOVA: $P < 0.01$; $F = 40.05$ at $df = 17$) and SUA (ANOVA: $P < 0.01$; $F = 108.22$ at $df = 17$), all dosages greater than and equal to 40 % piperine induced significantly higher mortalities than the control (Fig. 3b). There were no significant differences in

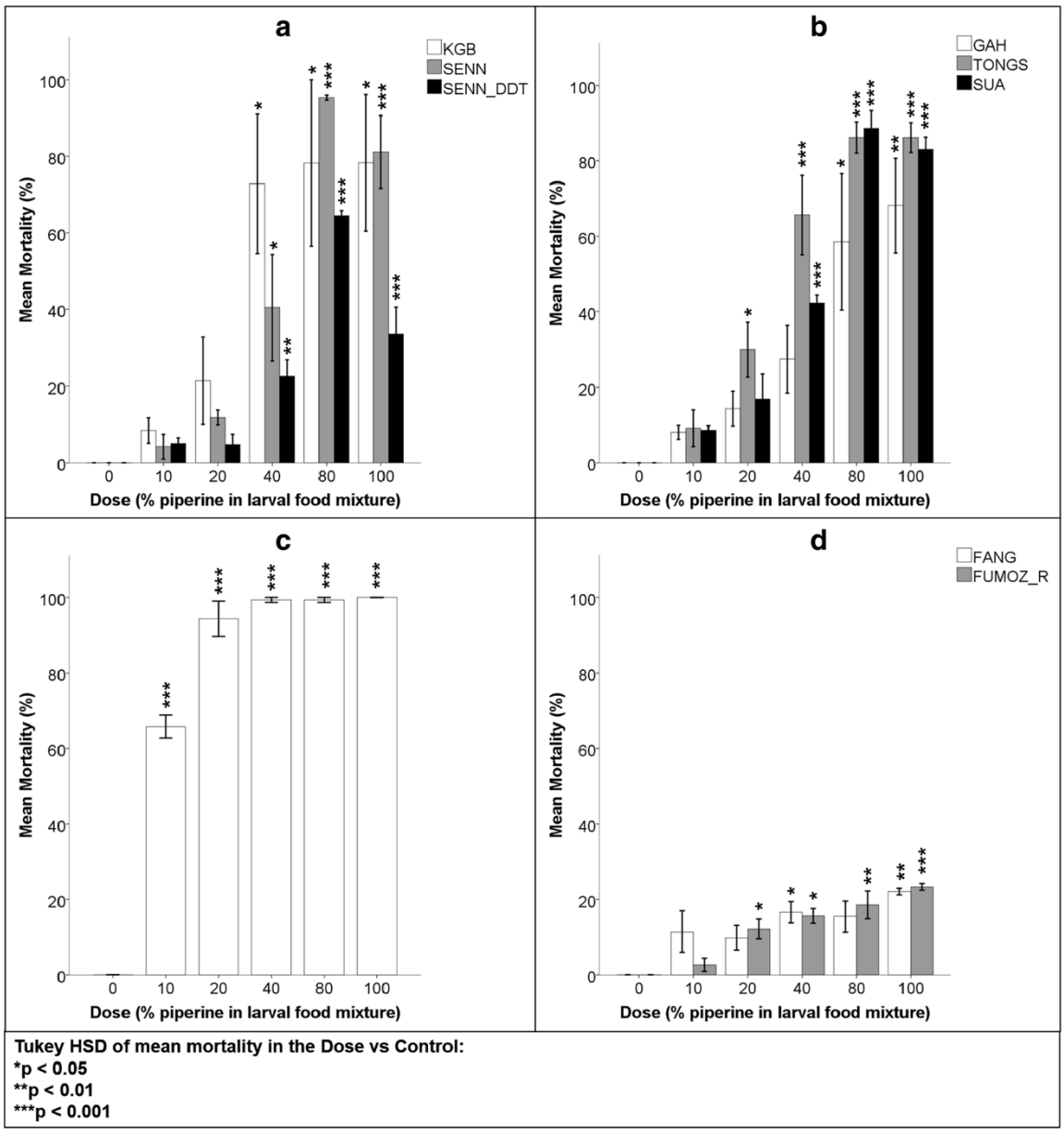


Fig. 3 Mean mortalities of laboratory-reared *Anopheles* larvae 48 h after being fed powdered piperine. The dose value represents the percentage of piperine in a 50 mg treatment mixture. 0 % represents the control group which was fed standard larval food only and 100 % represents a treatment comprised of piperine only. A One-way ANOVA and Tukey HSD *post-hoc* comparisons were used for each strain to determine significant differences in mortality at each dose compared to that of the relevant control at 95 % confidence. **a** *Anopheles arabiensis* strains. **b** *Anopheles gambiae* (GAH& TONGS) and *An. coluzzii* (SUA) strains. **c** *Anopheles quadriannulatus*. **d** *Anopheles funestus* strains

mortality induced by piperine between either GAH and TONGS or GAH and SUA (Table 4).

Anopheles quadriannulatus

SANGWE larvae were more susceptible to the piperine than other *An. gambiae* complex strains (Table 4). Up to 100 % mortality was induced in larvae exposed to 100 % piperine and even 10 % piperine induced more than 60 % mortality. All of the piperine-inclusive doses induced significantly higher mortality than the control (ANOVA: $P < 0.01$; $F = 292.01$ at $df = 17$) (Fig. 3c).

Table 4 Tukey HSD *post-hoc* analysis of *Anopheles* larval mean mortalities 48 h post-exposure to piperine

	Strain	Mean mortality	Standard deviation
1	KGB	$51.83^{7**,8–9*}$	39.23
2	SENN	46.58^{7***}	39.33
3	SENN-DDT	26.05^{7***}	23.61
4	GAH	35.29^{7***}	29.44
5	TONGS	$55.43^{7*,8–9**}$	33.49
6	SUA	$47.89^{7**,8–9*}$	34.65
7	SANGWE	$91.75^{1,6**,2–4,8–9***,5*}$	14.11
8	FANG	$15.09^{1,6*,5**,7***}$	6.91
9	FUMOZ-R	$14.46^{1,6*,5**,7***}$	7.96

A one-way ANOVA ($P < 0.01$; $F = 10.83$ at $df = 134$), supplemented with a Tukey HSD *post-hoc* test, was used to determine significant differences in the overall mean mortalities induced between *Anopheles* strains indicated by superscript numbering at 95, 99 and 99.9 % confidence
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Anopheles funestus

FANG larvae only showed significantly higher mortalities than that of the control in the 40 and 100 % piperine dosages (ANOVA: $P < 0.01$; $F = 5.14$ at $df = 17$). However, the highest mean mortality, which was induced by 100 % piperine, was only 22 %. The mortalities induced by piperine in FUMOZ-R larvae were significantly higher than that of the control in all dosages exceeding 10 % piperine (ANOVA: $P < 0.01$; $F = 17.88$ at $df = 17$) although the highest mean mortality achieved was low at only 24 % using 100 % piperine (Fig. 3d). There was no significant difference in the mortality induced by piperine between FANG and FUMOZ-R (Table 4).

Black pepper versus piperine

The SILC and TONGS strains were excluded from the statistical analyses comparing mortalities induced by black pepper versus piperine as neither were exposed to both. Mortality among the *An. gambiae* complex strains 24 h after being fed black pepper was significantly higher than that induced by piperine 48 h post-feeding (Student's *t*-test: $P < 0.01$; $t = 8.34$ at $df = 178$). Although it appeared that black pepper induced higher mortality than piperine in the *An. funestus* strains as well, the difference is not statistically significant at 95 % confidence (Student's *t*-test: $P = 0.07$; $t = 1.87$ at $df = 58$).

Discussion

Prior to this study, *Anopheles* larval toxicity caused by the ingestion of either black pepper or piperine had not been explicitly investigated. To date, the evaluations of the insecticidal activity of *P. nigrum* has been focussed mainly on the constituent alkaloids and their effects on a variety of insect species ranging from those of economic importance [18, 19] to disease vectors such as *Aedes aegytpi*, *An. stephensi* and *Culex quinquefasciatus* [20, 21]. Of the compounds comprising *P. nigrum*, piperine is the most recognized and is abundant in fully differentiated shoots of the plant [22, 23].

Commercial, ground black pepper and piperine showed marked toxic effects against late instar larvae of all the *An. gambiae* complex strains tested. Piperine ingestion was evidently less toxic to *Anopheles* larvae than black pepper, but did elicit larval mortality over 48 h and may serve as a synergist amongst the various amides constituting black pepper. Black pepper and piperine induced mortality in insecticide susceptible and resistant *An. gambiae* complex strains with no statistically significant differences between them. This suggests that insecticide resistance mechanisms play no role in the detoxification of black pepper and piperine in these species.

The *An. funestus* strains, however, showed markedly lower sensitivities to black pepper and piperine. This may reflect differences in their feeding habits and metabolism. Alternatively, their larval rearing conditions, which partially reflect the characteristics of their natural breeding sites, may have influenced their relative sensitivities. This is because the FANG and FUMOZ-R *An. funestus* strains are reared in water containing algae obtained from a nearby fish pond whereas the *An. gambiae* complex larvae are reared in water containing no additives other than larval food. However, it is not clear as to how or why this may have affected their responses to black pepper and piperine. One possibility is that food sources in the algae reduced their propensity to feed on the pepper/piperine treatments, raising the question as to what effect competing multiple food sources would have on the efficacy of such treatments in natural breeding sites.

Larviciding is one of four types of larval source management strategies, the others being habitat modification, habitat manipulation and biological control. It is recommended mainly in areas where larval habitats are few, fixed and findable [6]. Where implemented, LSM serves as a useful supplementary vector control measure, targeting mosquitoes at a stage of their life-cycle where their mobility is limited to the body of water they inhabit. Currently employed larvicides must comply with stringent criteria established by the WHO Pesticide Evaluation Scheme (WHOPES). These include assessments of how hazardous they are to humans and the environment, their storage requirements and shelf life, the susceptibility of local vectors and the associated costs of utilizing them [6]. In terms of these standards, plant-derived products such as pepper and piperine may be particularly advantageous, depending on their efficacy and residual activity under field conditions. These data give

no indication of the persistence of ground black pepper and piperine over time but it is envisaged that any pepper or piperine based control products would need to be formulated for increased persistence. Formulated products would also need to ensure adequate dispersal across a water surface, especially in the case of piperine which tended to clump.

A potential advantage of utilizing piperamides for larval control is that they act as neurotoxins, but in a manner distinct from pyrethroids, and so represent a novel mode of action [22], something urgently required for alternative methods of vector control [24]. They also have an inhibitory effect on enzymes and have shown synergistic effects when used in conjunction with pyrethrum [25]. They have low mammalian toxicity and are not environmentally persistent, degrading quickly under full sunlight [18, 26].

Conclusions

Piper nigrum in the form of finely ground black pepper is highly toxic to members of the *An. gambiae* species complex including *An. gambiae* (*sensu stricto*), *An. coluzzii, An. arabiensis* and *An. quadriannulatus.* It appears markedly less toxic to *An. funestus.* Similarly, piperine, proved substantially more toxic to members of the *An. gambiae* species complex than to *An. funestus,* although piperine on the whole was less toxic than black pepper across all species and strains tested. There were no differences in response between insecticide-resistant and their corresponding insecticide-susceptible strains by species to intoxication by either black pepper or piperine, suggesting that insecticide resistance mechanisms have no effect on their relative susceptibilities to these compounds/derivatives. An appropriately formulated black pepper or derivative product may have potential as a larvicide for malaria vector control in settings where larval source management adds benefit under the auspices of integrated vector control. Assessments of the other piperamides present in black pepper may provide further insights into the larvicidal effects of this plant and its derivatives.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BDB and MC conceived the project, designed the experiments and finalised the manuscript. MS and SVO helped design and conducted the experiments. MS and SVO analyzed the data and drafted the first version of the manuscript. All authors read and approved the final version of the manuscript.

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APPENDIX B

Samuel, M., Oliver, S.V., Wood, O.R., Coetzee, M. and Brooke, B.D., 2015. Evaluation of the toxicity and repellence of an organic fatty acids mixture (C8910) against insecticide susceptible and resistant strains of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae). *Parasites and vectors*, ***8***:321.

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Parasites & Vectors

RESEARCH Open Access

Evaluation of the toxicity and repellence of an organic fatty acids mixture (C8910) against insecticide susceptible and resistant strains of the major malaria vector *Anopheles funestus* Giles (Diptera: Culicidae)

Michael Samuel[1,2], Shüné V. Oliver[1,2], Oliver R. Wood[1,2], Maureen Coetzee[1,2] and Basil D. Brooke[1,2*]

Abstract

Background: Malaria vector control relies principally on the use of insecticides, especially pyrethroids. Because of the increasing occurrence of insecticide resistance in target vector populations, the development of new insecticides, particularly those with novel modes of action, is particularly important, especially in terms of managing insecticide resistance. The C8910 formulation is a patented mixture of compounds comprising straight-chain octanoic, nonanoic and decanoic saturated fatty acids. This compound has demonstrated toxic and repellent effects against several arthropod species. The aims of this study were to measure the insecticidal effects of C8910 against an insecticide susceptible (FANG) and a pyrethroid resistant (FUMOZ-R) laboratory strain of *An. funestus* as well as against wild-caught *An. funestus* material from Zambia (ZamF), and to investigate the repellent effects of two formulations of C8910 against these strains.

Methods: Toxicity against adult females was assessed using a range of concentrations based on the CDC bottle bioassay method and repellence of three different C8910 formulations was assessed using standard choice-chamber bioassays.

Results: C8910 proved equally toxic to adult females of the FUMOZ-R and FANG laboratory strains, as well as to adult females of the wild-caught (ZamF) sample. None of the C8910 formulations tested gave any conclusive indication of repellence against any of the strains.

Conclusion: C8910 is equally effective as an adulticide against pyrethroid resistant and insecticide susceptible *An. funestus*. However, the formulations tested did not show any consistent repellence against laboratory reared and wild-caught female samples of this species. Nevertheless, C8910 shows potential as an adulticide that can be used for malaria vector control, particularly in those instances where insecticide resistance management is required.

Keywords: *Anopheles funestus*, C8910 toxicity, C8910 repellence, malaria vector control

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Background

The World Health Organization (WHO) estimates that 198 million cases of malaria and 584,000 resultant deaths occurred globally in 2013 [1]. The vast majority of malaria cases occur in the Afrotropical region.

Anopheles funestus Giles is a primary malaria vector species in the Afrotropical region [2, 3] and is the nominal member of the Funestus Subgroup which comprises four species: *An. funestus, An. parensis, An. vaneedeni* and *An. confusus*. These are almost morphologically indistinguishable at all life stages [3, 4]. Of these, only *An. funestus* has been implicated in malaria transmission. *Anopheles funestus* females are highly anthropophilic and endophilic, and often take multiple blood meals. These characteristics combined with a relatively high longevity make *An. funestus* populations especially efficient at malaria transmission, and often show higher *Plasmodium falciparum* sporozoite infection rates than other vector species [5].

A strong tendency toward endophily makes *An. funestus* populations especially susceptible to control by indoor spraying of residual insecticides (IRS). Currently, only insecticides belonging to the pyrethroid, carbamate, organophosphate and organochlorine (DDT only) classes are available for malaria vector control. These collectively target only two insect neurological sites (the sodium ion channel and acetylcholinesterase) [6], which makes the development of resistance and cross-resistance between classes a likely prospect in regions where insecticide selection is suitably intense. The rate of resistance development is also exacerbated by the use of these insecticides for agricultural pest control [7]. Several populations of *An. funestus* have developed resistance to insecticides including pyrethroids (type I and II), carbamates (bendiocarb and propoxur), the organochlorine DDT and the cyclodiene dieldrin (reviewed by Coetzee & Koekemoer [8]).

The many instances of insecticide resistance in *An. funestus* highlight a significant problem facing insecticide based vector control strategies, the consequences of which are exemplified by the malaria epidemic of 1996–2000 experienced in northern KwaZulu-Natal, South Africa [9, 10]. During this epidemic, pyrethroid and carbamate resistant *An. funestus* were able to expand their ranges into areas that were under pyrethroid-based IRS control [11]. The epidemic was subsequently halted by a range of interventions including the re-introduction of DDT into the IRS programme as a resistance management option [10]. Insecticide resistance in malaria vector populations has become so widespread that malaria vector control is synonymous with resistance management, and has led to the development of the Global Plan for Insecticide Resistance Management (GPIRM) [12].

Since the introduction of pyrethroids in the 1990s, no new classes of insecticide have been approved by the WHO for use in public health [13]. The development of new insecticides, particularly those with novel modes of action, is particularly important, especially in terms of managing insecticide resistance [14].

In combination with insecticide applications, topical and spatial insect repellents are potentially useful in malaria vector control [15, 16]. Several candidate repellents have recently been identified [16]. However, their use as alternatives is not widespread, and a reduction in the efficacy of repellents can occur over time [17].

Fatty acids found naturally on human skin have shown repellent effects against *Aedes aegypti* mosquitoes including those attempting to bite or oviposit [18–20]. The C8910 formulation is a patented mixture of compounds comprising straight-chain octanoic, nonanoic and decanoic saturated fatty acids (C8, C9 and C10) [21]. This formulation has demonstrated repellent effects against biting and non-biting flies as well as ticks [22]. In addition, an incapacitating and toxic effect against several mosquito species, including the dengue vector *Ae. aegypti* [20] and several malaria vector species including *An. gambiae, An. dirus, An. farauti, An. freeborni, An. minimus* and *An. stephensi* [23], has been observed. The mode of action of C8910 has not been fully elucidated but likely involves respiratory inhibition [21].

The aims of this study were to measure the insecticidal effects of C8910 against insecticide susceptible and pyrethroid resistant laboratory strains of *An. funestus* as well as against wild-caught *An. funestus* from Zambia, and to investigate the repellent effects of two formulations of C8910 against these strains.

Methods

Mosquito strains

All of the mosquito strains/samples used in this study are housed in the Botha De Meillon Insectary (BMDI) at the National Institute for Communicable Diseases (NICD) in Johannesburg.

Ethical clearance for the use of mosquitoes for research purposes has been obtained from the Human Research Ethics Committee (medical) of the University of the Witwatersrand, Johannesburg (ref: W-CJ-100510-1).

Laboratory-reared colonies

FANG: This *An. funestus* colony originated from southern Angola and has been kept in colony since 2003. It is fully susceptible to insecticides. Specifically, adult males and females exposed to 0.75 % permethrin for 1 h consistently show 100 % mortality 24 h post exposure.

FUMOZ-R: This *An. funestus* colony has been selected for pyrethroid resistance from the base colony (FUMOZ) which originated from southern Mozambique and has been kept in colony since July 2001 [24].

Wild-caught samples

ZamF: Samples of indoor-resting *An. funestus* Clade I, highly resistant to pyrethroids and carbamates yet susceptible to DDT and organophosphates, were collected in the Nchelenge District of Zambia during the first quarter of 2014 [25]. Females were transported live to the BDMI where they were induced to lay eggs. Hatched larvae were reared through to adulthood and samples of F1 adult progeny (as representative of the wild population) were used for the toxicity and repellence tests detailed below.

Toxicity assays

The CDC bottle bioassay protocol of Brogdon & Chan [26] was used to assess the insecticidal effects of C8910 on the *An. funestus* colonies/wild-caught samples. C8910 was supplied in liquid form by the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia) and diluted in acetone to obtain a series of dilutions at a concentration range of 50-400 μg a.i./ml. 1 ml of each C8910 solution was used to treat each bottle and controls included bottles treated with acetone only. Twenty to twenty-five adult female mosquitoes (aged 1–5 days old) were gently introduced into each bottle. Knockdown, which was defined as a mosquito on its back and unable to right itself, was recorded at 15 min intervals over a 2 h exposure period. The entire concentration range plus a control was assessed through 3 replicates per colony/wild-caught sample. Following exposure, mosquitoes were removed from the bottles and placed into holding cups with access to 10 % sucrose solution. Environmental controls were conducted concurrently with unexposed mosquitoes directly inserted into a holding cup with access to sucrose solution. Final mortalities were recorded at 24 h post-exposure.

All statistical analyses were done using IBM SPSS (Armonk, NY). The mean Lethal Concentrations inducing 50 % mortality (LC50s) were determined for each colony/wild-caught sample using regression lines of log-transformed mortality data. Analysis of variance (ANOVA) was used to compare LC50s between the three data sets. As all control deaths were below 10 %, no mortality data were corrected.

Repellence tests

Repellence tests were conducted using standard choice-chamber bioassays. Initially, two C8910 repellent formulations developed by Stratacor Inc. (Richmond, CA) were supplied. Each of these consisted of 15 % C8910 as the active ingredient, suspended in a mineral oil carrier and scented with original green leafy (formulation 1) and modified green leafy (formulation 2) fragrances. A C8910 fatty acid blend produced by Emery Oleochemicals (Cincinnati, OH) was suspended in mineral oil to create a third formulation, also comprising 15 % C8910. This formulation was used for the repellence tests on the ZamF strain.

In order to avoid residual repellents affecting subsequent bioassays, disposable cloths were treated with 5 ml of each formulation and partially wrapped around the treatment forearm of each investigator. A cloth treated with 5 ml DC345 silicon oil was wrapped around each investigator's alternate arm as a control. Positive control tests on each colony/wild-caught sample were conducted using a commercial product containing the insect repellent DEET (N, N-diethyl-meta-toluamide-19.5 %) as its active ingredient. The DEET product was applied to the cloth of the treatment arm of each investigator as described above. Three investigators participated in the assays for C8910 formulations 1 and 2. Owing to limited wild-caught material, only two investigators participated in the assays for C8910 formulation 3.

Forty to fifty non blood-fed adult females per strain/sample per replicate were starved of sugar solution for 12 h and then gently inserted into the choice-chamber. They were left to acclimatize for 20 min in the dark in climate-controlled conditions of 25 ± 2 °C and 75 % humidity. The investigator concerned then placed the treatment and control arms at either end of the choice-chamber, so that each arm was 2 cm from the gauze-covered end of the chamber. The number of mosquitoes landing and attempting to blood-feed (hereafter referred to as the number of landings) on either arm was recorded at 3 min intervals for a period of 15 min. During counts, a red light was used to illuminate the chamber so as to minimize disturbance. The repellence of each formulation was assessed for each colony/sample through three replicates per investigator.

Variation in the total number of landings on the control versus treatment arms at each time interval was tested for significance using paired t-tests. These analyses were done using IBM SPSS with significance set at 95 % confidence.

Results

C8910 toxicity

The dose-mortality response curves for each sample are shown in Fig. 1. There was no statistically significant difference in response between the three strains (one-way ANOVA: $F = 0.19$, $p = 0.83$). In general, mortality increased with increasing C8910 concentration for all samples. However, this trend was least obvious in the wild-caught ZamF sample, especially at the lower doses (50-250 μg/ml a.i). A Tukey HSD post-hoc test of the ZamF 50-250 μg/ml a.i dose range shows that the significant trend ($F = 3.4$, $p = 0.04$) indicated by one-way ANOVA does not reflect a significant difference in mean mortalities.

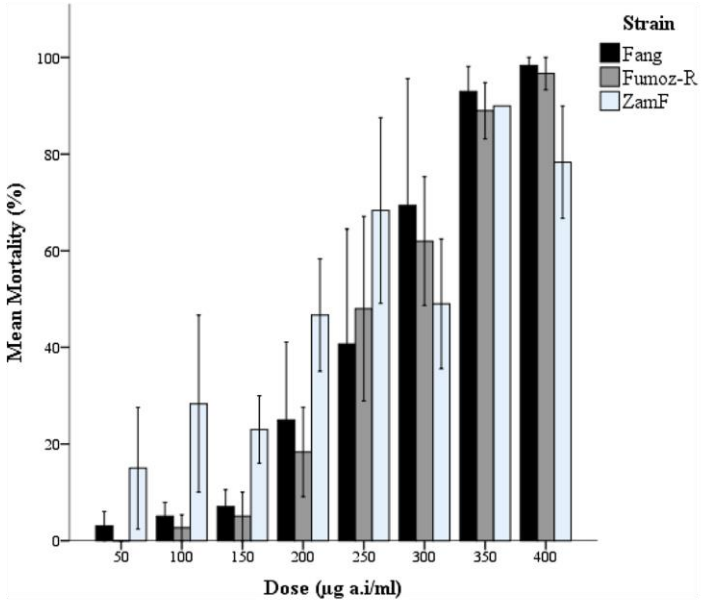


Fig. 1 Mean percentage mortality of *Anopheles funestus* strains 24 h post-exposure to C8910. FANG (insecticide susceptible) and FUMOZ-R (pyrethroid resistant) colonies as well as ZamF (wild caught) samples were exposed to C8910-treated bottles in accordance with the CDC bottle bioassay protocol. C8910 exposure lasted 2 h, after which, the mosquitoes were removed and placed in non-treated holding containers. Mortality was recorded 24 h after the initial exposure began. Three replicates were completed per dose per strain. Only one replicate of ZamF was achieved at 350 µl/ml a.i bottle

The mean lethal concentrations inducing 50 % mortalities (LC50s) in each sample are shown in Fig. 2. Although FUMOZ-R (254.39 ± 26.4 µg/ml a.i.) showed the highest mean LC50 followed by FANG (241.25 ± 39.4 µg/ml a.i.) and ZamF (202.80 ± 56.3 µg/ml a.i.), there was no significant variation in mean LC50 between samples (one-way ANOVA: F = 0.4, $p = 0.69$).

Repellence tests

C8910 formulation 1 induced marked variation in mean landing responses between investigators (Table 1). For the FANG strain, significantly fewer landings were recorded on the control arm than the treatment arm of investigator 1. The reverse was true for investigator 2 for whom significantly fewer landings were recorded on the treatment arm than the control arm, while there was no significant difference in the mean numbers of landings on each arm of investigator 3. For the FUMOZ-R strain, no significant difference in the mean numbers of landings on each arm for any of the investigators was recorded.

C8910 formulation 2 also induced marked variation in mean landing responses between investigators (Table 2). For the FANG strain, significantly fewer landings were recorded on the control arm than the treatment arm of investigator 1. The reverse was true for investigator 2 for whom significantly fewer landings were recorded on the treatment arm than the control arm, while there was no significant difference in the mean numbers of landings on each arm of investigator 3. For the FUMOZ-R strain, significantly fewer landings were recorded on the treatment arm than the control arm of investigator 2 only. There were no significant differences in the mean numbers of landings on each arm of investigators 1 and 3. For the ZamF sample, there were no significant differences in the mean numbers of landings on each arm of both investigators using C8910 formulation 3 (Table 3).

Repellence tests using DEET showed zero landings on the treatment arms of all investigators whereas multiple landings were recorded on the respective control arm of each investigator (Table 4).

Discussion

In general, C8910 proved equally toxic to adult females of the pyrethroid resistant (FUMOZ-R) and insecticide susceptible (FANG) *An. funestus* laboratory strains, as well as to adult females of the wild-caught (ZamF) *An. funestus* sample. It should be noted, however, that the responses recorded for ZamF were more variable than those recorded for the laboratory strains. This likely reflects greater genetic heterogeneity in the wild-caught sample compared to the laboratory strains which are

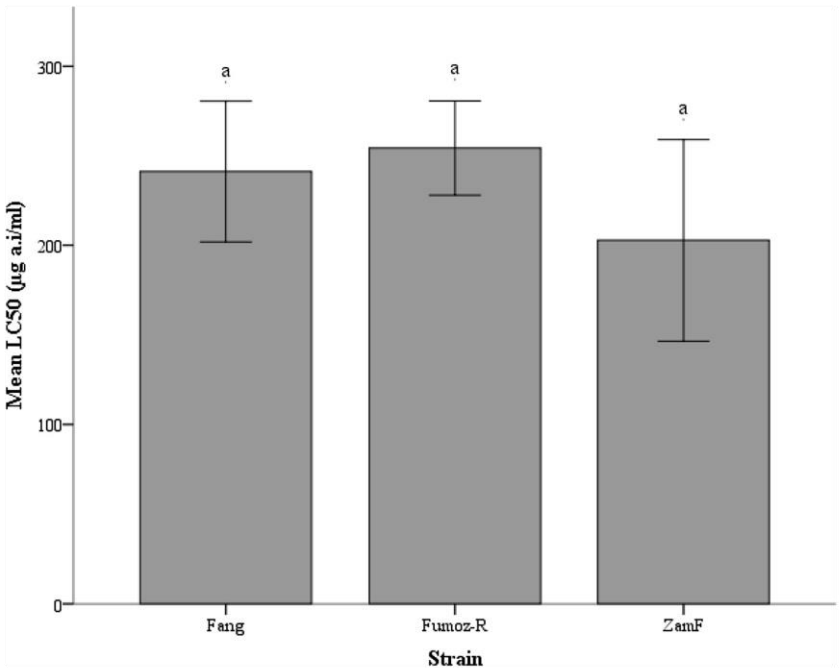


Fig. 2 Mean lethal concentrations inducing 50 % mortality (LC50s) in *Anopheles funestus* strains 24 h post-exposure to C8910. Following C8910 exposure as per the CDC bottle bioassay protocol, LC50s were determined for each colony/wild-caught sample using regression lines of log-transformed mortality data. Analysis of variance (ANOVA) was used to compare LC50s between the three data sets

expected to show reduced genetic variation, and thereby reduced heterogeneity in response to insecticide intoxication, owing to laboratory colonisation and the effects of bottle-necking. The LD50 values recorded here for *An. funestus* are reasonably comparable to those recorded for other anophelines including *An. gambiae* (91.76 ug a.i./bottle), *An. dirus* (132.59 ug a.i./bottle), *An. farauti* (118.17 ug a.i./bottle), *An. freeborni* (119.87 ug a.i./bottle), *An. minimus* (55.44 ug a.i./bottle) and *An. stephensi* (112.58 ug a.i./bottle) [23], allowing for

Table 1 Mean number of mosquito landings per arm recorded during C8910 formulation 1 repellence assays

	Mean ± SE		*p*
	Control	Treatment	
FANG			
Investigator 1	10.4 ± 1.2	15.0 ± 1.1	0.01[a]
Investigator 2	17.4 ± 1.3	8.6 ± 1.4	0.03[a]
Investigator 3	9.0 ± 2.1	11.6 ± 1.5	0.44
All Investigators	12.3 ± 1.3	11.7 ± 1.0	0.80
FUMOZ-R			
Investigator 1	2.0 ± 0.9	3.2 ± 1.0	0.54
Investigator 2	4.6 ± 0.8	6.2 ± 1.5	0.47
Investigator 3	3.0 ± 0.3	1.0 ± 0.6	0.08
All Investigators	3.2 ± 0.5	3.5 ± 0.81	0.79

Standard choice-chamber bioassays were performed on three investigators using the laboratory-reared *Anopheles funestus* FANG (insecticide-susceptible) and FUMOZ-R (pyrethroid-resistant) strains. Each arm of an investigator acted as a control and treatment respectively. Results (p) of paired t-tests comparing control vs treatment arms are given

[a]indicates significant difference at 95 % confidence

Table 2 Mean number of mosquito landings per arm recorded during C8910 formulation 2 repellence assays

	Mean ± SE		*p*
	Control	Treatment	
FANG			
Investigator 1	2.4 ± 0.8	6.6 ± 0.9	0.02[a]
Investigator 2	8.6 ± 1.0	6.0 ± 0.6	0.03[a]
Investigator 3	8.6 ± 0.9	7.2 ± 1.2	0.42
All Investigators	6.5 ± 0.92	6.7 ± 0.62	0.85
FUMOZ-R			
Investigator 1	3.4 ± 1.0	4.0 ± 1.0	0.75
Investigator 2	6.4 ± 1.3	1.2 ± 0.5	0.03[a]
Investigator 3	2.4 ± 0.5	3.0 ± 0.5	0.21
All Investigators	4.1 ± 0.7	2.7 ± 0.5	0.22

Standard choice-chamber bioassays were performed on three investigators using the laboratory-reared *Anopheles funestus* FANG (insecticide-susceptible) and FUMOZ-R (pyrethroid-resistant) strains. Each arm of an investigator acted as a control and treatment respectively. Results (p) of paired t-tests comparing control vs treatment arms are given

[a]indicates significant difference at 95 % confidence

Table 3 Mean number of mosquito landings per arm recorded during C8910 formulation 3 repellence assays

	Mean ± SE		*p*
	Control	Treatment	
ZamF			
Investigator 1	3.6 ± 0.3	4.2 ± 0.4	0.21
Investigator 2	5.2 ± 0.7	6.6 ± 1.3	0.43
All investigators	4.4 ± 0.4	5.4 ± 0.8	0.24

Standard choice-chamber bioassays were performed on two investigators using the wild-caught *Anopheles funestus* ZamF samples. Each arm of an investigator acted as a control and treatment respectively. Results (p) of paired t-tests comparing control vs treatment arms are given

the fact that the exposure times differed substantially (30 min for *An. gambiae, An. dirus, An. farauti, An. freeborni, An. minimus* and *An. stephensi* vs two hours for *An. funestus*). Nevertheless, these data affirm the toxicity range of C8910 against *Anopheles* mosquitoes in general.

Based on CDC bottle bioassays similar to those used in the experiments described here, adult FUMOZ-R females show a 76.1 fold decrease in susceptibility to pyrethroids compared to FANG [27]. Pyrethroid resistance in FUMOZ-R is primarily mediated by the detoxifying capabilities of at least two monooxygenase P450s [8, 28–30] and the resistance phenotype is further enhanced by thickened cuticles [31]. The population from which the ZamF sample was derived also carries high levels of pyrethroid resistance [25]. Although the resistance mechanisms in this population have not been fully characterised as yet, preliminary data derived from synergist bioassays show that monooxygenases play a fundamentally important role. Collectively, the C8910 mortality data presented here suggest that monooxygenase-mediated pyrethroid resistance in *An. funestus* offers no protection against the toxic effects of C8910, and that this mixture of compounds therefore shows potential as an adulticide and as a "resistance breaker" for malaria vector control directed against *An. funestus*.

Table 4 Mean number of mosquito landings per arm recorded during DEET repellence assays

	Mean ± SE		*p*
	Control	Treatment	
FANG			
Investigator 2	5.0 ± 0.6	0	0.01[a]
FUMOZ-R			
Investigator 1	4.0 ± 1.3	0	0.04[a]

Standard choice-chamber bioassays were performed on investigators using the laboratory-reared *Anopheles funestus* FANG (insecticide-susceptible) and FUMOZ-R (pyrethroid-resistant) strains. Each arm of an investigator acted as a control and treatment respectively. Results (p) of paired t-tests comparing control vs treatment arms are given

[a]indicates significant difference at 95 % confidence

None of the C8910 formulations tested gave any conclusive indication of repellence against *An. funestus*. Although two of the formulations were scented (2 % original green leafy and 2 % modified green leafy fragrances), it is unlikely that the scent confounded the experiments because the unscented formulation gave similarly ambiguous results. It is possible that the 15 % formulations are too weak to induce repellence. These tests also suggest an investigator effect [32, 33] because only the data for investigator 2 indicated some repellence. However, compared to the data obtained using DEET, which showed complete repellence against *An. funestus* regardless of investigator, none of the tests using C8910 suggested a repellent effect. These data are surprising considering the demonstrations of repellence against other arthropods including *Aedes* and *Culex* mosquitoes [18–20, 22], and further investigations against *An. funestus* and other malaria vector species are required.

The fatty acids comprising C8910 have been approved by the U.S. FDA and are categorized as "Generally recognized as safe" [23]. They have low mammalian toxicity and are unlikely to pose significant environmental concerns. Further studies will be required to assess the residual activity of C8910 as an *An. funestus* adulticide. A promising mechanism for prolonging residual activity is micro-encapsulation, and various strategies are currently underway to produce formulations with extended activity [23]. The potential and efficacy of C8910 as a larvicide also requires investigation, and studies are currently underway to establish these for *An. funestus*.

Conclusions

It is concluded that C8910 is equally effective as an adulticide against pyrethroid resistant and insecticide susceptible *An. funestus*. However, it did not show any consistent repellence against laboratory reared and wild-caught female samples of this species. Nevertheless, C8910 shows potential as an adulticide that can be used for malaria vector control, particularly in those instances where insecticide resistance management is required. However, future deployment as a public health intervention, most likely in conjunction with traditional insecticides in a mosaic or rotational strategy, will depend of further toxicity assessments followed by formulation, phased trial and commercialisation processes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BDB and MC conceived the project and designed the experiments. MS, SVO and ORW helped design and conducted the experiments. MS and SVO analyzed the data and produced the first drafts of the manuscript. BDB guided the analysis and interpretation of data and produced the final version of the manuscript. All authors read and approved the final version of the manuscript.

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