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BIONOMICS OF *ANOPHELES* MOSQUITOES IN EHLANZENI DISTRICT MUNICIPALITY, MPUMALANGA

Kayla Noeth

(2525673)

This thesis is submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2025

DECLARATION

I, Kayla Noeth, declare that this Thesis Report is my own unaided work. It is being submitted for the Doctor of Philosophy degree at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



Kayla Noeth

5th day of September 2025

DEDICATION

To my Dad

Whose absence throughout this journey was deeply felt. Your support, in life and beyond, has shaped my journey in more ways than I can express. This is for you.

PRESENTATIONS ARISING FROM THIS RESEARCH PROJECT

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2. Noeth, K.P., Chen, C.-Y., Misser, S., Ismail, S., Munhenga, G. and Oliver, S. V. 'The effect of sub-lethal exposure of temephos on the longevity and gut microbiota of the major malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae).' For submission to Acta Tropica.

ABSTRACT

Malaria, vectored by *Anopheles* mosquitos, remains a persistent public health threat in South Africa, where it is endemic in the provinces of Limpopo, Mpumalanga, and KwaZulu-Natal. Efforts to eliminate malaria are hindered by the presence of residual transmission. This study aimed to characterise selected aspects of *An. gambiae* complex vector bionomics in the Ehlanzeni district. Surveillance data collected from 2009 to 2021 in the Ehlanzeni district was analysed to understand vector bionomics in the province better. The dominant vector species recorded in the province was *Anopheles merus*, followed *An. arabiensis*. There was no significant correlation between vector abundance and climate at a broad scale (across 13 years). However, significant correlations may exist on a smaller scale (on a year-by-year basis). Further exploration of the data revealed important information regarding annual and seasonal population dynamics, and the efficacy of various collection methods. This study further aimed to characterise the chemical and bacterial composition of *Anopheles gambiae* complex larval habitats in Mpumalanga, as well as examining their effects on the gut microbiota of wild *An. gambiae* complex from the region. In addition, standardised bioassays were performed on adults originating from larvae from two sentinel sites at Vlakbult and Block A in the Ehlanzeni district to determine their insecticide resistance profiles. The adults emerging from larvae collected from Vlakbult and Block A were fully susceptible to both DDT and deltamethrin. Diversity of the bacterial samples from larval breeding sites were affected by the source. Larvae were found to harbour microbes potentially involved in insecticide degradation. The water samples were remarkably high in heavy metal pollution, but a range of pollutants were present. This study also aimed to investigate the potential of four common agricultural pollutants to cause hormetic effects in an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) laboratory strain of *An. arabiensis* under controlled conditions. The results show that there is a possibility that common agri-pollutants, particularly chloride, may induce hormetic responses in *An. arabiensis* when reared under laboratory conditions. This may have implications for *An. gambiae s.l.* vector populations in the field. Finally, this study aimed to investigate the effects of sub-lethal exposure of a common larviciding agent, temephos, on the gut microbiota composition and adult longevity of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) laboratory strain of *An. arabiensis*. Treatment affected the abundance of bacteria associated with both pesticide degradation and *Plasmodium* protection. Furthermore, sub-lethal exposure

to temephos significantly shortened adult longevity in a strain specific manner. This study has shown that the fine-tuning of surveillance activities may be necessary to shift Mpumalanga from a control framework to an elimination framework. Chronic exposure to pollutants in breeding habitats may have implications for the development of insecticide resistance and alteration of key life-history traits of *An. gambiae s.l.* in Ehlanzeni. Insecticide resistance is a major barrier to malaria elimination programs. Surveillance activities should be tailored to a per-region basis and include monitoring of the effects of pollution on insecticide resistance, life-history traits, gut microbiota and vector competence. Understanding and accounting for these factors in control programs will assist in achieving South Africa's goal of eliminating malaria by 2028.

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ANOVA	Analysis of variance
CDNB	1-chloro-2,4,-dinitrobenzene
C	Celsius
CDC	Centre for Disease Control
CHARM	Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses
DDT	Dichlorodiphenyltrichloroethane
DALY	Disability Adjusted Life Years
et al	*Et alia*
GABA	Gamma-aminobutyric acid
GST	Glutathione S-transferase
g	Gram
GDP	Gross domestic product
IRS	Indoor residual spraying
IQR	Interquartile range
kdr	Knockdown resistance
LSM	Larval source management
L	Litre
LLIN	Long-lasting insecticide treated bed nets
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
ug	Microgram
mg	Milligram
ml	Millilitre
min	Minutes
M	Molarity
NICD	National Institute of Communicable Diseases
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCA	Principle Components Analysis
RH	Relative humidity
RMT	Residual malaria transmission
TSR	Target site resistance
VCRL	Vector Control Reference Laboratory
WHO	World Health Organization

CHAPTER 1
GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Vector Borne Diseases and The Burden of Malaria

Vector-borne diseases are illnesses that are transmitted from host to host via living organisms, such as ticks, mosquitoes and fleas World Health Organization, 2017). Vector-borne diseases in humans account for more than 17% of the total global mortality and morbidity (disease burden) caused by communicable diseases (World Health Organization, 2017).

Mosquito-borne diseases are of particular concern, since mosquito vectors transmit a range of pathogens to humans and these diseases have high disease burdens (World Health Organization, 2014). Malaria is a mosquito-borne protozoan disease caused by *Plasmodium* species and transmitted via *Anopheles* mosquitoes (Ross, 1897). Malaria is considered the most significant vector borne disease, with the global number of reported malaria cases in 2023 estimated at 263 million, of which 597 000 resulted in mortality (World Health Organization, 2024b). Sub-Saharan Africa accounted for approximately 94% of the reported malaria cases and approximately 95% of the reported malaria deaths (World Health Organization, 2024b).

Disease burden is measured as Disability Adjusted Life Years (DALY), with one DALY representing the loss of the equivalent of one year of full health (Murray, Lopez and Jamison, 1994). The global DALY for malaria in 2019 was 46.44 million; Sub-Saharan Africa accounted for 43.68 million of the global total (Shi *et al.*, 2023). By comparison, the global DALY of HIV in 2019 was 47.63 million (Tian *et al.*, 2023).

One of the most effective mechanisms of controlling vector-borne diseases is by limiting contact between the vector and host. This is known as vector control. In the context of *Anopheles* species, the primary mechanisms are indoor residual spraying (IRS) and long-lasting insecticide treated bed nets (LLIN) (World Health Organization, 2017). Despite the implementation of vector control interventions, elimination of malaria remains a challenge in Sub-Saharan Africa. This is in part impeded by a

complex vector system, the presence of insecticide resistant vector populations, and climate change.

1.2 A Complex Vector System

There are at least 41 primary human malaria vectors across the world (Sinka *et al.*, 2010), with as many or more secondary vectors of local significance (Coosemans *et al.*, 2008). These vectors all belong to the Anophelinae subfamily of the Culicidae family. Many member species consist of species complexes or species groups of phylogenetically related species or sub-species (Coosemans *et al.*, 2008; Sinka *et al.*, 2010).

Vectors are classified as either dominant or as secondary vectors based on their role in malaria transmission. Dominant vectors, also referred to as major or primary vectors, are considered to be the most significant in terms of malaria transmission. Secondary, or minor vectors, are of local importance in a particular geographical setting (Coosemans *et al.*, 2008). Secondary vectors increase or extend the transmission period of malaria by filling spatiotemporal niches of transmission unfulfilled or created by controlling primary vectors (Afrane, Bonizzoni and Yan, 2016; Hoi, Gilbert and Mideo, 2020). This may occur as a result of malaria control strategies targeting primary vectors that feed and rest indoors, while secondary vectors may feed and rest outdoors (Afrane, Bonizzoni and Yan, 2016). This was the case in Kenya where *An. funestus* was replaced by *An. rivulorum* following an intensive IRS campaign (Gillies and Smith, 1960).

Which vectors are considered primary or secondary varies on a regional basis. *Anopheles ziemannii,* for instance, is present in South Africa, but is not considered to be an important vector of transmission in this country (Dahan-Moss *et al.*, 2021). In contrast, *An. ziemanni* is locally important as a vector in Cameroon, where it is reported to play a more significant role in malaria transmission compared to *An. gambiae*, which is considered one of the primary vectors in the country (Tabue *et al.*, 2014). The classification of a “secondary” versus a “primary” vector is therefore fluid and depends on ecological conditions and malaria control strategies (Afrane, Bonizzoni and Yan, 2016).

The efficacy of a vector is influenced by feeding, resting and host selection behaviours. More efficient vectors tend to feed indoors and are highly anthropophilic (i.e. preferring human blood meals), such as *An. funestus s.s,* and *An. gambiae s.s.* (Githeko *et al.*, 1996; Cohuet *et al.*, 2004). However, some vector species may prefer feeding and resting outdoors, and may have a preference for animal blood meals e.g. *An. merus* and *An. arabiensis* (Mutero, Mosha and Subra, 1984; Tirados *et al.*, 2006). These feeding and resting behaviours can vary within closely related species and within the same species complex. Often species are able to adapt their feeding and resting behaviours. *Anopheles arabiensis* for example, changed its feeding and resting preferences from indoors to outdoors after IRS of dichlorodiphenyltrichloroethane (DDT) was conducted in Tanzania (Mnzava *et al.*, 1995) and in South Africa (Sharp and le Sueur, 1991).

In most of Africa, there are usually a range of anopheline species present, including primary and secondary vectors occurring in sympatry. These diverse biologies interacting with ecological conditions such as climate and breeding site characteristics complicate the interventions required for malaria control.

1.3 Malaria Vectors in South Africa

In South Africa, at least 26 anopheline species have been identified across the three malaria endemic provinces (Limpopo, Mpumalanga and KwaZulu-Natal). Fourteen (14) of these species have either been implicated as potential secondary vectors or have been confirmed as primary or secondary vectors of malaria (Burke *et al.*, 2017, 2019; Braack *et al.*, 2020; Dahan-Moss *et al.*, 2021; Munhenga *et al.*, 2022) (Table 1.1). The most abundant vectors in South Africa are members of the *An. gambiae* species complex and the *An. funestus* species group.

Table 1.1. Current known and implicated *Anopheles* malaria vector species found in South Africa.

Data obtained from Burke *et al*. (2017, 2019), Braack *et al*. (2020), Dahan-Moss *et al*. (2021) and Munhenga *et al*. (2022). NI = Not implicated in South Africa.

Anopheles species complex, group or other	Species	Vector status	Comment	References
An. gambiae complex	*An. arabiensis*	Dominant	Confirmed	1, 2, 3
	An. merus	NI	Potential	1, 2, 3
	An. quadriannulatus	None		1, 2, 3
An. funestus group	*An. leesoni*	Secondary	Potential	1, 2, 3
	An. parensis	Secondary	Confirmed	4
	An. rivulorum	Secondary	Potential	1, 2, 3
	An. rivulorum-like	No data		1, 2, 3
	An. vaneedeni	Secondary	Confirmed	5
Other *Anopheles* species	*An. coustani*	NI	Potential	1, 2, 3
	An. demeilloni	NI	Potential	1, 2, 3
	An. maculipalpis	No data		1, 2, 3
	An. marshalli complex	NI	Potential	1, 2
	An. pharoensis	NI	Potential	1, 2
	An. pretoriensis	NI	Potential	1, 2, 3
	An. rhodesiensis	No data		1
	An. rufipes	NI	Potential	1, 2, 3
	An. squamosus	NI	Potential	1, 2
	An. tenebrous	No data		1
	An. ziemanni	NI	Potential	2
	An. listeri	No data		1
	An. theileri	No data		1
	An. longipalpis	No data		1
	An. flavicosta	No data		1
	An. cinereus	No data		1
	An. natalensis	No data		1
	An. nili	No data		1

[1]Braack *et al*. (2020); [2]Munhenga *et al*. (2022); [3]Dahan-Moss *et al*. (2021); [4]Burke *et al*. (2019); [5]Burke *et al*. (2017)

The *An. gambiae* species complex originally consisted of six members species: *An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus, An. merus*, *An. melas* and *An. bwambae* (White, 1974, 1985; Mattingly, 1977). Later, four more species were added to the complex: *An. coluzzii* (Coetzee, Hunt, *et al.*, 2013), *An. amharicus* (Coetzee, Hunt, *et al.*, 2013), *An. fontenillei* (Barrón *et al.*, 2019) and *An. tengrela* (Tennessen *et al.*, 2021). In South Africa, only *An. arabiensis*, *An. quadriannulatus* and *An. merus* are found, and only *An. arabiensis* has been implicated as a vector (Dandalo *et al.*, 2017).

The *An. funestus* species group consists of ten members: *An. funestus s.s.*, *An. vaneedeni*, *An. rivulorum*, *An. rivulorum-like*, *An. leesoni*, *An. confusus*, *An. parensis*, *An. brucei*, *An. aruni* and *An. fuscivenosus* (Coetzee and Koekemoer, 2013; Mouatcho *et al.*, 2018). In South Africa, only *An. vaneedeni*, *An, rivulorum*, *An. rivulorum-like*, *An. leesoni* and *An. parensis* occur (Dahan-Moss *et al.*, 2021). Only *An. vaneedeni* and *An. parensis* have been implicated as secondary vectors (Burke *et al.*, 2017, 2019).

Several other *Anopheles* species have been implicated, but not confirmed, as potential secondary vectors. This includes *An. leesoni, An. rivulorum, An. coustani, An. demeilloni, An. marshalii, An. pharoensis, An. pretoriensis, An. rufipes, An. squamosus* and *An. ziemannii* (Dahan-Moss *et al.*, 2021). For several species no data on vector status is available (Braack *et al.*, 2020) (Table 1.1). Several of these have been implicated as secondary vectors in other African countries, such as *An. ziemannii* (Cameroon), *An. coustani* (Kenya), *An. squamosus* (Zambia) and *An. rivulorum* (Tanzania) (Gillies and Smith, 1960; Wilkes, Matola and Charlwood, 1996; Mwangangi *et al.*, 2013; Tabue *et al.*, 2014; Stevenson *et al.*, 2016). The presence of local secondary vector species and their potential contribution to residual malaria transmission hampers elimination efforts of the disease (Afrane, Bonizzoni and Yan, 2016; Hoi, Gilbert and Mideo, 2020; Carnevale and Manguin, 2021).

1.4 The Challenge of Residual Malaria Transmission

Residual malaria transmission (RMT) refers to the low-level, often outdoor transmission of malaria that persists after full coverage has been achieved with convectional vector control strategies such as IRS and LLIN (Killeen, 2014).

Several factors contribute to the residual transmission of malaria. These include the types of control tactics employed, vector species composition, vector behaviours, insecticide resistance, climate, and breeding site characteristics (Durnez and Coosemans, 2013). These factors often interplay concurrently, which further complicates the elimination of malaria.

Exclusive use of indoor based vector control interventions in a given setting contributes to RMT. Such an approach may cause indoor feeding and resting vectors to shift their

behaviours to outdoor biting, and/or from human to animal blood meals (Darriet *et al.*, 1984). Indoor control strategies may also cause a change in species composition within the same species complex or group. Following an intensive IRS programme using the organochloride dieldrin, *An. rivulorum* was observed to have completely replaced *An. funestus s.s.* in Kihurio, Tanzania (Gillies and Smith, 1960). Another way indoor vector control interventions may contribute to RMT is by increasing the importance of local secondary vectors after successful elimination or reduction of a primary vector. This was observed in the Ndop health district in Cameroon, where *An. ziemanni* replaced *An. gambiae s.s.* as the primary vector following an intensive LLIN campaign (Tabue *et al.*, 2014).

Climate and the larval breeding environment have been found to play a role in RMT as well. Climatic factors such as rainfall and relative humidity have been found to be major drivers of malaria in South Africa, with temperature having the least influence (Abiodun *et al.*, 2018). Increased temperatures accelerate larval development and increased rainfall provide a greater number of suitable breeding habitats (Bayoh, 2001; Mbogo *et al.*, 2003). Pollutants present in the larval breeding habitat have been shown to influence life-history traits and contribute to the development of insecticide resistance (Nkya *et al.*, 2014), as discussed below.

1.5 The Compounding Effect of Insecticide Resistance

Insecticide resistance refers to a heritable change in the susceptibility to a particular class of insecticide. This resistance can occur as a result of a direct selection pressure, such as exposure to an insecticide at sub-lethal concentrations. Resistance can also occur through an indirect selection pressure, such as exposure to non-insecticidal xenobiotics, e.g. agrichemicals, heavy metals or microplastics (Nkya *et al.*, 2014). Insecticide resistance contributes to RMT by reducing the efficacy of insecticide-based control interventions such as LLIN and IRS (Omondi *et al.*, 2017; Glunt, Coetzee, *et al.*, 2018; Nwankwo, 2021). As such, this emphasises that monitoring and controlling for the development of insecticide resistance is essential to safeguard utility of current limited vector control tools. This ultimately reduces the risk of further outbreaks of malaria.

Sub-lethal doses of insecticide can result in the selection for insecticide resistance in mosquito populations. This can occur as a result of a variety of mechanisms, including an overexpression of detoxification enzymes (Brogdon *et al.*, 1999; Vulule *et al.*, 1999; Main *et al.*, 2018). This may come at fitness cost, such as decreased adult size, longevity and fecundity (Rivero *et al.*, 2011). Sub-lethal exposure of an insecticide at the larval stage may cause resistance in the adult life stage. The sub-lethal exposure of *An. gambiae s.s.* larvae to a mix of of insecticides, fungicides and herbicides resulted in increased tolerance to clothianidin and a clothianidin and deltamethrin mix in adult females (Zoh *et al.*, 2022). Exposing a pyrethroid-resistant *An. arabiensis* strain to sub-lethal doses of pyriproxyfen in larval habitats in Tanzania significantly reduced pyrethroid-related mortalities in the subsequent adult generation (Opiyo *et al.*, 2021). In addition, mortality rates of adults to Olyset® LLINs were significantly reduced after pre-exposure of the adults to pyriproxyfen at larval stage (Opiyo *et al.*, 2021).

Sub-lethal exposure of insecticides also induces behavioural changes in mosquitoes (Carnevale and Manguin, 2021). These include deterrent, repellent, interference, preventative and knock-down effects (as reviewed in Carnevale and Manguin (2021)). Changes in behavioural patterns allow for the increased survival of mosquito populations and thus contribute to the transmission of malaria, particularly in an outdoor setting.

Non-insecticidal xenobiotics may confer insecticide resistance by modulating detoxification enzyme activity and gene expression. Exposure to heavy metal pollutants such as cadmium, copper and lead has been linked to an increased tolerance of malathion and deltamethrin in *An. arabiensis* (Oliver and Brooke, 2018b). This increased tolerance was mediated by elevated cytochrome P450 and β-esterase activity (Oliver and Brooke, 2018b). Similarly, *Aedes aegypti* larvae exposed to atrazine, copper and permethrin showed an increased tolerance to temephos, a common larviciding agent (Poupardin *et al.*, 2008). Here, cytochrome P450 activity was increased after exposure to permethrin, fluoranthene and copper, while glutathione S-transferase (GST) activity increased after exposure to fluoranthene. Further analyses through quantitative polymerase chain reaction (qPCR) confirmed the induction of multiple cytochrome P450 genes and one carboxylesterase gene (Poupardin *et al.*, 2008). Riaz *et al.* (2009) showed multiple detoxification and reduction/oxidation genes

were induced when exposing *Ae. aegypti* larvae to sub-lethal concentrations of glyphosate and benzo-alpha-pyrene.

Another recently discovered driver of insecticide resistance is the presence of gut bacteria with the capacity to degrade pesticides. Bacteria such as *Stenotrophomonas* spp. and *Lysinibacillus* spp. were found to be associated with deltamethrin resistance in an *An. coluzzii* insecticide resistant strain from Ivory Coast (Pelloquin *et al.*, 2021). Supplementing an insecticide resistant strain of *An. arabiensis* with gram-negative bacteria (*Escherichia coli*) or gram-positive bacteria (*Streptococcus pyrogenes*) via sugar pads increased its deltamethrin and malathion resistance profile, while antibiotic treatment of the same strain showed decreased tolerance to the same insecticides (Barnard *et al.*, 2019). Another study in Kenya showed that resistance in *An. gambiae s.s.* was significantly associated with pyrethroid-degrading bacteria such as *Lysinibacillus* spp. and *Streptococcus* spp. (Omoke *et al.*, 2021). This developing field of research remain understudied in malaria vector species from South Africa.

The knowledge of insecticide resistance profiles in South African *Anopheles* populations is rudimentary. The last information on insecticide resistance in Mpumalanga was published in 2014 (Ramalwa *et al.*, 2014). This study tested the susceptibility of 30 *An. arabiensis* mosquitoes to 4% DDT (n = 22) and 0.05% deltamethrin (n = 8) (Ramalwa *et al.*, 2014). Results showed full susceptibility to both insecticides. However, the samples sizes were too small to make significant conclusions, as the World Health Organization (WHO) recommends exposing a minimum of 100 mosquitoes per insecticide to draw meaningful conclusions on insecticide resistance (World Health Organization, 2016). Similarly, the last published data on insecticide resistance in Limpopo was in 1996 (Gericke, Govere and Durrheim, 2002). In this study, the susceptibility of *An. arabiensis* from two sites, Thomo and Malahlapanga, was tested on four different insecticides (4% DDT, 0.05% deltamethrin, 1% fenitrothion and 0.1% propoxur). The authors generally found full susceptibility to all insecticides tested, however the mosquito sample size of susceptibility tests for Thomo was less than the WHO recommendation (< 100 mosquitoes for each of the four insecticides were tested) (Gericke, Govere and Durrheim, 2002). In KwaZulu-Natal a more thorough study was recently completed, where wild-caught *An. arabiensis* was exposed to five different insecticides: 4% DDT; 0.25% pirimithos-methyl, 0.75% permethrin, 0.1% bendiocarb and 0.05% deltamethrin (Munhenga *et al.*,

2022). Here, a sample size of at least 200 mosquitoes was used for each assay. The authors found low levels of pyrethroid resistance, but warned against the development of cross-resistance and recommended frequent monitoring of insecticide resistance in the province (Munhenga *et al.*, 2022).

The development of non-insecticidal driven insecticide resistant *Anopheles* populations may be of particular importance in Mpumalanga, due to the sizeable agricultural, mining, manufacturing and forestry industries (Young, 2022), which produce large amounts of agrochemical, heavy metal, and plastic pollution. The high levels of pollution in the province may play an important role in the development of resistant vector populations and the persistence of malaria transmission.

1.6 The Impact of The Climate and Environment on Life-History Traits and Insecticide Resistance of Malaria Vectors

Several studies have demonstrated the effects of the exposure of non-insecticidal xenobiotics on the life-history traits of *Anopheles* populations. Organic fertiliser, used as a proxy for cattle manure, significantly increased the larval development rate, adult longevity and adult size of *An. arabiensis*, *An. quadriannulatus* and *An. merus* larvae (Jeanrenaud, Brooke and Oliver, 2019). This has recently been shown to be specific for cattle manure, as opposed to chicken manure (Hardy *et al.*, 2022). Nitrogen, phosphorous and potassium (NPK) fertilisers generally decreased the time to pupation of a susceptible *An. arabiensis* colony, while urea increased the time to pupation and number of eggs laid (Samuel, Brooke and Oliver, 2020). Rearing *An. arabiensis* larvae in metal polluted water such as cadmium, copper and lead decreased adult fecundity and longevity of the first filial (F1) generation (Jeanrenaud, Brooke and Oliver, 2020). Exposure of larvae to the herbicide glyphosate significantly decreased the time to pupation of a resistant *An. arabiensis* strain, as well as significantly increasing the adult longevity (Oliver and Brooke, 2018a). Kibuthu *et al*. (2016) showed that exposure to xenobiotics can change the adaptive responses of mosquitos to insecticide selection pressures, thereby shaping the long-term selection of metabolic mechanisms that result in insecticide resistance.

Climatic factors such as relative humidity (RH) and temperature have been shown to have an impact on insecticide toxicity and may impact resistance if toxicity declines.

Hadaway and Barlow (1963) investigated the effect of RH on *An. stephensi* mortality when exposed to soils impregnated with DDT, dieldrin and carbamate insecticides. Mortality was significantly higher at 95% RH after a 2-minute contact period vs 43% RH after a 4-hour contact period (Hadaway and Barlow, 1963). Another study found that bendiocarb (a carbamate-based insecticide) exposure showed higher toxicity at increasing temperatures for two laboratory strains of *An. funestus* (Glunt, Oliver, *et al.*, 2018). Of interest is the variable effects of temperature and toxicity on mosquito strains that differ in insecticide resistance phenotype. The study by Glunt, Oliver, *et al*. (2018) found that toxicity of deltamethrin (pyrethroid) on a susceptible strain of *An. arabiensis* decreased at increasing temperatures, while for a resistant strain toxicity was significantly higher at low (18°C) and high (30°C) temperature extremes, compared to the standard rearing temperature (25°C). Hodjati and Curtis (1999) found similar results for resistant and susceptible strains of *An. gambiae* and *An. stephensi* after exposure to permethrin at increasing temperatures. Oliver and Brooke (2017) investigated the underlying mechanisms of heat exposure on insecticide toxicity. The authors concluded that heat shock proteins augment pyrethroid resistance, and that detoxification enzymes may be involved, however the enzyme assay results were not clear enough to draw meaningful conclusions. A complex mathematical model showed that regions with pronounced changes in climatic factors such as temperature and rainfall are at an increased risk of the development of insecticide resistance (Agboka *et al.*, 2024). It is therefore critical to consider climatic factors when planning and implementing vector control interventions.

1.7 Context and Rationale

Malaria has been a consistent public health threat in South Africa since the early 1900's. The disease is endemic in the Limpopo, Mpumalanga and KwaZulu-Natal provinces. Several epidemics occurred in the 20th century, the most lethal of which resulted in approximately 22 000 deaths during the 1929 - 1933 epidemic (Coetzee, Kruger, *et al.*, 2013). During an epidemic in 2000, more than 64 000 cases of malaria were recorded (constituting 0.14% of the then population of South Africa) (Coetzee, Kruger, *et al.*, 2013). This epidemic occurred in the wake of replacing DDT with other pyrethroid insecticides for IRS interventions. Relaxed border control between northern KwaZulu-Natal and Mozambique, good rains and parasite resistance to the drug sulphadoxine/ pyrimethamine likely played a role as well in exacerbating the epidemic

(Coetzee, Kruger, *et al.*, 2013). Critically, the re-emergence of insecticide-resistant *An. funestus* in northern KwaZulu-Natal was a significant factor that contributed to the severity of the outbreak, as this rendered IRS control efforts ineffective (Coetzee, Kruger, *et al.*, 2013). To combat this, the use of DDT in IRS programmes was reintroduced and since 2000, there has been a steady decline in the number of cases and deaths due to malaria (World Health Organization, 2020; Moonasar *et al.*, 2021). Most recently, the number of confirmed malaria cases increased from 7 280 in 2022 to 9 890 in 2023 and the number of malaria-related deaths increased from 29 to 113 during the same period (World Health Organization, 2024b). Despite the low transmission rates, elimination of the disease has not yet been achieved. This includes Mpumalanga, where the disease burden remains high, especially in the Ehlanzeni district (National Department of Health, 2019).

The elimination of malaria in South Africa has been hampered by a poor understanding of vector bionomics and the various factors contributing to residual malaria transmission. Critical factors relating to vector bionomics to consider during the implementation of effective control strategies include vector species composition and larval breeding site characteristics. Particularly, the effects of pollution and sub-lethal exposures of mosquitoes to insecticides and life-history traits are some of the factors that need to be considered. Another critical factor to consider is the insecticide resistance profiles of *Anopheles* vectors. Insecticide resistance is a major challenge to elimination programs (Quiñones *et al.*, 2015). The importance of the midgut microbiome to insecticide resistance and vector life-history traits is another factor to take into consideration. Finally, the impact of climate change on vector population dynamics needs to be considered as well.

The vectors responsible for malaria transmission in Mpumalanga have only been circumstantially incriminated. Recent surveillance reports record *An. arabiensis* and *An. merus* to be the most abundant *An. gambiae* complex members present in Mpumalanga, with *An. quadriannulatus*, a non-vector, present at lower levels (Dahan-Moss *et al.*, 2019, 2020). *Anopheles arabiensis* was implicated as a vector in KwaZulu-Natal and Mpumalanga (Dandalo *et al.*, 2017), in addition to *An. vaneedeni* (Burke *et al.*, 2017) and *An. parensis* (Burke *et al.*, 2019). *Anopheles merus*, which has been implicated as a vector in neighbouring countries Mozambique (Cuamba and Mendis, 2009) and Madagascar (Tsy *et al.*, 2003), has not been directly implicated as a vector

in South Africa. It is critical to identify the major and minor vectors driving the ongoing malaria transmission in Mpumalanga.

Additionally, larval breeding sites in Mpumalanga have not been well characterised. This includes investigation into the microbiota present within larval breeding habitats. This is despite the sizeable agricultural and mining industries in Mpumalanga, which might result in significant pollution in water systems including mosquito larval breeding habitats. It is unclear what downstream impact this has, if any, on the insecticide resistance profiles and life-history traits of *An. gambiae s.l.* populations from this province. It is also unclear what the current insecticide resistance status of anopheline malaria vector populations from the province are. In addition, to the best of our knowledge, no study has investigated the gut microbiota of wild *An. gambiae s.l.* in Mpumalanga and the role of the microbiome in the life-history traits, insecticide resistance profiles and vector competence of vectors.

Characterising these factors for *Anopheles* vector populations in Mpumalanga will assist with the development of an integrated control strategy that targets different vector behavioural traits, which will reduce residual malaria transmission. This study therefore adds to the body of knowledge of vectors in the Ehlanzeni district in Mpumalanga.

1.8 Aims and Objectives

The aim of this study is to characterise the population dynamics, breeding habitats, and insecticide resistance of *Anopheles gambiae* complex mosquitoes in Ehlanzeni, Mpumalanga, in order to better understand their role in malaria transmission. The study also aims to investigate the effects of sub-lethal xenobiotic and insecticide exposure on the life-history traits of two related strains of *An. arabiensis* that differ in insecticide resistance profiles under controlled conditions.

Specific objectives of the study are to:

1. Determine vector species composition and population dynamics in the malaria endemic district of Ehlanzeni, Mpumalanga.
2. Determine the insecticide susceptibility status of *An. gambiae s.l.* populations from Ehlanzeni, Mpumalanga.

3. Characterise the chemical and bacterial composition of larval breeding sites in Ehlanzeni, Mpumalanga.
4. Characterise the bacterial composition of the midguts of *An. gambiae s.l.* in Ehlanzeni, Mpumalanga.
5. Investigate the effects of sub-lethal heavy metal, agri-pollutant and plastic exposure, as well as exposure to a common larvicide, temephos, on the life-history traits and gut bacteria of two laboratory strains of *An. gambiae s.l*. that differ in insecticide resistance profiles.

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 Ethical Clearance

An ethical clearance waiver was applied for and granted (29-02-2024-O) (Appendix B).

The Section 20 Permit for permission to conduct research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No 34 of 1984) is available in Appendix C.

2.2 Mosquito Strains Used in Laboratory Studies

The laboratory reared mosquito strains used in this study are two strains of *Anopheles arabiensis* that differ in insecticide susceptibility. The SENN strain originated from Sennar, Sudan, and has been maintained in colony at the National Institute of Communicable Diseases (NICD) since 1980. SENN is an insecticide un-selected strain which initially had incipient resistance for DDT and malathion (Hemingway, 1983).

The second strain is SENN DDT, which was selected from the SENN strain through continuous exposure to 4% DDT. Selection for DDT resistance was initiated in 1995 and continues until present. SENN DDT exhibits confirmed resistance to DDT, deltamethrin, permethrin, malathion (results shown in Chapter 5), as well as lambda-cyhalothrin (Oliver and Brooke, 2017). This strain carries the knockdown resistance (*kdr*) L1014F mutation and exhibits elevated levels of cytochrome P450, general esterases and GST activity (Oliver and Brooke, 2013).

2.3 Insectary Conditions

Both mosquito strains are housed in the Botha de Meillon insectary of the NICD, Johannesburg, South Africa. Ambient temperature is kept at 25˚C (± 2˚C) and relative humidity at 80% (± 5%), as described by Hunt *et al*. (2005). A 12-hour light/ dark cycle is maintained with a 30-minute dusk/dawn cycle (Hunt *et al.*, 2005).

2.4 Protocol for Development Assays of Laboratory Reared Mosquito Strains

2.4.1 Rearing protocol

Larvae were reared in reverse osmosis water and fed a mixture of powdered Beano™ dog biscuits and Brewer's yeast (3:1) three times daily, prepared as per Hunt *et al* (2005). The larval food was stored at room temperature. Once larvae pupated, they were transferred to adult rearing cages and allowed to emerge. Adults were provided with *ad libitum* access to a 10% sucrose solution which was refreshed twice a week. Females and males were kept in the same cage, but females were not allowed blood for the duration of their lifetime.

2.4.3 Measurements

The number of larvae successfully pupating was counted for each treatment. Time to pupation was measured as the number of days between egg hatching and pupation. Mortality of the adults was scored daily, and cadavers were removed daily. Mortality was monitored until all individuals in the cage were dead.

2.4.2 Experimental setup

Four (4) rectangular plastic containers (19.5 cm x 7.5 cm x 13.5 cm) were each filled with 1 L of distilled water. One bowl served as a control and did not receive any experimental treatments, while the remaining three bowls served as experimental treatment bowls. One hundred (100) first instar larvae (less than 24 hours after hatching) of an insecticide-unselected strain of *An. arabiensis* (SENN) were added to each bowl. This constituted one replication. This represented the generalised pattern for larval development, where time to pupation was used as a proxy for larval development (Samuel, Brooke and Oliver, 2020). Larval development time assays were replicated three times from larvae originating from three different egg batches. The same protocol was followed for the insecticide-selected strain (SENN DDT).

2.5 Bioassays for Characterisation of SENN and SENN DDT Insecticide Resistance

Baseline characterisation of insecticide resistance present in SENN and SENN DDT was done using the standard WHO tube bioassays protocol (World Health Organization, 2016).

Six (6) holding tubes, each lined with clean untreated paper, were prepared. Twenty (20) to twenty-five (25) female mosquitoes were aspirated into each holding tube using an aspirator and allowed to acclimatise for one (1) hour. Each holding tube contained twenty-five (25) mosquitoes that were then transferred directly into a corresponding experimental tube. Five (5) experimental tubes were prepared, each lined with insecticide-treated papers (Vector Control Research Unit, Universiti Sains Malaysia) as follows: 4% DDT (product code PD400), 0.05% deltamethrin (product code PDE005), 0.75% permethrin (product code PPE075), 0.25% pirimiphos methyl (product code PPI025), and 5% malathion (product code PMA500). A sixth experimental tube was prepared as an untreated environmental control
.
To prevent cross-contamination, each holding tube was labelled with the same identifier as its paired experimental tube. Thus, mosquitoes from a given holding tube were only transferred into their designated experimental tube and subsequently returned to the same holding tube after exposure. Mosquitoes were exposed to insecticide papers for one (1) hour. Survivors were then transferred back into their original holding tubes and provided ad libitum access to a cotton wool ball soaked in 10% sucrose solution.

For each treatment, the number of dead females was recorded. Mortality was calculated as the mean percentage of dead females per treatment, with the standard error reported for each mean. This process was replicated at least four times or until at least 100 mosquitoes were exposed for each insecticide.

2.6 Common Statistical Methods

All data were analysed for normality using a Shapiro-Wilk test (Shapiro and Wilk, 1965). The significance level (α) for all analyses was set at 0.05 (95% confidence level).

Longevity and time to pupation data were analysed using a Kaplan-Meier estimator (Kaplan and Meier, 1958) and statistically significant differences between treatments were determined using a log-rank test (Harrington and Fleming, 1982). All analyses were performed in R Studio (RStudio Team, 2023).

CHAPTER 3

HISTORICAL TRENDS IN *AN. GAMBIAE* COMPLEX COMPOSITION, COLLECTION TRENDS AND ENVIRONMENTAL EFFECTS FROM 2005-2020 IN MPUMALANGA PROVINCE, SOUTH AFRICA

3.1 Introduction

3.1.1 Malaria risk in South Africa, with special reference to Mpumalanga

Ten percent (10%) of South Africa's population remains at risk for contracting malaria (Raman *et al.*, 2016). The disease is largely restricted to the low-altitude regions in the North and East of the country. In 2023, there were 9 890 reported cases of malaria in South Africa, and 113 deaths as a result of the disease (World Health Organization, 2024b).

Mpumalanga is one of the three malaria endemic provinces targeted for elimination by 2028 (National Department of Health, 2024). In 2019, Mpumalanga reported 4 418 cases of malaria (44% of the national total for 2019); the majority of which were imported (3 321 cases) (Moonasar *et al.*, 2021). The imported cases likely originated from Mozambique and eSwatini Click or tap here to enter text.(Raman *et al.*, 2016). Local cases accounted for 568 of the reported cases (Moonasar *et al.*, 2021). Within Mpumalanga province, Ehlanzeni is the only malaria endemic district (National Department of Health, 2019), and consists of four local municipalities, namely Bushbuckridge, Nkomazi, Mbombela and Thaba Chwew.

The population of Ehlanzeni in 2019 was 1 856 753 (~67 people per km^2) in 514 000 households (Department of Cooperative Governance and Traditional Affairs (COGTA), 2020). The largest portion of the population falls within the 0 - 14 age category (33%), followed by the 25 - 44 age category (31.5%) (Department of Cooperative Governance and Traditional Affairs (COGTA), 2020). Malaria incidence (population per 1000 at risk for malaria) in the province in 2018 was 1.54 for the Bushbuckridge local municipality, 0.44 for Nkomazi, 0.34 for Mbombela and 0.16 for Thaba Chwew (Moonasar *et al.*, 2021). The majority of the population at risk for malaria are therefore school-going children and the young working force.

3.1.2 Malaria vectors in Mpumalanga

3.1.2.1 Species composition and vector status

The dominant *Anopheles* species in Mpumalanga during a survey between 1997 and 1998 was *An. coustani* (55.8%), *An. funestus s.l.* (27.9%), *An. gambiae s.l.* (8.6%), and *An. pretoriensis* (5.2%) (Govere *et al.*, 2000). Other species included *An. demeilloni*, *An. rufipes*, *An. squamosus*, *An. longipalpis*, *An. marshallii* and *An. maculipalpis* (2.6%) (Govere *et al.*, 2000). By 2020, the proportion of *An. funestus s.l.* captured during the annual Vector Control Reference Laboratory (VCRL)/ NICD surveillance program declined to 14.8%, while the proportion of *An. gambiae s.l.* increased to 64.7% (Dahan-Moss *et al.*, 2021). Other species such as *An. coustani* (4%), *An. demeilloni* (0.2%), *An. maculipalpis* (2.9%), *An. pretoriensis* (10.5%) and *An. rufipes* (6.3%) were present at much lower levels than the survey in 1997/1998 (Dahan-Moss *et al.*, 2021).

Anopheles merus was the most abundant member of the *An. gambiae* complex during both the 1997/1998 survey (56%) and the 2020 survey (52.8%), with *An. arabiensis* occurring at much lower levels (13.6% in 1997/1998 and 8.4% in 2020). This in contrast to KwaZulu-Natal, where *An. arabiensis* is the most abundant member of the *An. gambiae* complex (Dandalo *et al.*, 2017; Munhenga *et al.*, 2022). *Anopheles gambiae s.s.* and *An. funestus s.s.* were not present during either the 1997/1998 or the 2020 surveys, as both species were largely eliminated from South Africa during the 1950's as a result of IRS control efforts (De Meillon, 1986; Sharp and le Sueur, 1996).

Only *An. arabiensis* of the *An. gambiae* complex and *An. parensis* and *An. vaneedeni* of the *An. funestus* group have been successfully incriminated as vectors in South Africa (Burke *et al.*, 2017, 2019; Dandalo *et al.*, 2017). Other anopheline species in South Africa have not yet been incriminated as malaria vectors, but are suspected to contribute to transmission due to their vector status in other African countries. This includes *An. coustani* in Kenya and Madagascar (Mwangangi *et al.*, 2013; Goupeyou-Youmsi *et al.*, 2020), *An. squamosus* and *An. rufipes* in Zambia (Stevenson *et al.*, 2016; Saili *et al.*, 2023) and *An. marshallii* in Cameroon (Antonio-Nkondjio *et al.*, 2006).

3.1.2.2 Brief summary of An. gambiae complex vector bionomics

Since *An. funestus s.l.* has declined significantly in Mpumalanga per the most recent vector surveillance report (Dahan-Moss *et al.*, 2021), and none of the other anopheline species have been implicated in transmission in South Africa, this section will focus on vector bionomics of the *An. gambiae s.l.* complex.

Anopheles arabiensis

An. arabiensis is a highly versatile and adaptable vector of malaria and has been observed to rest and feed both indoors and outdoors (White, 1972; Bøgh *et al.*, 2001; Duchemin *et al.*, 2001; Taye *et al.*, 2006; Tirados *et al.*, 2006; Mahande *et al.*, 2007). *Anopheles arabiensis* shows the ability to adapt its feeding behaviour depending on the environment in which it lives (Sharp and le Sueur, 1991; Mnzava *et al.*, 1995). In a study investigating the behavioural adaptation of *An. arabiensis* in KwaZulu-Natal, researchers showed that in houses sprayed with DDT, the human-blood index of indoor-resting mosquitoes was 31%, compared to over 90% for unsprayed houses (Sharp and le Sueur, 1991). This shows that *An. arabiensis* was able to adapt its feeding behaviour from human feeding to animal feeding when DDT was introduced into its feeding environment.

At first, *An. arabiensis* was thought to be predominantly zoophilic, however anthropophily has increasingly been observed (Bøgh *et al.*, 2001; Tirados *et al.*, 2006). This may not as much be due to *An. arabiensis* changing its feeding preferences, but rather it being opportunistic in its feeding behaviours. Bøgh *et al.* (2001) showed that the human-blood-index of *An. arabiensis* collected indoors from houses with cattle within 20 m of the homestead was 52%, compared to 82% from houses with cattle at least 50 m away from the homestead. This demonstrates that *An. arabiensis* is capable of utilising both human and animal hosts, depending on which hosts were readily available.

This behavioural plasticity makes *An. arabiensis* a difficult vector to control, particularly since it has been shown that LLINs are not effective in controlling *An. arabiensis* (Kitau *et al.*, 2012). Additionally, insecticide treatments such as IRS are biased towards eliminating indoor resting mosquitoes. This necessitates both indoor and outdoor control strategies to effectively control this important vector species.

Anopheles merus

Anopheles merus was identified as the “salt-water *An. gambiae* variant”, due to its immature stages being found predominantly in saline water bodies and its inability to breed with freshwater *An. gambiae s.l.* species (Iyengar, 1962; Paterson, 1964; White, 1974). Increasingly, *An. merus* has been found in more freshwater habitats as well as increasingly inland (Coetzee *et al.*, 1993; Kloke, 1995; La Grange, 1995; Masendu *et al.*, 2005; Mbokazi *et al.*, 2018). This is due to its greater osmotolerance compared to other members of the *An. gambiae* complex (White *et al.*, 2013). Their osmotolerance has a genetic and physiological underpinning involving expression of inducible differential genes between saltwater and freshwater environments, which increases as the salt concentration increases (White *et al.*, 2013; Uyhelji, Cheng and Besansky, 2016).

Anopheles merus shows some behavioural plasticity with regards to its feeding and resting behaviours. While outdoor-feeding and resting has predominantly been recorded for this species, indoor- feeding and resting has also been observed (Iyengar, 1962; Paterson, 1964; Mutero, Mosha and Subra, 1984). Although *An. merus* is predominantly zoophilic, it is also opportunistic and will readily feed on humans when available (Gillies and de Meillon, 1968; Mwangangi *et al.*, 2003; Kipyab *et al.*, 2013).

The abundance of *An. merus* in Mpumalanga compared to other *An. gambiae s.l.* members, along with its shifting distribution in South Africa (Govere *et al.*, 2000; Mbokazi *et al.*, 2018), raises concerns about its impact on malaria transmission in the region. While *An. merus* has not yet been implicated as a vector in South Africa, it has been found to play a role in transmission in Mozambique, Tanzania, Madagascar and Kenya (Temu *et al.*, 1998; Mbogo *et al.*, 2003; Tsy *et al.*, 2003; Cuamba and Mendis, 2009).

3.1.3 Climatic parameters associated with vector life-history traits

Temperature, rainfall and humidity are important climatic factors that affect mosquito life-history traits. Temperature affects larval development and adult survival (Bayoh, 2001). Elevated temperatures can decrease development time and increase hatchability of eggs (Gaaboub, El-Sawaf and El-Latif, 1971). Temperature extremes,

however, can halt development on the low end (< 12°C) and can be fatal at the high end (> 38°C) (Bayoh, 2001). Rainfall typically increases the number of breeding habitats available, thus increasing vector densities (Mosha and Mutero, 1982; Govere *et al.*, 2000). However, prolonged rains may flood breeding sites and decrease vector densities (Govere *et al.*, 2000; Paaijmans *et al.*, 2007). Relative humidity influences longevity and activity of vector populations, whereby low humidity levels cause desiccation stress and levels below 10% are typically fatal (Gaaboub, El-Sawaf and El-Latif, 1971; Yamana and Eltahir, 2013). Humidity levels below 10%, however, are unusual, found mostly in desert regions (Howe *et al.*, 1968). Bayoh (2001) found that a temperature and relative humidity combination of 15 - 25°C and 60 - 100% relative humidity had the highest survival of *An. gambiae s.s.* mosquitoes.

Ehlanzeni District Municipality has a moist, subtropical climate (Laduma Tapp Town and Regional Planners, 2010). Rainfall occurs predominantly in the summer months of October to March, with an average of 579 mm per year between 1991 and 2021 (Laduma Tapp Town and Regional Planners, 2010; Climate-Data.org, 2023). The average daily temperature during summer between 1991 and 2021 was 24°C, with average maximum temperatures of 30°C and average minimum temperatures of 20°C (Climate-Data.org, 2023). During the winter months the average daily temperature between 1991 and 2021 was 19°C, with average maximum temperatures of 25°C and average minimum temperatures of 12°C (Climate-Data.org, 2023). Relative humidity in Ehlanzeni between 1991 and 2021 ranged from approximately 50% in winter to approximately 70% in summer (Climate-Data.org, 2023). These climatic conditions make Ehlanzeni District Municipality an ideal habitat for mosquitoes and facilitate their survival through the colder winter months.

3.1.4 Challenges for malaria elimination

Despite the implementation of effective control measures, transmission of malaria in Ehlanzeni remains persistent, albeit at low levels. Malaria elimination in the province face several challenges.

According to a review conducted by Raman *et al.* (2016), most malaria cases in Mpumalanga are imported, likely originating from neighbouring countries such as Mozambique and eSwatini (formerly Swaziland). Often, high levels of asymptomatic

cases further complicate the diagnosis, treatment and elimination of malaria (Raman *et al.*, 2020). Malaria elimination cannot be realised in the face of ongoing importation of cases (El Hassan *et al.*, 2015; Li *et al.*, 2016), and as such elimination efforts should be focused on a regional basis as opposed to being country-delineated (Wangdi *et al.*, 2015). Despite the success of initiatives such as the Lebombo Spatial Development Initiative (LSDI), the more recent Mozambique-South Africa-Swaziland (MOSASWA) Initiative and the Elimination 8 project in reducing cross-border transmission in southern Africa (Sharp *et al.*, 2007; Maharaj *et al.*, 2016; Elimination 8, 2019; Raman *et al.*, 2021), imported cases remain a challenge in South Africa. This is particularly true for Mpumalanga, where imported cases account for the majority of reported malaria cases (Raman *et al.*, 2016).

In addition to imported cases, local transmission of malaria still occurs (Raman *et al.*, 2016). This local transmission may be compounded by an incomplete understanding of local vector populations and vector bionomics. While both *An. parensis* and *An. vaneedeni* have been implicated in malaria transmission in South Africa, both species are strongly zoophilic (Burke *et al.*, 2017, 2019) and thus unlikely to be the major vector in Mpumalanga. Control efforts in Mpumalanga have largely focused on IRS, which are ineffective against outdoor- resting mosquitoes such as *An. arabiensis.* This is a contributing factor to the ongoing residual transmission of the disease. Vector incrimination of other primary and secondary vectors such as *An. merus* will assist in refocusing control efforts to target outdoor-resting species and assist with reaching zero malaria cases in Mpumalanga.

Insecticide resistance is another challenge that threatens malaria elimination goals. Information on insecticide susceptibility of vector population in Mpumalanga is rare, with the last publicly available data published in 2014 (Ramalwa *et al.*, 2014). Resistance to insecticides used in national control programs hinder malaria elimination goals. This can be seen in the resurgence of *An. funestus* in KwaZulu-Natal due to pyrethroid resistance and the subsequent malaria epidemic in 2000 (Hargreaves *et al.*, 2000).

3.1.5 The value of surveillance in an elimination context

Strong epidemiological and entomological surveillance is essential to disrupt the transmission of malaria (Kaiser, 1966). Poor surveillance will prevent countries from shifting from a sustained control framework to an elimination framework and prevent implementing targeted interventions in endemic areas (Lourenço *et al.*, 2019). Surveillance, when conducted consistently and over a long-term period, can address knowledge gaps regarding the life-history traits of vector populations, can inform control interventions to be more effective and can identify vector control challenges and opportunities (Killeen *et al.*, 2018).

Entomological surveillance data that focus on vector populations can be particularly useful when overlaying it with other data layers such as historical climatic data and malaria case data. These can be useful predictors of future malaria outbreaks and may contribute to early warning systems. This is essential when shifting from control to elimination (Midekisa *et al.*, 2012; Schincariol *et al.*, 2021).

3.1.6 Rationale

Ehlanzeni is a key surveillance area in the Mpumalanga province, as it is a malaria endemic area. Sustained surveillance has been conducted in the province since at least 2008, and as such a large database has been curated on vector population life-history traits at the VCRL at the NICD in Johannesburg, South Africa. Information on vector species composition, seasonal collections, capture methods and locations have been recorded in this database. Analysis of the data in this database could provide useful information on historical trends of vector population dynamics, capture methods and vector abundance associated with location. Additionally, these data could be combined with historical climatic data to provide insight on how vector abundance and climate are associated.

3.1.7 Aims

The specific aims of this chapter are to investigate trends in vector abundance between 2005 and 2020 in Ehlanzeni using the database curated by the VCRL/ NICD. Specifically, the following will be investigated and retrospectively analysed:

1. Annual *Anopheles* species composition.

2. Seasonal species composition of the *An. gambiae* complex.

3. Efficiency of collection methods employed for each species of the *An. gambiae* complex present in Ehlanzeni.

4. Distribution of *An. merus* in Ehlanzeni over the surveillance period.

5. *An. gambiae* diversity associated with each sampling location.

6. Impact of climate on *An. gambiae* complex abundance trends.

3.2. Materials and Methods

3.2.1 Overview

The dataset summarised in this study is curated by the VCRL at the NICD in Johannesburg, South Africa. The data was collected in collaboration with the Mpumalanga Provincial Malaria Control Programme of the National Department of Health of South Africa or as part of past research projects. The dataset consisted of the collection date, collection site, collection method and species identification. Data for the period 2005 - 2020 were analysed and summarised.

3.2.2 Mosquito collection methods

Anopheles larvae were collected by provincial entomological surveillance teams and personnel from the VCRL/NICD fieldwork teams. Larval sampling locations included natural and man-made pools, river shallows, stagnant ponds, water-filled cattle hoofprints and potholes, and other sources of still-standing water (Mbokazi *et al.*, 2018). Adult *Anopheles* species were collected using standard collections methods described by Dandalo *et al*. (2017), Braack *et al.* (2020) and Muirhead-Thomson (1951, 1958). These are described briefly below.

3.2.2.1 Human landing catches

Nighttime catches (after dusk) where legs or arms of consenting human volunteers were used as bait to lure female *Anopheles* to feed. Females were collected before biting.

3.2.2.2 Natural resting shelter

Adult *Anopheles* collected from natural shelters using aspirators, such as understory vegetation, cattle kraals, hollowed out trees or animal burrows.

3.2.2.3 Pit traps

The pit traps used in the Ehlanzeni surveillance program were modelled after pit shelters described by Muirhead-Thomson (1951, 1958). Man-made shelters were dug into riverbanks to provide shaded resting spaces for adult *Anopheles* species (Fig. 3.1).



Figure 3.1. Pit shelters dug into riverbanks used to collect adult *Anopheles* mosquitos in Ehlanzeni District, Mpumalanga. Photo courtesy of G. Munhenga.

3.2.2.4 Clay pots and modified plastic buckets

The use of clay pots and modified plastic buckets for the collection of *Anopheles* adults was first described by Dandalo *et al.* (2017). The size of both the clay pots and modified plastic buckets were standardised, with openings sufficiently large enough to facilitate easy entering and exiting of mosquitoes. These were placed at random in shady spots near livestock kraals or inhabited homesteads. Some pots and buckets were also placed close to mosquito breeding sites outside selected homesteads. The inside of the plastic bucket was scratched with sandpaper to facilitate tarsal contact of mosquitos and sprayed with black spray paint to increase the attractiveness of the bucket to resting mosquitos. Both the pots and the buckets were stationed permanently at sampling sites.

3.2.2.5 Carbon dioxide (CO_2) baited tent traps

Carbon dioxide (CO_2) baited tent traps are upright tent structures baited with dry ice as a CO_2 source (Fig. 3.2). These tents were erected just before sundown close to

human homesteads or livestock kraals/ shelters and mosquitoes were collected using aspirators within the tent throughout the night.

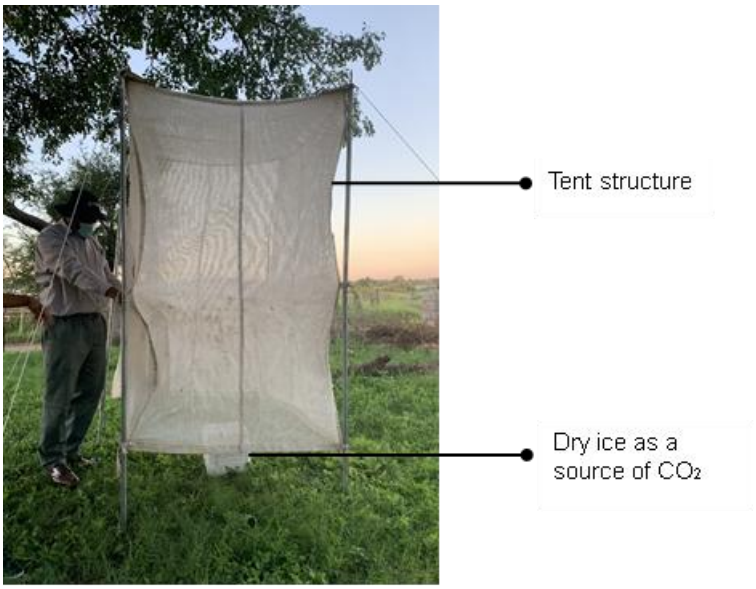


Figure 3.2. Carbon dioxide (CO_2)-baited tent traps used to collect adult *Anopheles* in Ehlanzeni District, Mpumalanga. Author's photograph.

3.2.2.6 Window traps

Window traps, described by Dandalo *et al.* (2017), consisted of a netted cage mounted outside a homestead over an open window of a bedroom. Mosquitoes leaving the room after feeding would be captured in the window trap and subsequently collected using aspirators.

3.2.2.7 Tyres

Disused or discarded tyres, sometimes containing accumulated rainwater, near homesteads or animal shelters were searched for resting adult *Anopheles* and collected using aspirators.

3.2.3 Mosquito identification methods

Once collected, adults were preserved on silica in 1.5 ml reaction tubes and identified using external morphological characteristics (Gillies and Coetzee, 1987). Specimens belonging to the *An. gambiae* complex were identified to species level using the

polymerase chain reaction (PCR) assay described by Scott *et al.* (1993), while specimens of the *An. funestus* group were identified to species level using the PCR assay described by Koekemoer *et al.* (2002). Larval specimens were reared to adulthood and the subsequent generation of adults were identified to species as described for adult collections.

3.2.4 Data collection and organisation

The data from surveillance collections were collated from 283 separate species identification sheets into a single datasheet in Microsoft Excel. Collection information that was captured included a unique identification number for each sample, date of the collection, the PCR or morphological species identity, location of the collection, sex of the collected specimen as well as the collection method used for collection. This datasheet consisted of 12 522 entries. Duplicate entries and entries that had no species identification, capture location or date of collection were removed from the datasheet, resulting in 9 728 entries (2 794 entries excluded).

The datasheet was cleaned by standardising the date format. The spelling of collection methods employed, location entries and species names were also standardised.

For ease of analysis, several new variables were added to the datasheet. A “Month” and “Year” column was added based on the “Date” column. A column for “Season” was added using the variables “Summer” (December, January, February), “Autumn” (March, April, May), “Winter” (June, July, August) and “Spring” (September, October, November). A “Municipality” column was added that grouped location entries into one of three district municipalities: “Mbombela”, “Nkomazi” and “Bushbuckridge”. A “Group” column was added that grouped species identification entries into one of three groups: “*An. gambiae* complex”, “*An. funestus* group” or “Other”. Finally, an additional 15 columns were added for each unique species identification entry, which converted the qualitative entry to a quantitative entry using 1’s and 0’s. A simplified diagram summarises the data organisation process in Figure 3.3.

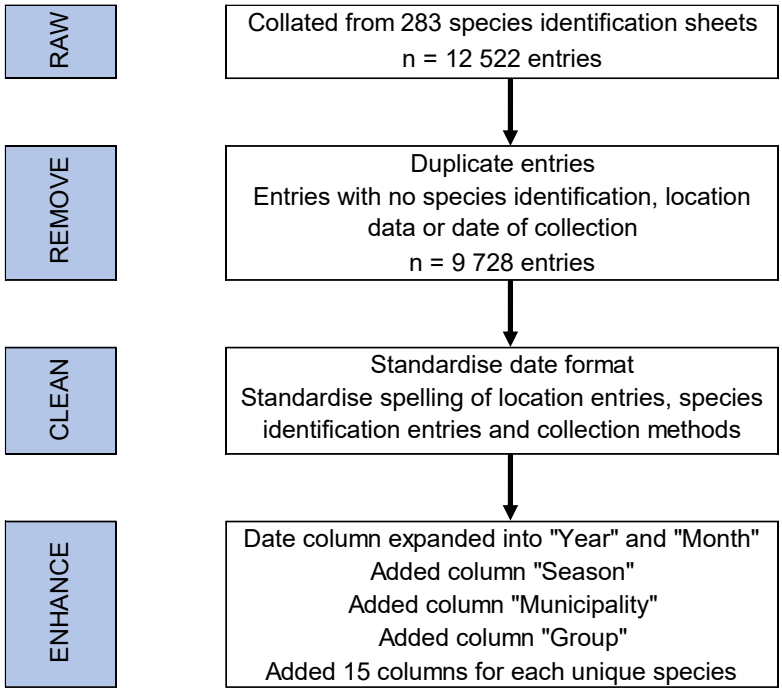


Figure 3.3. A simplified diagram summarising the data organisation process.

3.2.5 Specimen collection sites

Anopheles mosquitoes were collected from 79 sentinel sites in three municipal districts within the Ehlanzeni District (Fig. 3.4). These municipal districts include Bushbuckridge, Mbombela and Nkomazi. Maps were generated using Mapcreator© (*Mapcreator*, 2023).

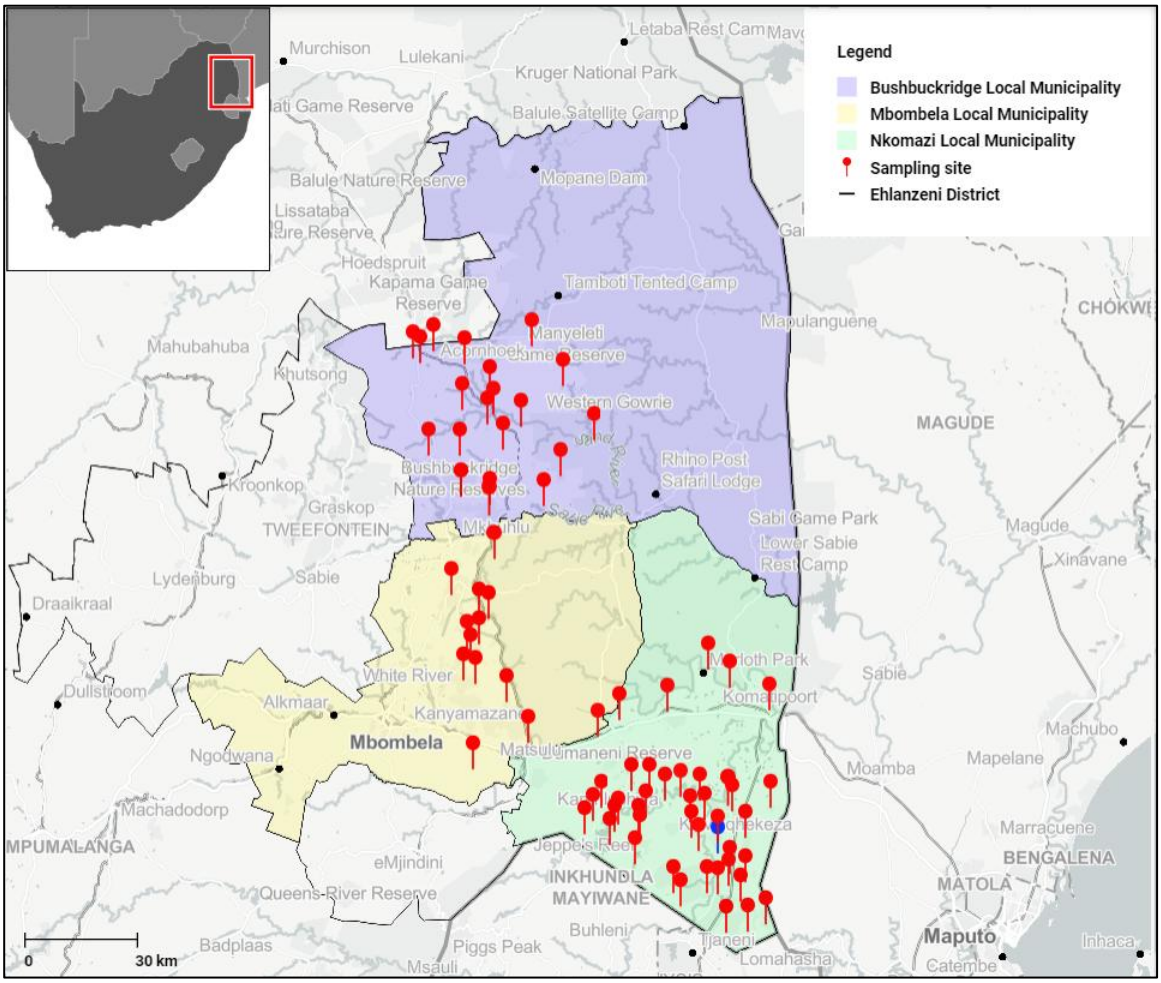


Figure 3.4. Surveillance sites in Ehlanzeni District (bordered in black) from which *Anopheles* mosquitoes were collected between 2005 and 2020.
Individual sites are marked by red pins. The Bushbuckridge municipal district is shaded in blue, Mbombela in yellow and Nkomazi in green. The blue pin indicates the approximate location of Block A in Nkomazi.

3.2.6 Climatic data

Data for the climatic analyses were retrieved from the National Aeronautics and Space Administration (NASA) Langley Research Centre (LaRC) POWER Project database (NASA Langley Research Center Atmospheric Science Data Center, 2022).

3.2.7 Data analysis

The total number of *An. gambiae s.l., An. funestus s.l.* and other *Anopheles* species were expressed as a percentage of total mosquito captures. Species composition of the *An. funestus* group was expressed as a percentage of the total number of *An.*

funestus s.l. mosquitoes collected. Species composition of the individual members from the *An. gambiae* complex was expressed as a percentage of the total number of *An. gambiae s.l.* mosquitoes collected. Proportional composition of all other *Anopheles* collected was expressed as a percentage of the total number of other *Anopheles* mosquitoes collected.

Further analyses were focused on the *An. gambiae* complex collected between 2009 and 2020, since this complex was the most abundant in the Ehlanzeni district. The total number of *An. gambiae* complex mosquitoes collected was stratified by species and by year and calculated for the entire study period. The total number of *An. gambiae* complex adults collected stratified by species by season was calculated, and the same was done for larval collections. The proportion of each species of the *An. gambiae* complex captured per collection method utilised over the study period was expressed as a percentage of total collections. The distribution of *An. merus* was visualised on a map generated by Mapcreator© (*Mapcreator*, 2023).Click or tap here to enter text.. Species richness was calculated for each municipality by counting the number of unique species present. Alpha diversity analyses compare species diversity and species evenness within a sample. The following alpha diversity indices were calculated for each municipality: 1) Simpson's, 2) Shannon-Wiener and 3) Pielou's Evenness. The Simpson Diversity Index estimates species diversity within a community and considers both the number of unique species present as well as the relative abundance of each species present (Simpson, 1949). The Shannon-Wiener Diversity Index estimates the species diversity within a community, considering the number of unique species present (Shannon, 1948). The Pielou Evenness Index measures whether species within a community are equally abundant (i.e. even) (Pielou, 1966). Calculations for each index are shown in Equations 1, 2, and 3, respectively (Fig. 3.5). A Shapiro Wilk test (Shapiro and Wilk, 1965) was conducted on the diversity index data to test for normality. A Kruskal-Wallis test (Kruskal and Wallis, 1952) was conducted to test for differences in diversity indices between municipalities.

$$D = \frac{\sum(n_i(n_i-1))}{(N(N-1))} \quad \text{Equation 1.}$$

$$H = -\sum_i \left(\frac{n_i}{N} . \ln\left(\frac{n_i}{N}\right)\right) \quad \text{Equation 2.}$$

$$J = \frac{H}{\ln(S)} \quad \text{Equation 3.}$$

where;

D = Simpson Diversity Index

H = Shannon-Weiner Diversity Index

J = Pielou's Evenness Index

S= total number of species in a sample, across all samples in the dataset

n_i=proportion of individuals of the i-th species in a whole community

Figure 3.5. Equations used to calculate Simpson's Diversity Index (D) (Equation 2), Shannon-Wiener Diversity Index (H) (Equation 2) and Pielou's Evenness Index (J) (Equation 3).

The impact of climate on *An. gambiae s.l.* abundance was investigated by correlating the number of mosquitoes captured in each district summed per year with the average temperature, precipitation and relative humidity for each year. The number of mosquitoes captured per district were also summarised per month across the collection period and correlated with mean monthly climatic variables. To investigate the impact of climate on a more microclimatic scale, the analysis focused on a single site in Nkomazi municipality, Block A (see Fig. 3.4, blue pin). Here, the number of mosquitoes captured at Block A was summed daily and correlated with daily climatic variables. The significance of the association between climatic variables and mosquito abundance were assessed using a Spearman's Rank Correlation test (Spearman, 1904). These analyses focused on Nkomazi and Bushbuckridge Municipalities, as the data for these two municipalities were more comprehensive than Mbombela Municipality.

Finally, sampling effort was expressed as the number of mosquitoes captured per sampling day by dividing the number of mosquitoes captured per year by the number of collection days for that year.

3.3 Results

3.3.1 Species composition

3.3.1.1 Overall species composition

Between 2005 and 2020, a total of 9 784 *Anopheles* species were collected, of which *An. gambiae s.l.* constituted 80.4% (n = 7 868), *An. funestus s.l.* constituted 13.6% (n = 1 327) and other *Anopheles* species constituted 6.0% (n = 589) (Fig. 3.6 A).

3.3.1.2 Anopheles funestus group composition

Anopheles rivulorum accounted for the majority of the *An. funestus s.l.* collections between 2005 and 2020 in Ehlanzeni District (76.6%, n = 1 017). This was followed by *An. vaneedeni* (12.0%, n = 159), *An. leesoni* (6.0%, n = 80) and *An. parensis* (5.4%, n = 71) (Fig. 3.6 B).

3.3.1.3 Anopheles gambiae complex composition

Anopheles merus accounted for the majority of *An. gambiae s.l.* collections during the surveillance period (50.3%, n = 3 933) (Fig. 3.6c). *Anopheles quadriannulatus* accounted for 30.1% of the *An. gambiae s.l.* collections (n = 2 348), while *An. arabiensis* was present at much lower numbers (19.6%, n = 1 531) (Fig. 3.6 C).

3.3.1.4 Other Anopheles species composition

Eleven (11) other *Anopheles* species were collected during the surveillance period between 2005 and 2020. The most abundant of these was *An. pretoriensis*, which accounted for 60.1% of other *Anopheles* species collected (n = 354) (Fig. 3.6 D.) *Anopheles rufipes* (15.1%, n = 89), *An. maculipalpis* (9.0%, n = 53), *An. coustani* (5.1%, n = 30), *An. marshallii* (4.9%, n = 29), *An. brunnipes* (2.2%, n = 13), *An. demeilloni* (1.7%, n = 10), *An. tenebrosus* (0.7%, n = 4), *An. pharoensis* (0.5%, n = 3), *An. squamosus* (0.5%, n = 3) and *An. theileri* (0.2%, n = 1) accounted for the other anophelines collected (Fig. 3.6 D).

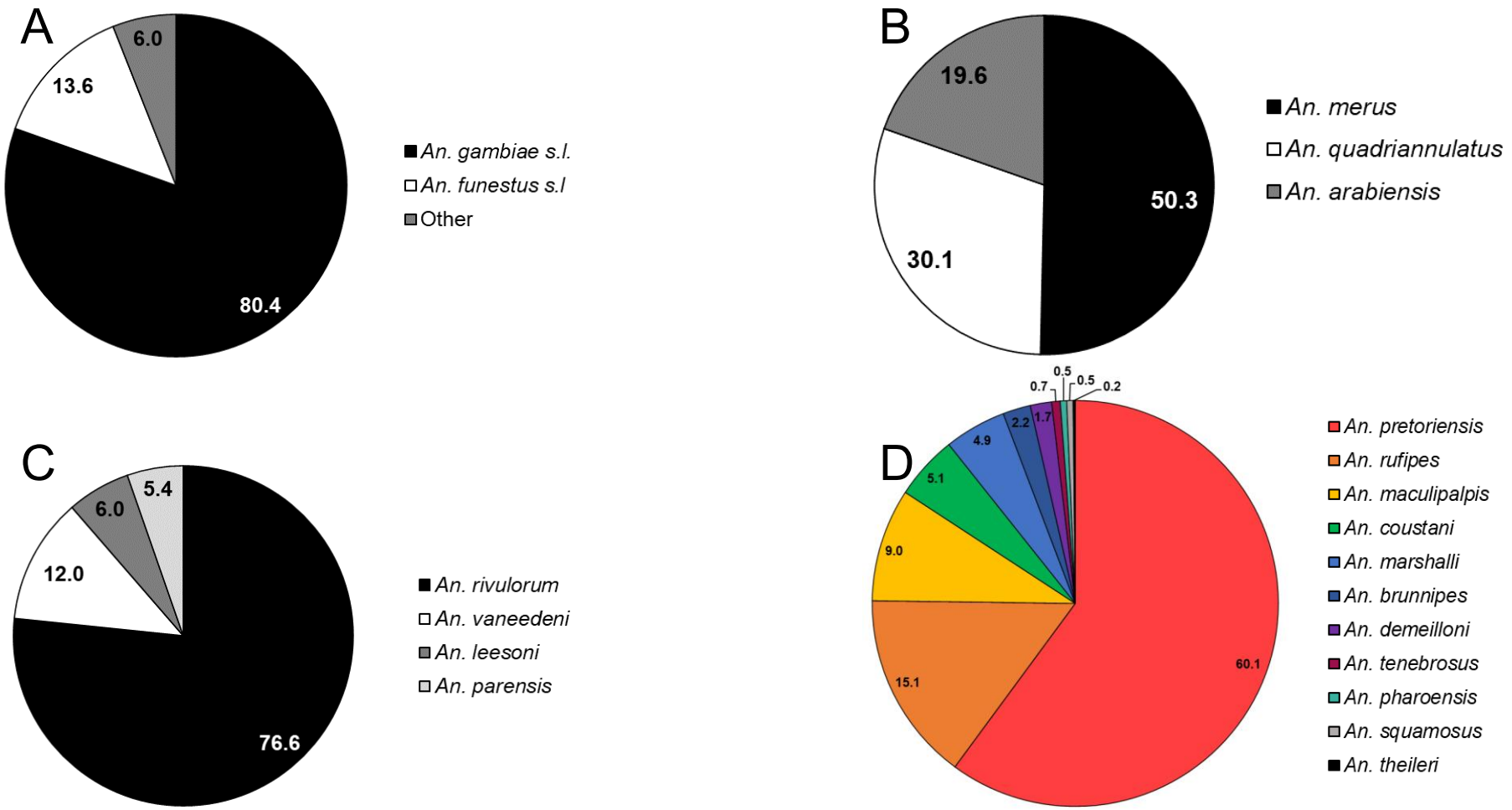


Figure 3.6. Species composition and proportion of *Anopheles* mosquitoes captured between 2005 and 2020 during surveillance activities in Ehlanzeni district, Mpumalanga.

A) Overall species composition and proportion by group/ complex; B) *An. gambiae s.l.* species composition and proportion; C) *An. funestus s.l.* species composition and proportion; D) species composition and proportion of other *Anopheles* species collected.

3.3.2 *Anopheles gambiae s.l.* annual abundance trends

Anopheles merus was the most abundant species of the *An. gambiae* complex between 2009 and 2020, with the exception of 2015 and 2017 (Fig. 3.7). *Anopheles arabiensis* was present at very low abundance during the earlier years of the surveillance period and gradually increased until it became the most abundant species in 2017 (Fig. 3.7). *Anopheles quadriannulatus* was the second most abundant species present throughout the surveillance period, except during 2015 when it was the most abundant (Fig. 3.7).

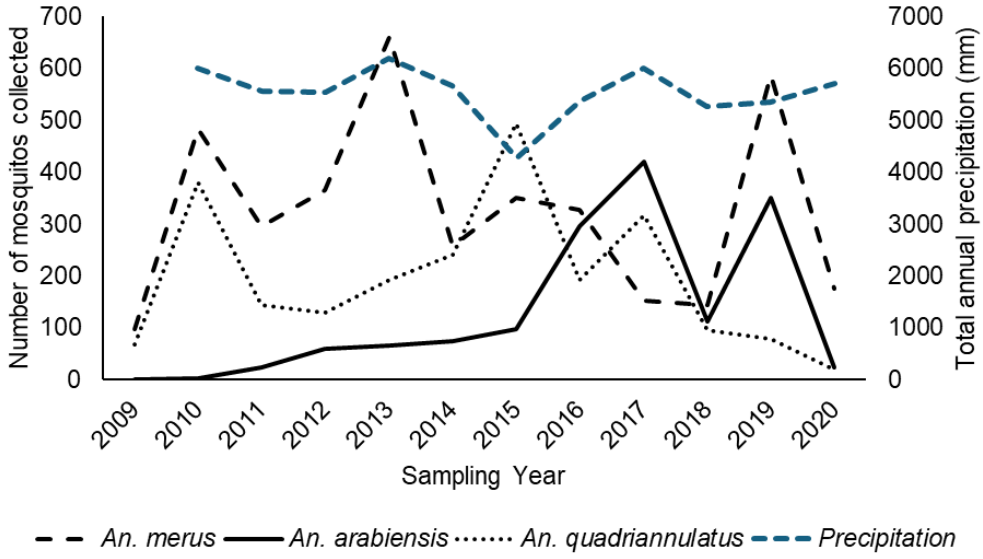


Figure 3.7. Annual abundance of *An. merus*, *An. quadriannulatus*, and *An. arabiensis* between 2009 and 2020 in Ehlanzeni District, Mpumalanga.
The total annual precipitation is shown by the dashed blue line.

3.3.3 *Anopheles gambiae s.l.* seasonal abundance trends

The majority of *An. gambiae s.l.* adults were collected in spring (n = 668) and *An. merus* accounted for the majority of the spring collections (n = 487) (Fig. 3.8). The most *An. arabiensis* adults were collected during autumn (n = 118), while being present at much lower numbers during spring (30) and summer (n = 9) (Fig. 3.8). *Anopheles quadriannulatus* was most abundant during spring (n = 151), followed by autumn (n = 72) (Fig. 3.8). During winter, a few adults of *An. merus* and *An. quadriannulatus* were collected (n = 5 and n = 7, respectively) (Fig. 3.8).

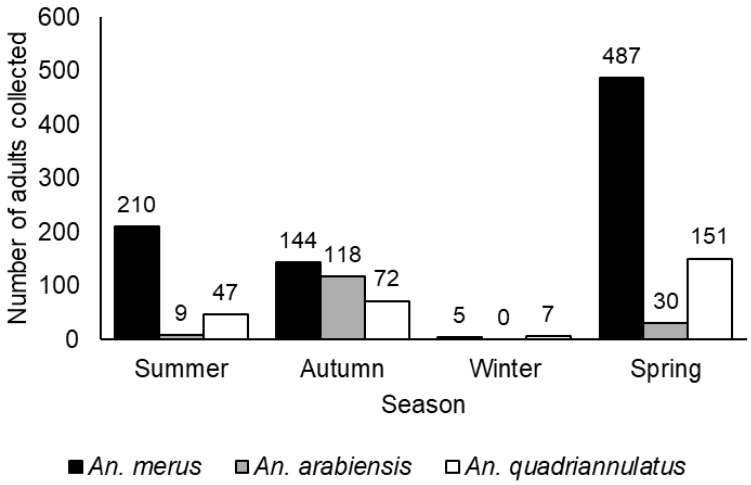


Figure 3.8. Seasonal abundance of *An. gambiae s.l.* adults between 2009 and 2020 in Ehlanzeni district, Mpumalanga.

The majority of *An. gambiae s.l.* larvae were collected in summer (n = 1 903) Fig. 3.9). *An. merus* accounted for the majority of larval collections during all four seasons (n = 3 087) (Fig. 3.9). *Anopheles arabiensis* larvae were most abundant during summer (n = 525) and were least abundant during spring (n = 178) (Fig. 3.9). *Anopheles quadriannulatus* larvae were relatively equally abundant during spring (n = 600), autumn (n = 593) and summer (n = 507) (Fig. 3.9). *Anopheles merus* and *An. quadriannulatus* accounted for the majority of the winter larval collections (n = 640 and n = 371, respectively) (Fig. 3.9).

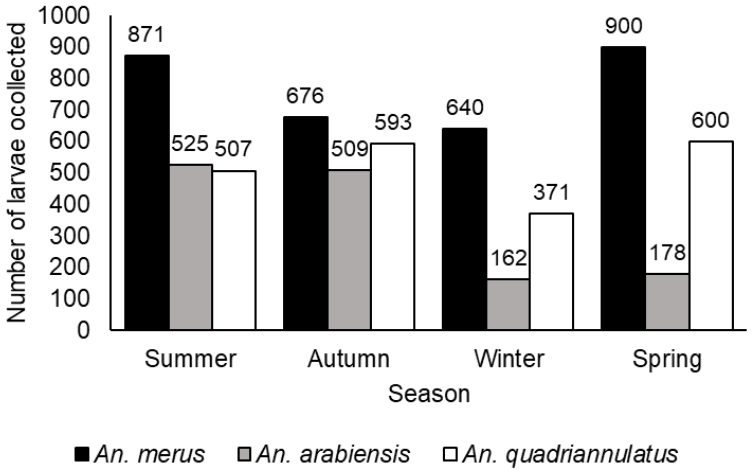


Figure 3.9. Seasonal abundance of *An. gambiae s.l.* larvae between 2009 and 2020 in Ehlanzeni district, Mpumalanga.

3.3.4 *Anopheles gambiae s.l.* collection method trends

Human landing catches accounted for the majority of *An. merus* and *An. arabiensis* adult collections, while pit traps accounted for the majority for *An. quadriannulatus* adults collected (Fig. 3.10). Human landing catches were not very effective at collecting *An. quadriannulatus* adults (Fig. 3.10). Carbon dioxide baited tent traps were moderately successful in collecting all three species (Fig. 3.10). Clay pots were not effective in collecting *An. gambiae s.l.* adults. However, the clay pots were more successful at collecting *An. arabiensis* compared to the other two species (Fig. 3.10). Other collection methods included disused tyres, window traps and pit toilets and were not very successful in collecting *An. gambiae s.l.* adults (Fig. 3.10).

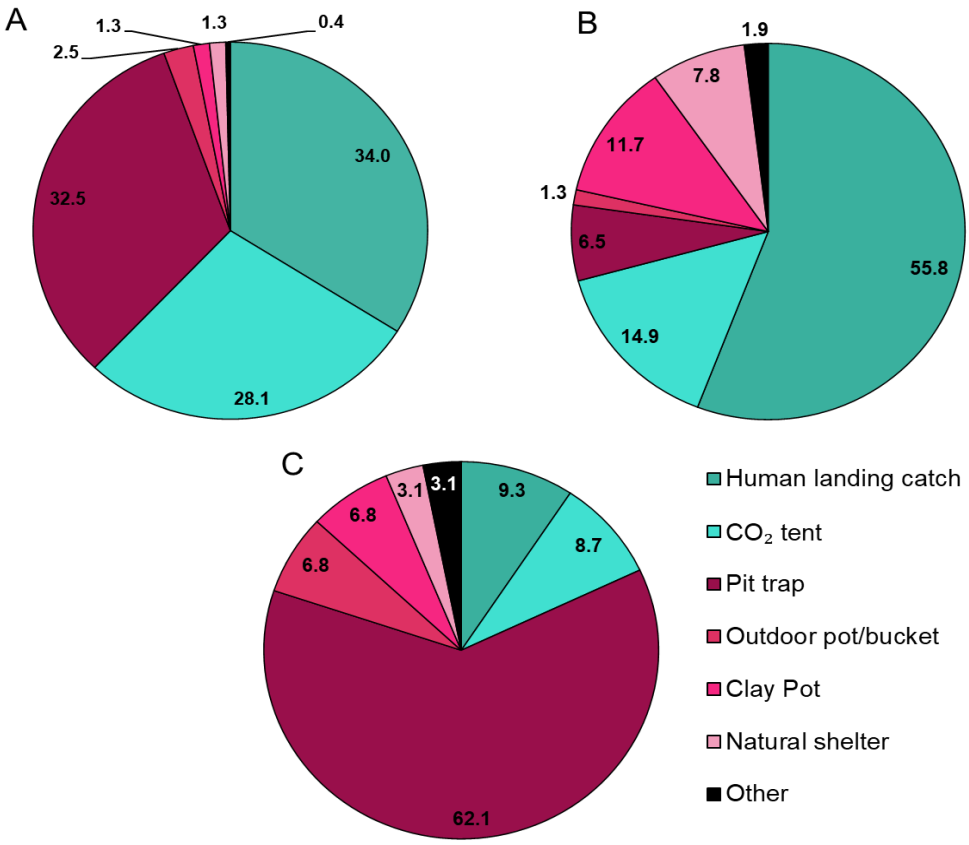


Figure 3.10. Proportion of adult *An. gambiae s.l.* adults collected using different methods between 2009 and 2020 in Ehlanzeni District, Mpumalanga.
A) *An. merus*; B) *An. arabiensis*; C) *An. quadriannulatus*. Shades of cyan indicate collection methods aimed at active mosquitoes, while shades of magenta indicate collection methods aimed at resting mosquitoes.

3.3.5 *Anopheles merus* distribution

Anopheles merus was collected from 40 sites across Ehlanzeni District (Fig. 3.11). *Anopheles merus* was most distributed in Nkomazi municipality (28 sites), compared to Bushbuckridge (7 sites) and Mbombela (5 sites). Notably, *An. merus* was found further inland, close to the border with Limpopo (Fig. 3.11)

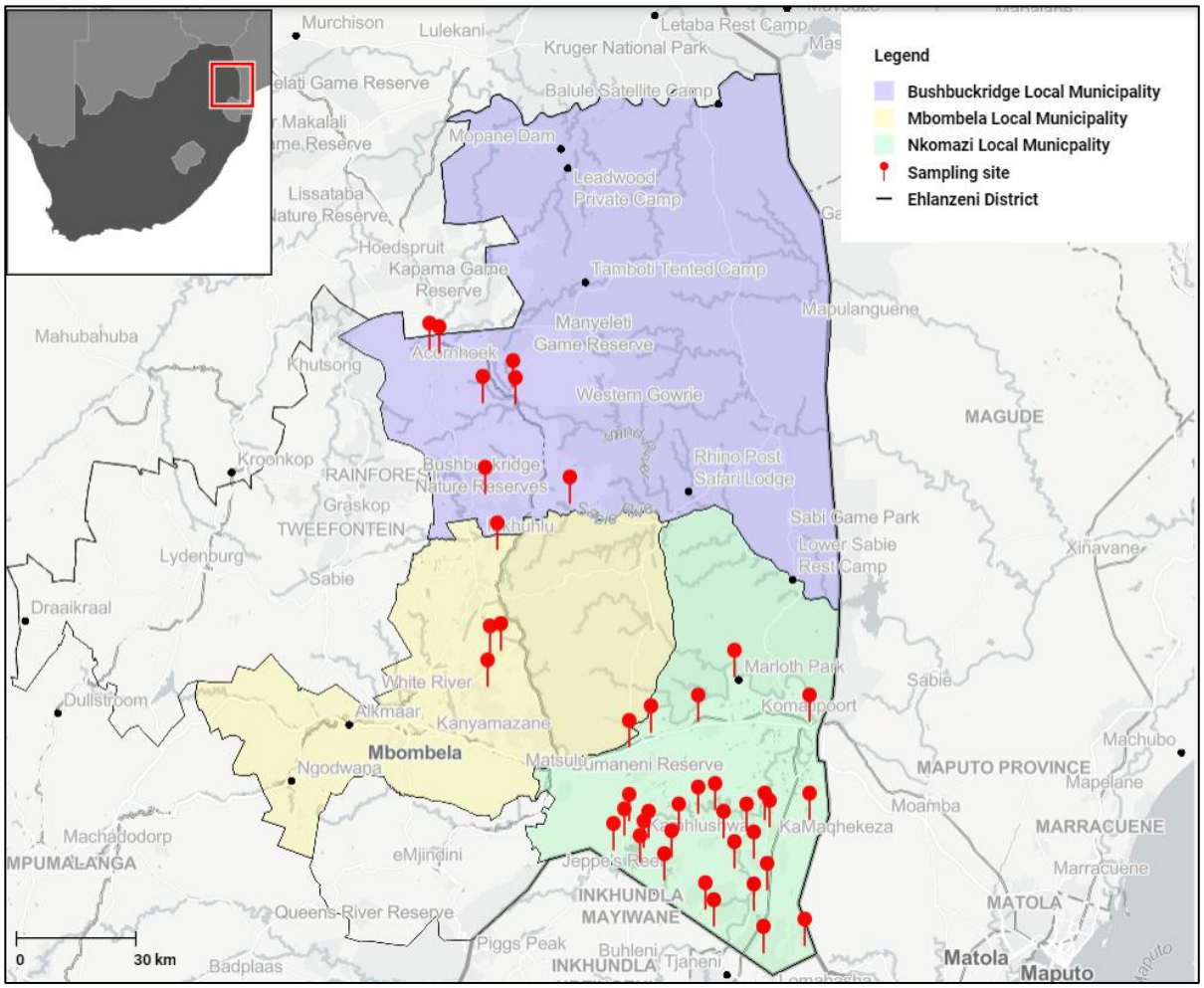


Figure 3.11. Distribution of *An. merus* in Ehlanzeni District by municipality between 2009 and 2020.

3.3.6 Diversity indices per sampling location

The median species richness (S) for Nkomazi, Mbombela and Bushbuckridge was 3.00 (interquartile range (IQR) = 1.00), 2.00 (IQR = 1.00) and 1.00 (IQR = 0.50), respectively (Fig. 3.12 A). The data was not normally distributed ($W_S = 0.78$, $p < 0.001$). Nkomazi had a significantly higher species richness compared to Mbombela and Bushbuckridge

and Mbombela had a significantly higher species richness than Bushbuckridge (S: $\chi^2(2, n = 71) = 25.97, p < 0.001$).

The median Pielou's Evenness Index (J) was 0.81 (IQR = 0.52), 0.33 (IQR = 0.80) and 0.00 (IQR = 0.08) for Nkomazi, Mbombela and Bushbuckridge, respectively (Fig. 3.12 B). The data was not normally distributed ($W_J = 0.80, p < 0.001$). Nkomazi had a significantly higher Pielou's Evenness Index compared to Mbombela and Bushbuckridge and Mbombela had a significantly higher Pielou's Evenness Index than Bushbuckridge (J: $\chi^2(2, n = 71) = 18.03, p < 0.001$).

The median Shannon-Weiner Diversity Index (H) was 0.69 (IQR = 0.52), 0.23 (IQR = 0.67) and 0.00 (± 0.058) for Nkomazi, Mbombela and Bushbuckridge, respectively (Fig. 3.12 C). The data was not normally distributed ($W_H = 0.85, p < 0.001$). Nkomazi had a significantly higher Shannon-Weiner Diversity Index compared to Mbombela and Bushbuckridge and Mbombela had a significantly higher Shannon-Weiner Diversity Index than Bushbuckridge (H: $\chi^2(2, n = 71) = 24.12, p < 0.001$).

The median Simpson's Diversity Index (D) was 0.52 (IQR= 0.36), 0.89 (IQR = 0.47) and 1.00 (IQR = 0.024) for Nkomazi, Mbombela and Bushbuckridge, respectively (Fig. 3.12 D). The data was not normally distributed ($W_D = 0.83, p < 0.001$). Bushbuckridge had a significantly higher Simpson's Diversity Index compared to Mbombela and Mbombela had a significantly higher Simpson's Diversity Index than Nkomazi (D: $\chi^2(2, n = 71) = 22.87, p < 0.001$).

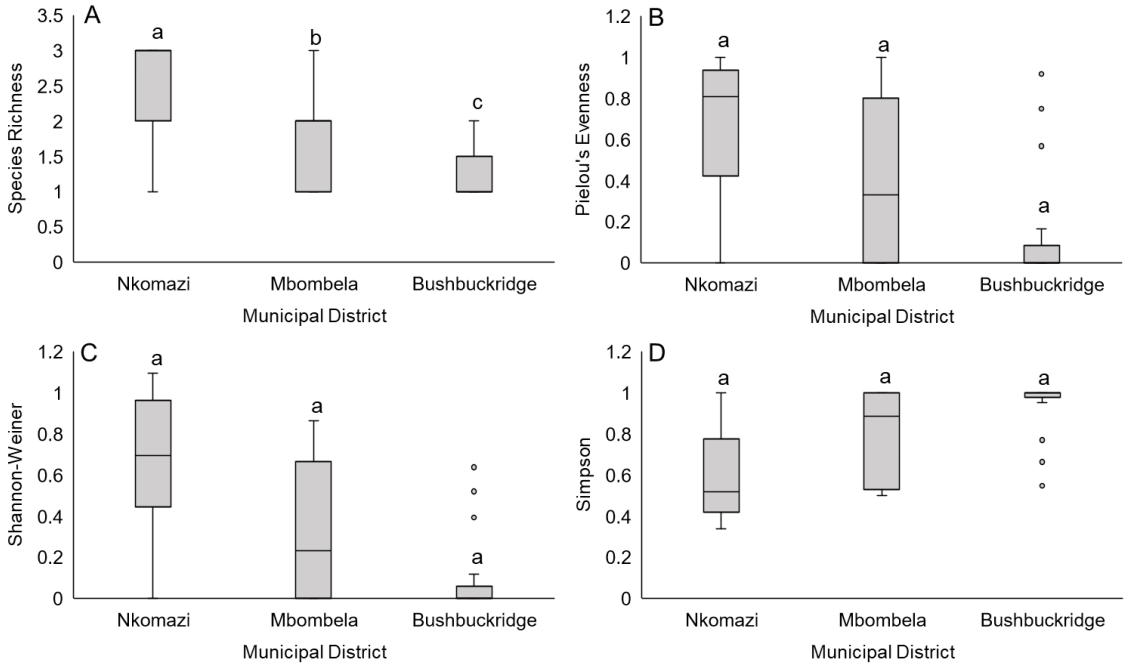


Figure 3.12. Alpha diversity indices for *An. gambiae s.l.* in Nkomazi, Mbombela and Bushbuckridge local municipalities within Ehlanzeni, Mpumalanga, between 2009 and 2020.

A) species richness; B) Pielou's Evenness Index; C) Shannon-Weiner Diversity Index and D) Simpson's Diversity Index. Letters indicate significant differences between municipalities.

3.3.7 Climate

There was a significant negative correlation between monthly *An. arabiensis* abundance and the average monthly temperature in Bushbuckridge (r_s = -0.7, p = 0.03). At a more microclimatic scale, there was a significant positive correlation between daily temperature at Block A, Nkomazi, and daily *An. arabiensis* abundance (r_s = 0.17, p = 0.02). There was a significant negative correlation between daily temperature and *An. quadriannulatus* abundance (r_s = -0.17, p = 0.02). No other correlations between climatic parameters and *An. gambiae s.l.* abundance was significant (p > 0.05 in all instances). The raw climatic data correlated to mosquito capture data is available in Appendix C.

3.3.8 Sampling effort

There was a clear sampling bias present as the number of *An. gambiae s.l.* mosquitoes sampled per day per year across the study period was not equal (Fig. 3.13). Sampling effort was highest during 2019, where 24.19 mosquitoes were collected per sampling day (Fig. 3.13). During 2020, sampling effort was the lowest (7.03 mosquito collected per sampling day) (Fig. 3.13).

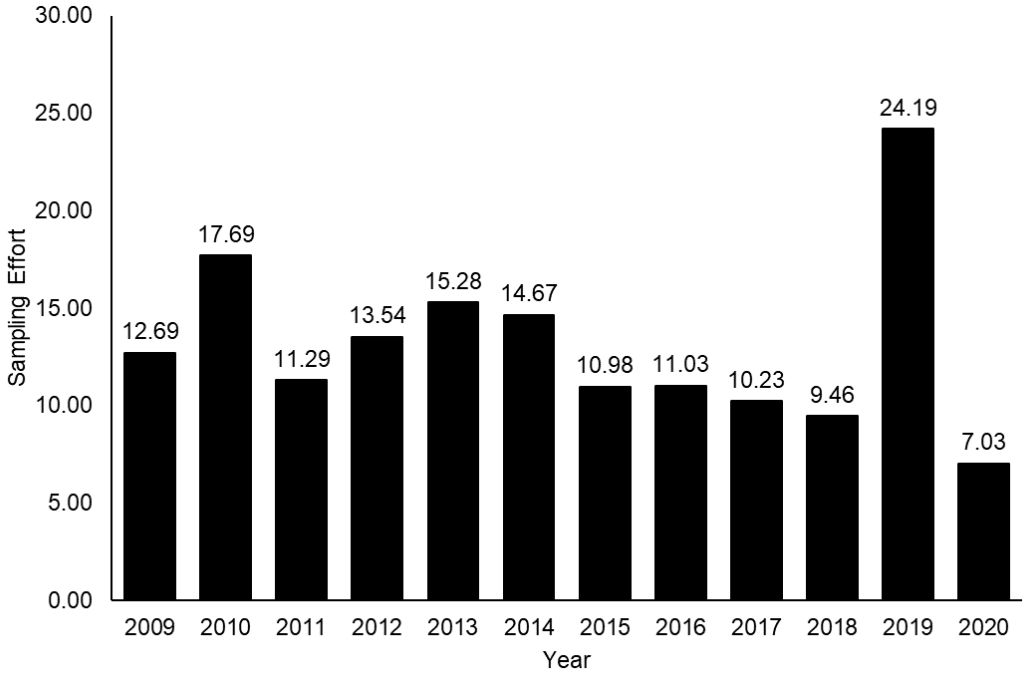


Figure 3.13. Number of *An. gambiae s.l.* mosquitoes sampled per day per year in Ehlanzeni District between 2009 and 2020.

3.4. Discussion

This was a retrospective analysis that investigated *Anopheles* vector abundance trends in malaria-endemic districts in Mpumalanga between 2005 and 2020. The results show changes in vector abundance and distribution over time that are not significantly influenced by climate at a macrogeographic scale.

According to the database, mosquitoes from the *An. gambiae* complex were the most abundant in the region compared to those from *An. funestus* group and other anophelines (Figure 3.6 A). *Anopheles funestus s.s.* and *An. gambiae s.s.*, primarily indoor and anthropophilic (human feeding) vectors (Gillies and de Meillon, 1968), have been absent from Mpumalanga, likely as a result of intensive IRS campaigns between the 1950's and 1980's (De Meillon, 1986; Sharp and le Sueur, 1996). The other members of the *An. funestus* group and *An. gambiae* complex tend more towards resting outdoors (exophily) (Gillies and de Meillon, 1968), which may explain their sustained presence in the province. *Anopheles funestus s.l.* is also one of the dominant vectors in Mozambique (De Meillon, 1941). Migration across the border into Mpumalanga is therefore possible.

Anopheles pretoriensis was the most abundant of the other *Anopheles* species that were collected. This species is not implicated as a vector in South Africa. A study in Zambia, however, found *An. pretoriensis* harboured *Plasmodium* spp. sporozoites and while not officially implicated as a vector, it likely plays an important epidemiological role in eastern Zambia (Lobo *et al.*, 2015). Gillies and de Meillon (1968) identified *An. pretoriensis*, *An. ziemanni*, *An. coustani* and *An. rufipes*, among others, as "incidental" vectors in parts of Africa with little or no epidemiological importance. The authors did concede that the epidemiological importance of these "incidental" vectors may change if the main vectors are successfully controlled or if community workers are exposed to exophilic species due to their occupation (Gillies and de Meillon, 1968).

Anopheles merus was the most abundant member of the *An. gambiae* complex in all three municipal districts within Ehlanzeni, followed by *An. quadriannulatus.* This is in accordance with previous studies in the region (Govere *et al.*, 2000; Mbokazi *et al.*, 2018). *Anopheles merus* and *An. quadriannulatus* primarily feed and rest outdoors (exophagy and exophily, respectively) (Pates *et al.*, 2001; Sinka *et al.*, 2010; Braack *et*

al., 2020). South Africa's malaria elimination control program focuses primarily on IRS (Brooke *et al.*, 2013), and this may account for the fact that *An. merus* and *An. quadriannulatus* has been able to increase in abundance in the region, while *An. arabiensis*, which is both endophilic (indoor resting) and exophilic (outdoor resting), is not as prevalent in Ehlanzeni (Gillies and de Meillon, 1968).

Anopheles merus has steadily been increasing its geographic range and relative abundance in the region (Ramalwa *et al.*, 2014; Mbokazi *et al.*, 2018), with the current study finding *An. merus* as far north as the border between Limpopo and Mpumalanga (Figure 3.11). This may be due to the increasing levels of pollution found in *Anopheles* breeding sites, which is associated with higher salinity than freshwater larval breeding sites (Emidi *et al.*, 2017). *Anopheles merus* is capable of normal development in salt concentrations as high as 70.40 g/L NaCl (Gillies and de Meillon, 1968; Le Sueur and Sharp, 1988), while *An. arabiensis* can only tolerate up to 17.5 g/L NaCl (Ould Lemrabott *et al.*, 2020). *Anopheles merus* may therefore be more adept at adapting to polluted breeding sites with higher salinity in Mpumalanga than *An. arabiensis*, which may explain their increased abundance and distribution.

Another reason for the increased distribution and abundance of *An. merus* may be a function of surveillance intensity. The study by Govere *et al.* (2000) visited only seven sampling sites in the Nkomazi district, while Ramalwa *et al.* (2014) focused on a single site at Vlakbult, Nkomazi. The study by Mbokazi *et al.* (2018) sampled from 52 larval breeding environments across Ehlanzeni. The current study identified 79 sites that were included in surveillance activities between 2009 and 2020 and included sites that were not visited by either Govere *et al*. (2000) or Mbokazi *et al.* (2018). It is therefore possible that *An. merus* has always been present across Ehlanzeni and its presence was not detected due to limited surveillance activities. Finally, it is also possible that successful IRS control interventions have resulted in population replacement of the endophagic (indoor feeding) *An. arabiensis* with the exophagic *An. merus*. This type of population replacement has been observed before in Kenya. Here, an intensive IRS campaign resulted in the replacement of *An. funestus*, a highly endophagic and anthropophilic species, with the more exophagic (outdoor feeding) and zoophilic (animal feeding) *An. rivulorum*, (Gillies and Smith, 1960). The wide distribution and high abundance of *An. merus* in Mpumalanga has important implications should *An. merus* be implicated as a vector of malaria in South Africa.

Although *An. merus* has been shown to be an important vector of malaria in Mozambique, Tanzania, Madagascar and Kenya (Temu *et al.*, 1998; Mbogo *et al.*, 2003; Tsy *et al.*, 2003; Cuamba and Mendis, 2009), it has not yet been implicated as a vector in South Africa (Dahan-Moss *et al.*, 2020). *Anopheles merus* likely plays a bigger role in malaria transmission in Mpumalanga than previously thought, given its abundance compared to *An. arabiensis* and the persistence of malaria cases in the province. Mbokazi *et al.* (2018) and Moonasar *et al.* (2013) also suggested that *An. merus* plays a role in malaria transmission in South Africa, based on the abundance of *An. merus* in the province and its incrimination in neighbouring countries.

Annual abundance trends showed that there were fluctuations in species abundance over time (Figure 3.7). Notably during 2017 *An. merus* abundance declined to a point where *An. arabiensis* was most abundant in 2017. Weather data from this study showed an increase in rainfall during 2016 - 2018. Rainfall reduces the salinity of semi-permanent breeding pools (Mosha and Mutero, 1982), which may have enabled *An. arabiensis* to utilise these as breeding habitats. The steady increase of *An. arabiensis* abundance may also be due to their behavioural plasticity, which allows them to adapt quickly to changing environments and helps them avoid IRS control efforts (Sinka *et al.*, 2010).

Seasonal fluctuations were present for all three species (Figures 3.8 and 3.9). *Anopheles merus* and *An. quadriannulatus* were most abundant during spring. This coincides with the start of the rainy season, which allows for a greater number of breeding habitats that are available. *Anopheles arabiensis* was least abundant during spring. This may be due to surveillance and control efforts that are more intensive during spring. While *An. merus* and *An. quadriannulatus* are predominantly exophilic, *An. arabiensis* tend towards both exo- and endophily (Gillies and de Meillon, 1968). Their reduced abundance during spring may be as a result of successful indoor control interventions. It may also be due to *An. arabiensis* being better adapted to drier seasons and climates (Gillies and Coetzee, 1987).

No significant correlation was found between climatic parameters and vector abundance when summarised on an annual or monthly basis. A significant correlation was found, however, when these parameters were summarised on a daily scale on a

site-specific level, as was the case for Block A. This finding is corroborated by studies conducted by Manyi *et al.* (2015), Mosha *et al.* (1982), Govere *et al*. (2000), Konwar *et al.* (2021) and Simon-Oke *et al.* (2015). These studies were generally short-term studies (less than one year and summarised monthly) and site-specific. Abiodun *et al.* (2018) and Zhou *et al.* (2004) recommends, however, that long term data is essential when assessing the impact of climate on malaria transmission. Since transmission is at least in part a function of vector abundance, it would therefore be essential to monitor the impact of climate on vector abundance on a long-term basis as well. Importantly, climatic parameters and vector abundance should then be summarised daily at a site-specific level, instead of summarising years of data across multiple sites together.

This study corroborates findings by Kelly-Hope *et al.* (2009) that show climate influences species differently. If the goal is to investigate the effect of climate on vector abundance to optimise malaria control programs, then analysing all species together may give false interpretations of observed trends. Konwar *et al.* (2021) and Simon-Oke *et al.* (2015) for example, analysed all vector species together instead of doing species-specific analyses, and this may have skewed their results.

There was a clear sampling bias in terms of collections where the majority of mosquitoes were collected as larvae. This may be due to the viability of this method compared to adult captures. Larval collections allow for long periods of collection during the day with no time restrictions, such as evening collections for adults. Pit traps, CO_2 tent traps and human landing catches were the most effective collection methods that focused on adult captures (Figure 3.10). Females of all three species (*An. arabiensis*, *An. merus* and *An. quadriannulatus*) engaged in anthropophily, however *An. arabiensis* was the most anthropophilic of the three species.

An interesting finding from this analysis was that clay pots were largely ineffective at capturing either of the three species. This is in contrast to the surveillance program in KwaZulu-Natal, where clay pots have been the most effective method for adult captures, and where *An. arabiensis* is the most abundant species (Dandalo *et al.*, 2017). This is likely due to the differences in species composition between the two provinces and their respective resting behaviours. While in Mpumalanga clay pots were largely ineffective, they were more effective in capturing *An. arabiensis*, which tend toward both endo- and exophily, whereas *An. merus* and *An. quadriannulatus*

tend more towards exophily (Gillies and de Meillon, 1968). Data on the efficacy of collection methods can therefore be extremely valuable in optimising annual surveillance programs by tailoring collection methods to suit the species composition of the area.

Diversity index analyses of the three districts showed that each district had unique diversity dynamics (Figure 3.12). Nkomazi had the highest diversity and evenness indices of the three municipalities, showing that all three species, *An. merus*, *An. arabiensis*, and *An. quadriannulatus*, occurred in the region at more or less even proportions. The other two municipalities, Bushbuckridge and Mbombela, had significantly less diversity and evenness, compared to Nkomazi. These two municipalities tended to have one dominant species in the area, i.e. *An. quadriannulatus*, with the other two species occurring at much lower abundance. Previous studies also found high diversity and species evenness present at Nkomazi (Govere *et al.*, 2000; Ramalwa *et al.*, 2014; Mbokazi *et al.*, 2018). Mbokazi *et al.* (2018) similarly found that species diversity was low for the Mbombela and Bushbuckridge districts. This finding suggests that blanket control efforts across a district should ideally be replaced with tailor-made solutions that speak to the species diversity and composition to reduce malaria transmission. Such an approach, however, has practical limitations, particularly in funding and available resources.

This was a retrospective analysis and therefore had several limitations. Sampling effort, defined as the number of mosquitoes captured per sampling day (see section 3.2.7) across the study period was not standardised (Figure 3.13) and information on the number of sampling personnel and sampling hours were not available to consider. The data collected was not always complete and as such 2 794 lines of data were excluded from the analyses. The various collection methods were not employed equally across the study period, for instance clay pots were only introduced in 2014 and CO_2 tents from 2018 onwards. Finally, information about the implementation of control interventions during the study period were not available to consider. These factors may have influenced the vector abundance patterns observed in this study.

The findings from this study, however, have laid a solid foundation for subsequent studies and have identified where the research gaps are. Future work should focus on

incriminating *An. merus* as a vector and further investigating the relationship between climate and vector abundance in greater detail.

3.5 Conclusion

Understanding vector bionomics and population dynamics is critical for malaria elimination efforts. Residual transmission remains a challenge within Mpumalanga, and as such, within South Africa. Historical and present surveillance data can be essential in achieving elimination goals. This study has shown that *An. merus* is the most abundant anopheline within the province and has been since at least 2009. While not yet implicated, *An. merus* is likely a potential vector of malaria within the Ehlanzeni district. The challenge of eliminating residual transmission relies on the challenge of implementing control strategies effective for outdoor resting- and feeding mosquitoes. Several such techniques were identified and reviewed by Sougoufara, Ottih and Tripet (2020) and includes sterile insect technique (SIT), paratransgenesis, larval source management, trapping of host seeking females and resting mosquitoes, attractive toxic sugar bait, *Wolbachia* releases and endectocides. Optimising the types of collection methods employed on a region-by-region basis and implementing control strategies at critical seasonal times, and pairing these data with climatic data, vector abundance data and malaria case data, will be particularly effective in the fight against malaria.

CHAPTER 4

IMPACT OF LARVAL HABITAT POLLUTION ON INSECTICIDE RESISTANCE PROFILE AND GUT MICROBIOTA COMPOSITION OF *AN. GAMBIAE* (GILES) FROM MPUMALANGA

4.1 Introduction

4.1.1 Global scale of insecticide resistance

The development of resistance to insecticides by malaria vector species is a challenge that threatens the efficacy of malaria control and elimination efforts globally (World Health Organization, 2018). The repeated use of the same insecticidal active ingredients in control interventions such as LLINs and IRS programs have led to the development of resistance in many *Anopheles* species that have been implicated in the transmission of malaria.

The most recent report on global insecticide resistance by the WHO identified 70 countries and 5 605 occurrences of confirmed insecticide resistance (i.e. where there was less than 90% mortality of mosquitoes after exposure to an insecticide) in *Anopheles* malaria vectors (World Health Organization, 2018). This includes resistance to four different classes of insecticides and 15 different active ingredients of insecticides (Table 4.1). The large majority of pyrethroid resistance cases were detected in Africa, where malaria is endemic to many countries (Figure 4.1) (World Health Organization, 2024a).

Table 4.1. Number of studies confirming insecticide resistance (< 90% mortality after exposure) in *Anopheles* vectors per insecticide class and type between 1978 and 2023, where 100 or more mosquitoes were used during bioassays (World Health Organization, 2018).

Insecticide class	Active ingredient	# Studies confirming resistance
Carbamate	Bendiocarb	334
Carbamate	Carbosulfan	33
Carbamate	Propoxur	64
Organochlorine	DDT	992
Cyclodiene	Dieldrin	34
Organophosphate	Fenitrothion	57
Organophosphate	Malathion	349
Organophosphate	Pirimiphos-methyl	3
Pyrethroid I	Etofenprox	60
Pyrethroid I	Permethrin	987
Pyrethroid II	Alpha-cypermethrin	273
Pyrethroid II	Cyfluthrin	122
Pyrethroid II	Deltamethrin	1881
Pyrethroid II	Lambda-cyhalothrin	416

Of particular concern is the widespread resistance to pyrethroids- one of the main insecticide classes used to control malaria in many countries and the major constituent of LLINs (World Health Organization, 2018). This necessitates the time-consuming practice of developing new active ingredients and insecticidal products from a limited number of insecticide classes available to public health (World Health Organization, 2018). Roll-out of these new active ingredients are slow and often takes several years (Hemingway, 2014). Understanding how insecticide resistance develops is essential for the development of new active ingredients that will be effective in the control and elimination of malaria vectors.

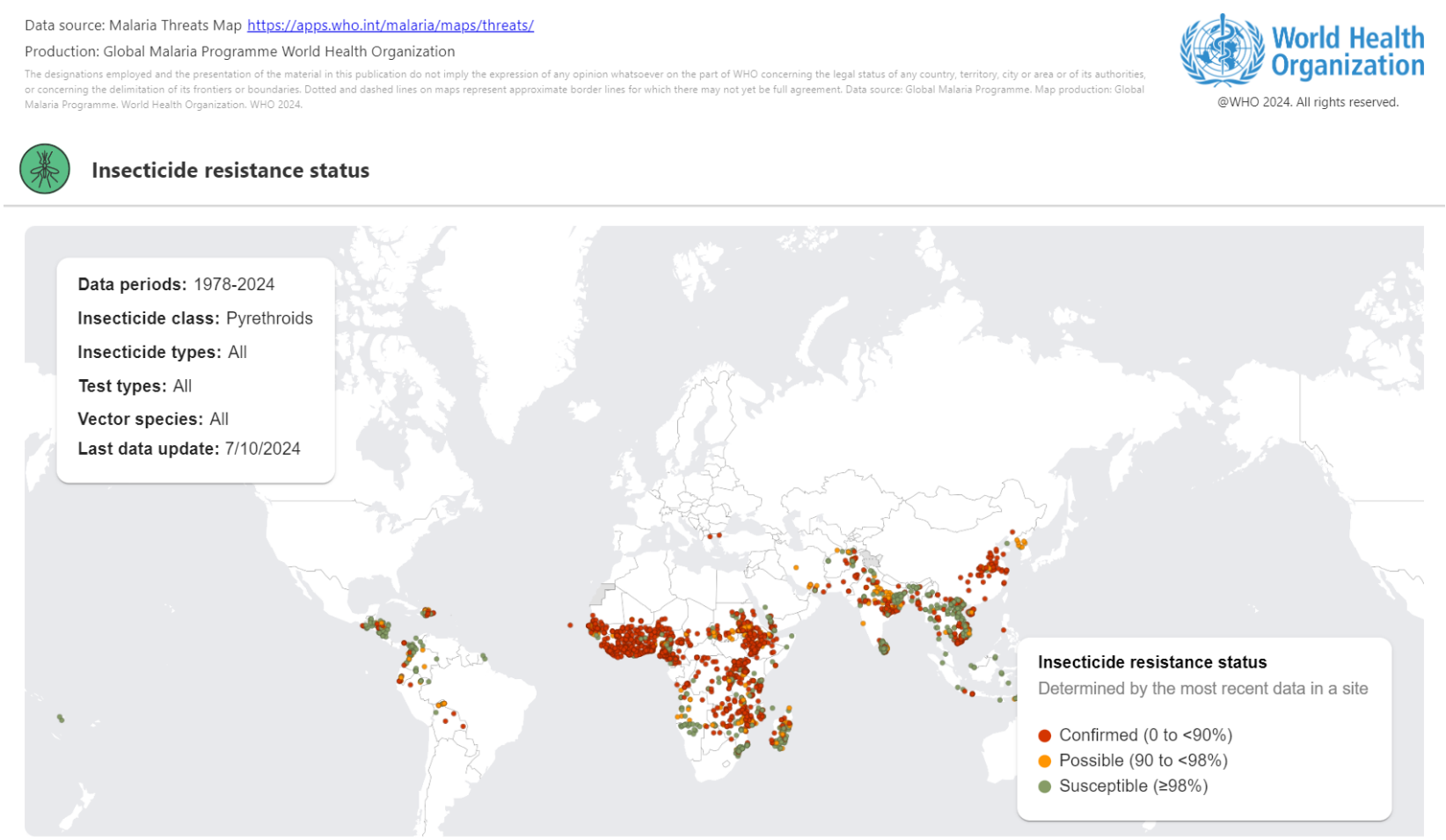


Figure 4.1. Global incidence of pyrethroid resistance between 1978 and 2023 occurring in *Anopheles* vector species (World Health Organization, 2024a).

Red dots indicate cases of confirmed resistance (0 – 90% mortality), yellow dots indicate cases of possible resistance (90 – 98% mortality) and green dots indicate cases of full susceptibility (≥ 98% mortality).

4.1.2 Mechanisms of insecticide resistance in insects

Several different types of mechanisms have been identified that contribute to insecticide resistance in insects. These mechanisms can range from broad, non-specific mechanisms to specific mechanisms, all of which reduce the amount of toxin absorbed by the insect. At the broadest level, mechanisms include behavioural resistance (Sparks *et al.*, 1989) and vigour tolerance (Davidson, 1958). Behavioural resistance involves avoidance of the toxicant, while vigour tolerance occurs as a result of increased body size, thereby requiring a larger dose of a xenobiotic before effects are triggered. The next broadest mechanism involves a reduction of the amount of toxin absorbed. This is mediated by thickened cuticles (Balabanidou *et al.*, 2016). A more specific mechanism involves defined enzyme systems that detoxify foreign compounds. This is known as metabolic resistance (Li, Schuler and Berenbaum, 2007). Another specific mechanism prevents the interaction of a pesticide with its neurological target, known as target site resistance (TSR) (Ffrench-Constant and Roush, 1991). Finally, a more recent driver of insecticide resistance has been associated with the gut microbiome (Siddiqui *et al.*, 2022). A comprehensive review of behavioural resistance, penetration resistance and vigour tolerance is available in Siddiqui *et al*. (2023). Metabolic resistance and TSR are two of the major mechanisms involved in insecticide resistance and are discussed briefly below. A brief discussion on bacterial resistance is also included.

4.1.3 Target site resistance

Most insecticides used in vector control are neurotoxic. Target site resistance refers to one or more mutations in a nerve targeted by conventional insecticides, typically resulting in the inhibition of the pesticidal action of the insecticide (Ffrench-Constant *et al.*, 1998). There are two typical examples of TSR, which involve mutations in the voltage-gated sodium channel (VGSC) and the gamma-aminobutyric acid (GABA) receptor.

The VGSC in insects plays a key role in the generation of action potentials, which are essential for neural communication and muscle contraction. This channel is activated when the neurotransmitter acetylcholine is released from presynaptic neurons and binds to nicotinic acetylcholine receptors located in the postsynaptic membrane. This

initiates an action potential in the postsynaptic membrane (Catterall, 1992). Activation of the VGSC allows for the influx of positively charged sodium ions into the neuron, resulting in the generation of an action potential (Catterall, 1992). Inactivation of the channel occurs shortly after the channel is opened, which blocks further influx of sodium ions into the neuron. This contributes to the membrane potential reaching a net negative state (repolarisation), which ultimately ends the generation of action potentials (Catterall, 1992). Insecticides that target the VGSC, such as pyrethroids, preferentially bind to open sodium channels and prevent them from inactivating (O'Reilly *et al.*, 2006), resulting in the continuous generation of action potentials, which ultimately leads to neural overload and death.

GABA acts as an inhibitory neurotransmitter which, when released from the presynaptic neurons, binds to and activates the GABA receptor (Sattelle, 1990) . This allows for the influx of negatively charged chloride ions into the neuron that facilitates the nerve returning to its natural negatively charged state and thereby preventing the generation of an action potential (Sattelle, 1990). Insecticides such as cyclodienes and phenylpyrazoles preferentially bind to the GABA receptor, preventing the binding of GABA (Ffrench-Constant and Roush, 1991; Bloomquist, 1994). This prevents the influx of negatively charged chloride ions and results in the continuous generation of an action potential, resulting in neural overload and death.

Mutations in the VGSC and the GABA receptor have been linked to insecticide resistance. A mutation from alanine to serine in the *rdl* gene, which codes for a subunit of the GABA receptor, renders the GABA receptor less sensitive to binding by cyclodienes and phenylpyrazoles, resulting in resistance to these insecticides (Ffrench-Constant *et al.*, 1993). There are numerous reports of mutations in the VGSC, all linked to the *para* and *Vssc* genes, which results in resistance to pyrethroids and DDT (Soderlund and Knipple, 1999). The most common mutation is the *kdr* mutation (L1014F) in the *para* gene locus (O'Reilly *et al.*, 2006). Several variations of the L1014F mutation have been identified (reviewed in Silva, Santos and Martins (2014)). In *Anopheles gambia*e, the L1014F (also known as *kdr* west) was detected in *An. gambiae* populations from west Africa (Martinez-Torres *et al.*, 1998). Later, a second mutation, L1014S, was identified in *An. gambiae* populations from east Africa, known as *kdr* east (Ranson *et al.*, 2000). The *kdr* mutations cause the VGSC to remain in a

closed state, thereby reducing the capacity of insecticides that target the VGSC to bind to the channel (O'Reilly *et al.*, 2006).

While TSR prevents the interaction of the pesticide with its target, it does not metabolise and remove the xenobiotic from the cell environment (Brooke, 2008). Metabolic detoxification enzymes facilitate this process. In conjunction with vigour tolerance and penetration resistance, these enzymes reduce the amount of xenobiotic present in the system. This results in reduced toxicity (Siddiqui *et al.*, 2023).

4.1.4 Metabolic resistance

One of the challenges insects face in the aquatic environment is the presence of toxic xenobiotics (any substance or synthetic chemical that is foreign to the body). Xenobiotics enter aquatic insect larvae either through the body from surface water or via the ingestion of sediment and food particles. Metabolic detoxification enzymes in resistant insects are often in a state of constant overexpression as a mechanism to cope with xenobiotic challenges (Li, Schuler and Berenbaum, 2007). There are also alternative mechanisms where slight variations in the enzyme results in more efficient detoxification (Mugenzi *et al.*, 2020). Metabolic enzymes involved in detoxification in insects include cytochrome P450's, general esterases and glutathione S-transferases (GSTs), and follows a three phasic cycle (Yu, 2008). Pest insects typically have higher levels of detoxification enzymes, for example mosquitoes have more than 100 identified cytochrome P450 gene sequences (Ranson *et al.*, 2002), versus 46 in honeybees (Claudianos *et al.*, 2006).

The first phase of metabolic detoxification involves oxidation, hydrolysis and reduction reactions whereby lipophilic xenobiotics are detoxified by decreasing their biological activity and making them water-soluble (Yu, 2008). Phase I typically involves cytochrome P450 enzymes, particularly enzymes in the CYP6 family in insects (Feyereisen, 2012; Ye *et al.*, 2022). If the resulting metabolites are sufficiently polar, they can be excreted and the detoxification process is complete; else metabolites are conjugated during the second phase with the aid of GSTs (Yu, 2008). This results in the formation of highly water-soluble mercapturic acids, which is then removed via active transport (Habig, Pabst and Jakoby, 1974). As such, the now water-soluble xenobiotic is unlikely to passively diffuse into the lipid rich cellular environment. The

third and final phase involve esterases which sequester and hydrolyse ester-containing xenobiotics (Hemingway and Ranson, 2000).

High levels of metabolic enzymes involved in xenobiotic detoxification have been linked to the development of resistance against several insecticides. Cytochrome P450 enzymes have been linked to an increased resistance in insects to several insecticide classes, such as pyrethroids (Penilla *et al.*, 2007), carbamates (Edi *et al.*, 2014), neonicotinoids (Zoh *et al.*, 2021), organophosphates (Smith *et al.*, 2019) and organochlorines (Fagbohun *et al.*, 2019). General esterases have been linked to an increased resistance in ester-containing insecticides such as organophosphates (Prasad *et al.*, 2017), carbamates (Mutunga *et al.*, 2015) and pyrethroids (Wu *et al.*, 2018), and GSTs have been shown to confer resistance to organophosphates, DDT and pyrethroids (Hemingway, Miyamoto and Herath, 1991; Vontas, Small and Hemingway, 2001; Penilla *et al.*, 2006).

Recent studies have shown that TSR can work in conjunction with metabolic detoxification enzymes to confer a resistant phenotype (Edi *et al.*, 2014; Mitchell *et al.*, 2014; Grigoraki *et al.*, 2021; Muhammad *et al.*, 2021). The study by Grigoraki *et al*. (2021) showed that a single *kdr* mutation introduced into a fully susceptible strain of *An. gambiae* via Clustered Regularly Interspaced Short Palindromic Repeats and CRISPER-associated protein 9 (CRISPR/Cas9) increased pyrethroid resistance. They also showed that when they induced the expression of GST, in addition to the *kdr* mutation, the strain's resistance to pyrethroids increased further (Grigoraki *et al.*, 2021).

4.1.5 The mosquito microbiome plays a role in insecticide resistance and immunity

A recently discovered driver of insecticide resistance has been associated with the microbiome in the midguts of insects. This resistance is facilitated by pesticide-degrading bacteria that occur within the midgut of the insect host (Dada *et al.*, 2018; Jaffar, Ahmad and Lu, 2022; Siddiqui *et al.*, 2022).

One of the main mechanisms of microbe-facilitated pesticide degradation occurs through the direct degradation of the pesticide via various metabolic pathways. These pathways involve several enzymatic reactions, including hydrolysis, dehalogenation,

oxidation, nitro-reduction, methylation and demethylation reactions (Kumar, Mukerji and Lal, 1996; Singh *et al.*, 1999; Ye, Dong and Lei, 2018). The bacterial enzymes that facilitate these reactions are reviewed by Russell *et al*. (2011) and Jaffar, Ahmad and Lu (2022).

The second mechanism of microbe-facilitated pesticide degradation occurs through microbial activity that changes their chemical and/ or physical environment, resulting in the indirect degradation of the pesticide. The main mechanisms here involve mineralisation, co-metabolism and synergistic metabolism (Tang, Ji and Hou, 2017; Ye, Dong and Lei, 2018). Mineralisation involves the degradation of the pesticide into an inorganic compound, which is then used as a source for growth (Ramya *et al.*, 2016; de Almeida *et al.*, 2017; Gomes, Omoto and Cônsoli, 2020). Co-metabolism occurs when a non-growth compound, such as a pesticide, is transformed or modified, but not utilised as a growth substrate (Dalton and Stirling, 1982; Wang *et al.*, 2010; Nzila, 2013; Sharma, Rajor and Toor, 2014). Synergistic metabolism refers to the joint metabolism of an organic pollutant by two or more bacteria, such as the metabolism of heavy metals by *Shewanella oneidensis* and *Lactobacillus rhamnosus* (Zhang *et al.*, 2022) and the degradation β-cyfluthrin by *Enterobacter* and several other species (Li *et al.*, 2022).

Factors that influence the degradation of pesticides by bacteria broadly involve environmental factors and the nature of the pesticide itself (Aislabie and Lloyd-Jones, 1995). Environmental factors include the pH, temperature, biological oxygen demand, nutrient availability, presence of other growth substrates within the water body, as well as light quality and intensity (Aislabie and Lloyd-Jones, 1995). The pesticide's structural composition, weight, functional groups, toxicity, concentration and solubility in water will affect its potential to be degraded by bacteria (Aislabie and Lloyd-Jones, 1995).

The gut microbiota not only potentially play a role in protecting the mosquito from synthetic chemical challenge, but also aid in modulating the insect host's immune system. Several studies have investigated the effects of the midgut microbiota in *Anopheles* species on the sporogenic development of *Plasmodium* species. Dong *et al.* (2009) showed that the midgut microbiota conferred resistance to infection by *Plasmodium* in *An. gambiae* by upregulating key anti-*Plasmodium* genes, whereas

mosquitoes that did not possess any midgut microbiota were more susceptible to *Plasmodium* infection. Gram-negative bacteria such as *Pseudomonas* spp., *Enterobacter* spp., *Escherichia coli*, *Ewingella americana, Serratia maracenses* and *Flavobacterium* spp. have been shown to partially or completely inhibit *P. falciparum* oocyst infection rates and densities in *An. gambiae* and *An. albimanus* (Pumpuni *et al.*, 1993, 1996; Gonzales-Ceron *et al.*, 2003). Exposure of *An. gambiae* and *An. stephensi* to the antibiotic gentamycin showed higher infection rates of *Plasmodium*, indicating that the removal of certain midgut bacteria may contribute to a higher susceptibility to *Plasmodium* infection (Beier *et al.*, 1994). These findings suggest that gut microbiota is a critical determinant of not only mosquito life-history in general, but also potentially in the adaptation to new environments, all of which may have epidemiological consequences.

4.1.6 Adaptation to polluted environments

Anopheles gambiae s.l. typically breed in clear, sunlit pools of still-standing water (Gillies and de Meillon, 1968). However, adaptation to polluted water has increasingly been seen in *An. gambaie s.l* (Awolola *et al.*, 2007; Kamdem *et al.*, 2017; King *et al.*, 2017) and *An. arabiensis* (Antonio-Nkondjio *et al.*, 2011; Jones, Toé, *et al.*, 2012; Azrag and Mohammed, 2018). Polluted aquatic environments are rich in toxic xenobiotics and pesticide-degrading bacteria. Adaptation to these polluted environments have been shown to be associated with insecticide resistance, as chronic exposure to toxic xenobiotics causes the continuous upregulation of metabolic detoxification enzymes (Li, Schuler and Berenbaum, 2007). Additionally, acquisition of pesticide-degrading bacteria from the aquatic environment have been shown for *Anopheles gambiae s.l.* (Boissière *et al.*, 2012). Breeding in polluted habitats therefore has the potential to challenge the efficacy of malaria elimination programs.

Both *An. gambiae s.s.* and *An. arabiensis* are efficient malaria vectors and their adaptation to urban breeding habitats, which are often polluted, drives local malaria transmission (Kamdem *et al.*, 2017). The increase in insecticide resistance due to pollutant exposure challenges the efficacy of malaria control programs.

4.1.7 Context and rationale

The Mpumalanga province has a diverse economic profile. In 2022, the region contributed 8% of the national gross domestic product (GDP). The main sectors and industries in the province are Mining (20% of the provincial GDP), Community Services (19% of the provincial GDP) Manufacturing (15% of the provincial GDP), Finance (14% of the provincial GDP), Trade (14% of the provincial GDP), Electricity (7% of the provincial GDP), Transport (6% of the provincial GDP), Agriculture (3% of the provincial GDP) and Construction (2% of the provincial GDP) (Young, 2022).

Agricultural activities in Mpumalanga include summer cereals, legumes, maize, soya, canola, sunflowers, fruits, sugarcane, citrus, nuts and livestock (sheep, cattle, chickens, pigs). A 2018 census recorded over 630 000 cattle, 375 000 sheep, 81 000 pigs and 22 million chickens (Statistics South Africa, 2020). Forestry produces paper, pulp, cellulose, saw timber, wood chips and wattle extract, with major facilities including PG Bison (Piet Retief), FX Group (Lothair) and Sappi's Ngodwana mill near Mbombela (Young, 2022). Mining includes gold, coal, chrome, platinum, iron ore, vanadium, manganese, granite and clay, with Mpumalanga producing 83% of South Africa's coal and housing 11 power stations that generate 80% of national electricity. Manufacturing focuses on Agri-processing (forestry products, sugar, fruits, nuts, condiments) and heavy industry (fuel, petrochemicals, chemicals, ferro-alloys, stainless steel). Sasol produces 60 million litres of fuel per day, Columbus Stainless Steel is Africa's only flat stainless-steel producer, and Samancor is the world's second-largest ferrochrome producer (Young, 2022). The province is also supported by two airports, an extensive railway network and the N4 highway, which links to the Maputo Corridor (Young, 2022).

These activities have contributed to extensive pollution. Mpumalanga is a global hotspot for nitrogen dioxide (Amritha *et al.*, 2024). and has high sulphur dioxide levels (Sangeetha and Sivakumar, 2019). Airborne pollutants travel long distances, depositing into aquatic ecosystems (Swackhamer *et al.*, 2004). Industrial pollution from coal mining has significantly impacted water quality of surface water in the province (Dzhangi and Atangana, 2023). Coal mining has also been found to have degraded water quality through acid mine drainage, as seen in Carolina in 2012 (McCarthy and Humphries, 2013), while wastewater pollution has affected the Crocodile River (Phungela *et al.*, 2022). Agricultural runoff of heavy metals and agrochemicals were

found to have polluted the Crocodile and Olifants River catchments (Heath, 1999; Oberholster, 2017).

It is possible that the pollution present in Mpumalanga has caused increased levels of pollution of *An. gambiae* larval breeding sites. If this is indeed the case, their increased adaptability to these pollutants may inadvertently also increase their resistance to commercially used insecticides. This could result in their range expansion from less polluted rural breeding habitats to more industrial areas which are heavily polluted. If this is true, this could potentially increase the risk for the transmission of malaria in the province.

The research questions this chapter will answer are whether the increasing levels of pollution in Mpumalanga, specifically Ehlanzeni District Municipality, is driving insecticide resistance and affecting life-history traits in the *An. gambiae* complex. The specific aims of the chapter are to:

1. Investigate chemical and bacterial composition of urban water bodies utilised by *An. gambiae s.l.* as breeding habitats in Ehlanzeni District.

2. Investigate the bacterial composition of *An. gambiae* complex larval midguts from Ehlanzeni District.

3. Investigate resistance enzyme profiles of wild *An. gambiae s.l.* adults and larvae from Ehlanzeni District.

4. Investigate susceptibility of wild *An. gambiae s.l.* adults from Ehlanzeni District to DDT and deltamethrin.

4.2 Materials and Methods

4.2.1 Sampling sites

Water from larval breeding sites and mosquito samples were collected from eight sites in Nkomazi and Bushbuckridge local municipalities. In Nkomazi, sampling sites were located at Vlakbult, Block A, Masibekela and Boschfontein (Fig. 4.2). In Bushbuckridge, sampling sites were located at Acornhoek, Saselani, Xanthia and Calcutta (Fig. 4.3). Maps were generated using Mapcreator© (*Mapcreator*, 2023).

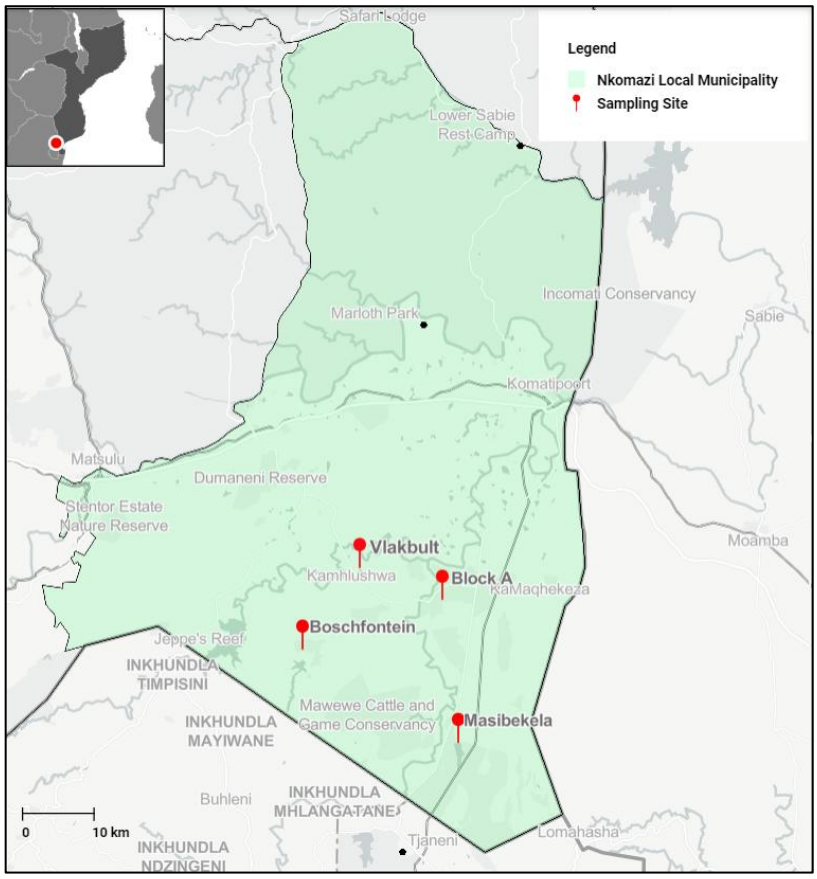


Figure 4.2. Sampling sites used for collection of water from larval breeding sites and *An. gambiae* mosquitoes in Nkomazi local Municipality, Mpumalanga, between December 2021 and March 2023.

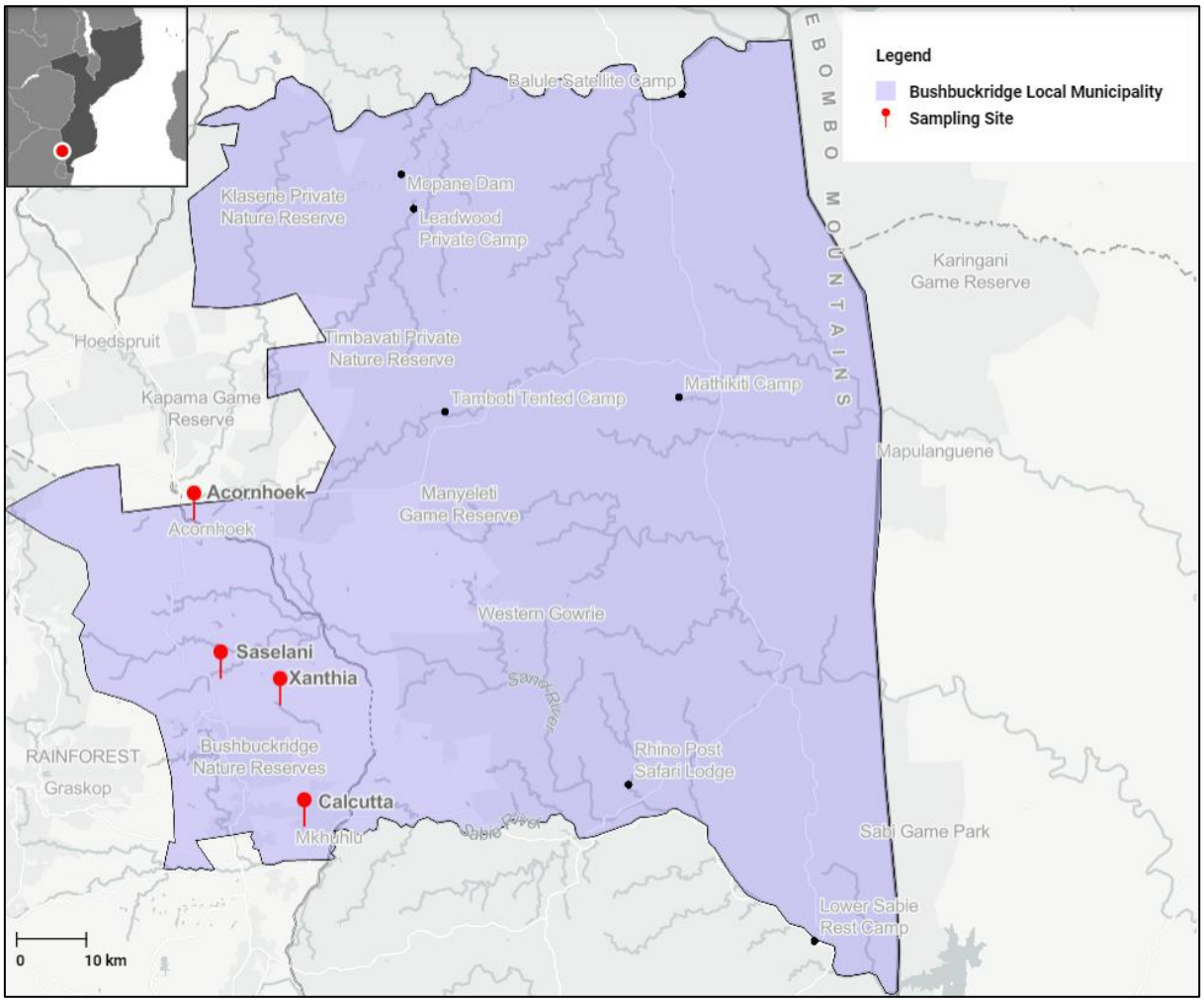


Figure 4.3. Sampling sites used for collection of water from larval breeding sites and *An. gambiae* mosquitoes in Bushbuckridge local Municipality, Mpumalanga, between December 2021 and March 2023.

4.2.2 Water chemical composition

Water samples of 350 ml were collected from the sampling sites at Vlakbult, Block A, Masibekela, Boschfontein and Calcutta between December 2021 and March 2023. The water was sampled in its natural state, with only macro debris removed in order to most accurately represent the breeding habitat composition. The sampling volume was based on volumes required for spectrophotometric water quality analyses. Samples were collected from sampling sites using 350 ml plastic bottles. Samples were stored at 2 - 4°C until ready for analysis.

The amount of pollutant present in each water sample was quantified using Macherey-Nagel NANOCOLOR® Tube Tests, as per manufacturer's instructions, and read using

a spectrophotometer (Macherey-Nagel NANOCOLOR® VIS, II serial number NVIS20305). The pollutants quantified and the details of the respective water quality tests used are detailed in Table 4.2. Each pollutant test was replicated three times for every water sample.

Table 4.2. Details of Macherey-Nagel water quality kits used for analysis of water samples collected at Nkomazi and Bushbuckridge sampling sites.

Test name	Product Number	Measuring range	Measuring unit	Validation
Manganese 10	985058	0.02 - 10.0	mg/L Mn	ISO 5725
Iron	91836	0.01 - 15.0	mg/L Fe	ISO 5725
Sulfate LR 200	985062	20.0 - 200.0	mg/L SO_4^{2-}	ISO 5725
Ortho- and total Phosphate 15	985080	0.30 - 15.0	mg/L P	ISO 5725
Sulfite 10	985089	0.05 - 10.0	mg/L SO_3^{2-}	ISO 5725
Nitrate 50	985064	0.3 - 22.0	mg/L NO_3^-N	ISO 23696-1, ISO 5725
Nitrite 2	985068	0.003 - 0.46	mg/L NO_2^-N	ISO 5725
Lead 5	985009	0.10 - 5.0	mg/L Pb^{2+}	ISO 5725
Cadmium 2	985014	0.05 - 2.0	mg/L Cd^{2+}	ISO 5725
Chloride 200	985019	5.0- 200.0	mg/L Cl^-	ISO 5725
Organic acids 3000	985050	30.0 - 3000.0	mg/L CH_3COOH	ISO 5725
COD 1500	985029	100.0 - 1500.0	mg/L O_2	ISO 15705, ISO 5725
Anionic surfactants 4	985032	0.2 - 4.0	mg/L MBAS*	ISO 5725
Cationic surfactants 4	985034	0.2 - 4.0	mg/L CTAB**	ISO 5725
Nonionic surfactants 15	985047	0.3 - 15.0	mg/L Triton®X-100	ISO 5725
Total-Nitrogen TNb 60	985092	3.0 - 60.0	mg/L N	ISO 23697-1, ISO 5725
Copper	91853	0.01 - 10.0	mg/L Cu^{2+}	ISO 5725

*MBAS = Methylene blue active substance
**CTAB= Hexadecyltrimethylammonium bromide

4.2.3 Bacterial composition

Water samples of 50 ml were collected from the sampling sites at Acornhoek, Calcutta, Vlakbult, Masibekela, Saselan, Block A and Boschfontein between September 2021 and March 2023. The sampling volume was based on volumes required for Matrix Assisted Laser Desorption Ionisation- Time of Flight (MALDI-TOF) mass spectrometry analyses. Samples were stored at 2 - 4°C until ready for analysis.

A total of 17 *An. gambiae s.l.* larvae were collected and used for analysis from the sampling sites at Masibekela (n = 12 larvae), Vlakbult (n = 3 larvae) and Block A (n = 2 larvae).

4.2.3.1 Breeding habitat water bacterial composition

A 1 ml sample of each water sample was added aseptically to 1 ml lysogeny broth in culturing tubes and incubated overnight at 37°C on a shaking platform at ~120 rotations

per minute. A negative control consisted of 0.1 M phosphate buffer saline (PBS) (pH 7.2).

After overnight incubation, the broth mixture was plated using the streak plating method to obtain single colonies (Sanders, 2012). The following media were used: chocolate agar, blood agar, brain heart infusion agar (BHI), tryptic soy agar (TSA), MacConkey agar and egg yolk agar (Diagnostic Media Products, South Africa). Chocolate, blood, and BHI agar were used for the isolation of fastidious and pathogenic bacteria, while TSA was chosen for its general non-selective properties. MacConkey agar was used for the selection of lactose-fermenting bacteria. Egg yolk agar was used for the isolation of general anaerobic bacteria. All plates were incubated overnight at 37°C, except the egg yolk plates. These plates were placed in an anaerobic chamber (Anaeropack™ Rectangular Jar 2.5L, Thermo Fischer Scientific) and used an oxygen absorbing/ carbon dioxide generating agent as a catalyst (AnaeroPack™-Anaero, Thermo Fisher Scientific). Egg yolk plates were incubated for 48 hours at 37°C.

Individual bacterial colonies were hand-selected from each plate based on differences in transparency, shape, colour, size and/ or growth pattern and applied in duplicate to a 96-well target plate (Bruker Daltonics: 8280784).

A working solution of 2.50 ± 0.30 mg α-Cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics: 8201344, CAS number 28166-41-8) dissolved in 250 µl standard solvent (50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid) (Sigma-Aldrich: 900666, CAS number 76-05-1) was prepared. One microlitre (1 µl) of the working solution was added to each occupied target plate position and left for 15 minutes to dry at room temperature.

Bacteria were identified by placing the target plate in the (MALDI) Biotyper® System, which has a benchtop Microflex™ LT/SH mass spectrometer (Bruker Daltonics). This system is located within the Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses (CHARM) at the NICD in Johannesburg, South Africa.

Bacterial identification relied on comparing 16s ribosomal ribonucleic acid (rRNA) spectra (maximum of 100 peaks used) with other peaks in the MBT 7854 MSP Library

(Bruker Daltonics, reference number 182903). Correct identification of bacteria was accepted if the identification score was ≥ 1.9, as per Seng *et al*. (2009).

4.2.3.2 Midgut bacterial composition

Larvae (n = 17) collected from the sampling sites at Block A, Vlakbult and Masibekela were cold-killed at -70°C for 3 - 5 minutes before dissection of the midguts. Strict sterile conditions were observed by sterilising all equipment with 70% ethanol and autoclaving at 121°C for 20 minutes at 15 psi.

Dissection of the midguts were done with an Olympus SZ61 dissection microscope using 40x magnification. Each midgut was suspended in 200 µl of 0.1M PBS (pH 7.2) and completely homogenised by mechanically grinding with a plastic pestle homogeniser. Each homogenised midgut was then added aseptically to 1 ml lysogeny broth in culturing tubes and incubated overnight at 37°C on a shaking platform (~120 rotations per minute). A negative control consisted of 1 ml PBS in 1 ml lysogeny broth (uninoculated).

After overnight incubation, the broth mixture was plated using the streak plating method to obtain single colonies (Sanders, 2012). Bacterial samples were identified through MALDI-TOF, as described for the water samples in section 4.2.3.1.

4.2.4 Resistance enzyme profiles

4.2.4.1 Mosquito collection

For the quantification of resistance enzymes present, *An. gambiae s.l.* larvae were collected from the sampling sites at Block A (n = 48), Xanthia (n = 36) and Boschfontein (n = 24). A further 24 *An. gambiae s.l.* larvae were collected at Block A and 12 larvae at Boschfontein and reared to adults. Samples were cold-killed and stored at -70° C until ready for analysis. The samples were killed at the age of three days and were not provided a blood meal. Only females were assayed.

4.2.4.2 Sample preparation

Larvae from each site were added in twos to a 96 well microplate and 50 µl PCR-grade water (without protease inhibitors) was added to each well. Samples were homogenised mechanically, and a further 250 µl PCR-grade water was added to each well.

Adults from each sampling site were placed individually into 1.5 ml reaction tubes and 300 µl PCR-grade water (without protease inhibitors) was added to each tube. Individual samples were homogenised using a Qiagen Tissue Lyser II (Whitehead Scientific/ Thermo Scientific, serial number 1213090746C) at 25 Hz for two minutes.

A laboratory strain of *An. arabiensis* (KGB) served as a positive control. Larvae and adults were prepared as above. This strain was used as it is a standard insecticide susceptible strain. PCR-grade water served as a negative control.

4.2.4.3 Protein quantification

To determine the quantity of protein present in each sample, the Bradford assay was performed using methodology outlined by Bradford (1976) as reference and applied to a microwell plate assay.

Bradford reagent (phosphoric acid/ Coomassie brilliant blue/ water; Bio-Rad: 5000205) and Bovine Serum Albumin (BSA) (Sigma-Aldrich: A7906, CAS number 9048-46-8) was used to calibrate a standard curve with the following concentrations: 10, 20, 30, 40, 50, 100, 200, 300, 400 and 500 µg/ml.

The optical density of the samples was determined using a Spectramax® ABS Plus 96-well plate reader (Molecular Devices) by reading absorbance at 595 nm. The amount of protein in mg/ml present in each sample was calculated automatically in SoftMax® Pro version 7.1.0 (Molecular Devices).

All subsequent enzyme quantities (Cytochrome P450, α- and β esterase and GST were calculated automatically in SoftMax® Pro version 7.1.0 (Molecular Devices).

4.2.4.4 Cytochrome P450 assay

The Cytochrome P450 assay was performed using methodology outlined by Brogdon, McAllister and Vulule (1997) and Penilla *et al*. (1998) as a reference, where haeme peroxidase activity is used as a measure of Cytochrome P450 activity.

A 0.01 M solution of cytochrome-C (Sigma Aldrich: C2867, CAS number 9007-43-6) was used to calibrate a standard curve with the following concentrations: 0, 0.25, 0.5, 1, 2, and 4 M.

A final concentration of 1.38 x 10^{-8} M 3,3′5,5′ tetramethyl benzidine (Sigma Aldrich: T2885, CAS number 54827-17-7) dissolved in a solution consisting of 24 ml methanol and 72 ml sodium acetate (pH 5.0) was added to 20 µl of each homogenised sample. To this solution, 80 µl of 0.0625 M potassium phosphate (pH 7.2) was added and the reaction was allowed to proceed. Finally, a final concentration of 0.70 M 3.0% hydrogen peroxide (Sigma Aldrich: 88597, CAS number 7722-84-1) was used to stabilise the reaction, which was allowed to proceed for two (2) hours at room temperature.

The optical density of the samples was determined by reading absorbance at 650 nm. The concentrations of cytochrome-C equivalent were determined by calibration to a standard curve. An R^2-value of 0.95 or more on the standard curve was required to calibrate the assay results.

4.2.4.5 Alpha- (α) and beta (β) esterase assay

The esterase assay was performed using methodology outlined by Penilla *et al*. (1998).

A 0.01 M solution of α-naphthol (Sigma: N1000, CAS number 90-15-3) was used to establish a standard curve with the following concentrations 0, 2.5, 5, 10, 20 and 40 mM. The same was done with a solution of β-naphthol (Sigma: 185507, CAS number 135-19-3). The solution was stained with 50 µl of the staining solution described below.

A solution of α-naphthyl acetate (Sigma: N8505, CAS number 830-81-9) with a final concentration of 22 mM in 120 µl sodium phosphate buffer (pH 7.2) was added to 20 µl of each homogenised sample. This was repeated using a solution of β-naphthyl acetate (Sigma: N6875, CAS number 1523-11-1) instead of α-naphthyl acetate.

The solution was allowed to react on ice for 30 minutes before adding a staining solution consisting of $7.18x10^{-7}$ M Fast Blue RR salt (Sigma: F0500, CAS number 14726-29-5) dissolved in a solution with a final concentration of 0.1 M sodium phosphate buffer (pH 7.0). The staining reaction was allowed to proceed for five (5) minutes at room temperature. The reaction was terminated by the addition of 50 µl of 0.1 M sodium phosphate (pH 7.0) supplemented with 5.0% (w/v) sodium dodecyl sulfate (Sigma: 436143, CAS number 151-21-3). The optical density of the samples was determined by reading absorbance at 570 nm.

The concentrations of α- or β naphthol were determined by calibration to a standard curve. An R^2-value of 0.95 or more on the standard curve was required to calibrate the assay results.

4.2.4.6 Glutathione S-transferase assay

The GST assay was performed using methodology outlined by Habig, Pabst and Jakoby (1974) and Penilla *et al*. (1998) as a reference.

A working solution of 1.56 x 10^{-8} M 1-Chloro-2,4-dinitrobenzene (CDNB) (Sigma: 237329, CAS number 97-00-7) dissolved in 1.2 ml methanol was added to a solution of 4.92 x 10^{-8} M reduced glutathione (Sigma: G4251, CAS number 70-18-8) in 0.25 M sodium phosphate buffer (pH 6.5). This solution was added to 10 µl of each homogenised sample.

The optical density of the samples was determined by monitoring the rate of CDNB conjugation kinetically at 340 nm for five (5) minutes, as described by (Brogdon and Barber, 1990). GST activity was calculated as the rate of CDNB conjugation over five (5) minutes (Equation. 4.1). The assay results were only accepted if the GST conjugation was in the linear range. The enzyme activity was only considered successful if the amount of product formed exceeded that of the uncatalyzed reactions of the blank wells in the microplate.

Equation 4.1. Calculation of GST-activity.

$$\frac{\Delta A340\ min^{-1} \times 0.2\ ml\ Reaction\ Volume}{0.0096\ \mu mol^{-1}cm^{-1} \times 1000\ ml \times 0.524\ cm \times A}$$

$$= 0.0396 \times A340\ min^{-1} \times Sample\ Dilution / A\ (\mu mol/min/mL)$$

Where:
$0.0096\ \mu M^{-1}cm^{-1}$ = 1-S-glutathionyl-2,4-dinitrobenzene (GS-DNB) extinction coefficient
A = sample volume in reaction well (ml)
0.524 cm = light path of the 0.2 ml Reaction Volume in a 96 well plate (cm)

4.2.5 Insecticide susceptibility bioassays

A total of 326 *An. gambiae s.l.* larvae were collected from the sampling sites at Vlakbult (n = 149) and Block A (n = 177) and reared to adults under standard insectary conditions (see Chapter 2 General Materials and Methods: Rearing protocol). Three-day old non-bloodfed adults were collected for insecticide bioassays following the standard WHO bioassay protocol (World Health Organization, 2016).

For Vlakbult, 75 adults were exposed to 4% DDT and 59 adults were exposed to 0.05% deltamethrin. Fifteen (15) adults were used as a pyrethroid control. For Block A, 68 adults were exposed to 4% DDT and 71 adults were exposed to 0.05% deltamethrin, A pyrethroid solvent control consisted of 38 adults. An environmental control consisted of unexposed adult mosquitoes from a susceptible laboratory strain of *An. arabiensis* (KGB) (n = 20).

Adult mosquitoes were collected after exposures and processed through PCR for molecular species identification, as per Scott, Brogdon and Collins (1993).

4.2.6 Data analysis

Pollutant quantities in the water samples were averaged per site. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was conducted to determine whether the data were normally distributed. A Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to test for

differences in pollutant quantities between sites, and a post-hoc Dunn test (Dunn, 1964) was used to determine differences in pairs where statistical differences in groups were observed. A standard value of maximum allowable quantities for freshwater ecosystems was obtained from literature as a reference value for degree of pollution for each pollutant tested. References for each pollutant is available in the Results section below.

A qualitative list containing each species of bacteria isolated from the water samples was tabulated for each sampling site and an UpSet plot was generated in R Studio (RStudio Team, 2023). The same was done for bacterial species isolated from the midguts of *An. gambiae s.l.* larvae and adults.

Enzyme levels of *An. gambiae s.l.* larvae and adults were averaged per site. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was conducted to determine whether the data were normally distributed. A Kruskal-Wallis test (Kruskal and Wallis, 1952) was used to test for differences in enzyme levels between sites, and a post-hoc Dunn test was used to determine where the differences were observed. Mean cytochrome P450, α- and β-esterase and GST levels were obtained and compared to a reference mean for an insecticide susceptible laboratory strain (KGB). Values lower than the mean values of KGB suggested a lack of increased detoxification activity.

The number of adult *An. gambiae s.l.* mosquitoes used for the insecticide bioassays were recorded for each insecticide per site by sex.

4.3 Results

4.3.1 Chemical composition of water bodies

4.3.1.1 Heavy metals

All sites were substantially polluted with heavy metals (Fig 4.4). The South African water quality guideline for aquatic ecosystems for manganese is ≤ 370.00 µg/L, ≤ 3.00 µg/L for cadmium, ≤ 0.50 µg/L for lead, ≤ 10.00 µg/L for iron and ≤ 5.00 µg/L for copper (Department of Water Affairs and Forestry, 1996). Calcutta, Masibekela, and Boschfontein contained iron levels which exceeded the detection limit of the spectrophotometer. This indicates that these sites had at least 2.00 mg/ml of iron at minimum. Similarly, copper levels at Masibekela were above the instrument's detection range, confirming a minimum amount of 2.00 mg/ml copper.

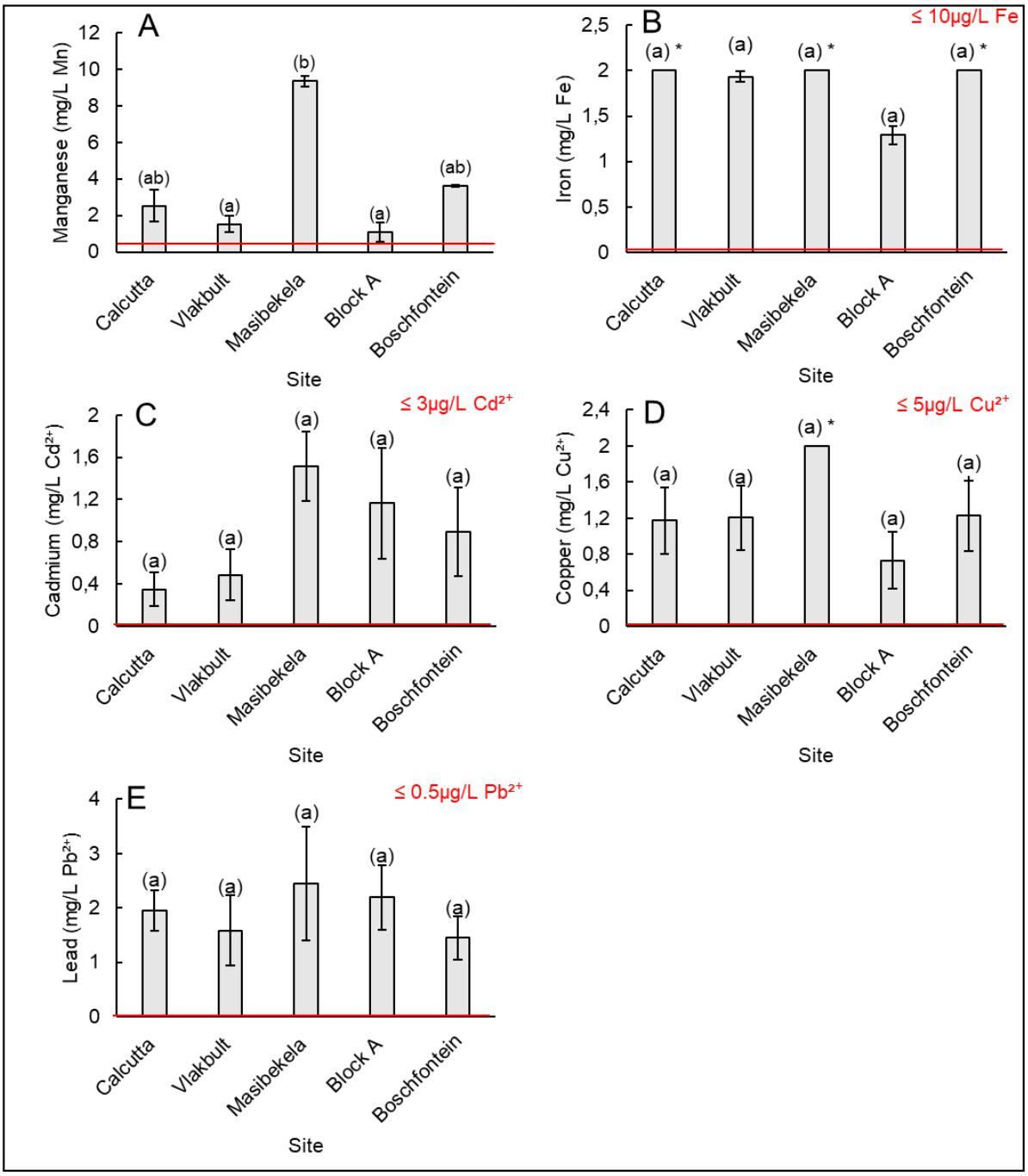


Figure 4.4. Concentration of heavy metals in mg/ml present in water samples from *An. gambiae s.l.* larval breeding sites in Ehlanzeni.

A) manganese, B) iron, C) cadmium, D) copper, and E) lead. Letters indicate significant differences between sites. Asterisks (*) indicate a site had a pollutant present higher than the detectable amount, and the value therefore represents the minimum amount of pollutant present for the site. The red line and red text indicate the maximum allowable amount of each respective pollutant for freshwater ecosystems/ invertebrates. Error bars represent the standard error of the mean.

4.3.1.2 Agri-pollutants

Water samples from Calcutta and Boschfontein had sulfate levels below the detectable amount (Fig. 4.5 A). Samples from Vlakbult and Block A had sulfate levels below the 128.00 mg/L SO_4^{2-} limit for freshwater invertebrates, as recommended by Elphick *et al*. (2011), while water samples from the site at Masibekela were above this level (Fig. 4.5 A).

All sampling sites had low levels of sulfite present and were well below the maximum amount of 173.20 mg/L SO_3^{2-} for freshwater invertebrates, as recommended by Dowden and Bennett (1965) (Fig. 4.5 B).

Substantial phosphate contamination was present in samples from Calcutta, Masibekela and Boschfontein (Fig. 4.5 C). While there is no limit set for aquatic ecosystems in South Africa, the standard limit for phosphates in sewage effluent is 1.00 mg/L (Government Gazette, 1984).

Nitrate contamination was present at all sites. Samples from Calcutta, Masibekela and Boschfontein had nitrate levels above the recommended level for freshwater invertebrates (2.00 mg/L NO_3^-N), per Camargo, Alonso and Salamanca (2005) (Fig. 4.5 D). Nitrate contamination at Vlakbult and Block A were below this recommended level (Fig 4.5 D).

Nitrite contamination was present in water samples from all sites, however nitrite levels were all below the recommended level of 1.00 – 1.50 mg/L for freshwater invertebrates (specifically stoneflies), as per Soucek and Dickinson (2012) (Fig. 4.5 E).

Water samples from all sites had substantial nitrogen contamination, with levels above the 10.00 mg/L recommended level for aquatic ecosystems in South Africa (Department of Water Affairs and Forestry, 1996) (Fig. 4.5 F).

Water samples from Calcutta, Vlakbult and Boschfontein had relatively low levels of chloride contamination (Fig. 4.5 G), however this was still more than the recommended levels for freshwater invertebrates in soft water (64.00 mg/L), as per Elphick, Bergh and Bailey (2011). Chloride levels at Masibekela and Block A were above the

detectable amount, therefore these two sites had a minimum amount of 200.00 mg/L chloride (Fig. 4.5 G).

Organic acids, measured as acetic acid (CH_3COOH), were present in water samples from all sites (Fig. 4.5 H). Water samples from the sites at Calcutta, Vlakbult, Block A and Boschfontein were well below the recommended level of 1500.00 mg/L for *Culex* larvae, as per (Dowden and Bennett, 1965) (Fig. 4.5 H). The site at Masibekela, however, had substantially elevated levels of acetic acid (2 417.00 mg/L) (Fig. 4.5 H).

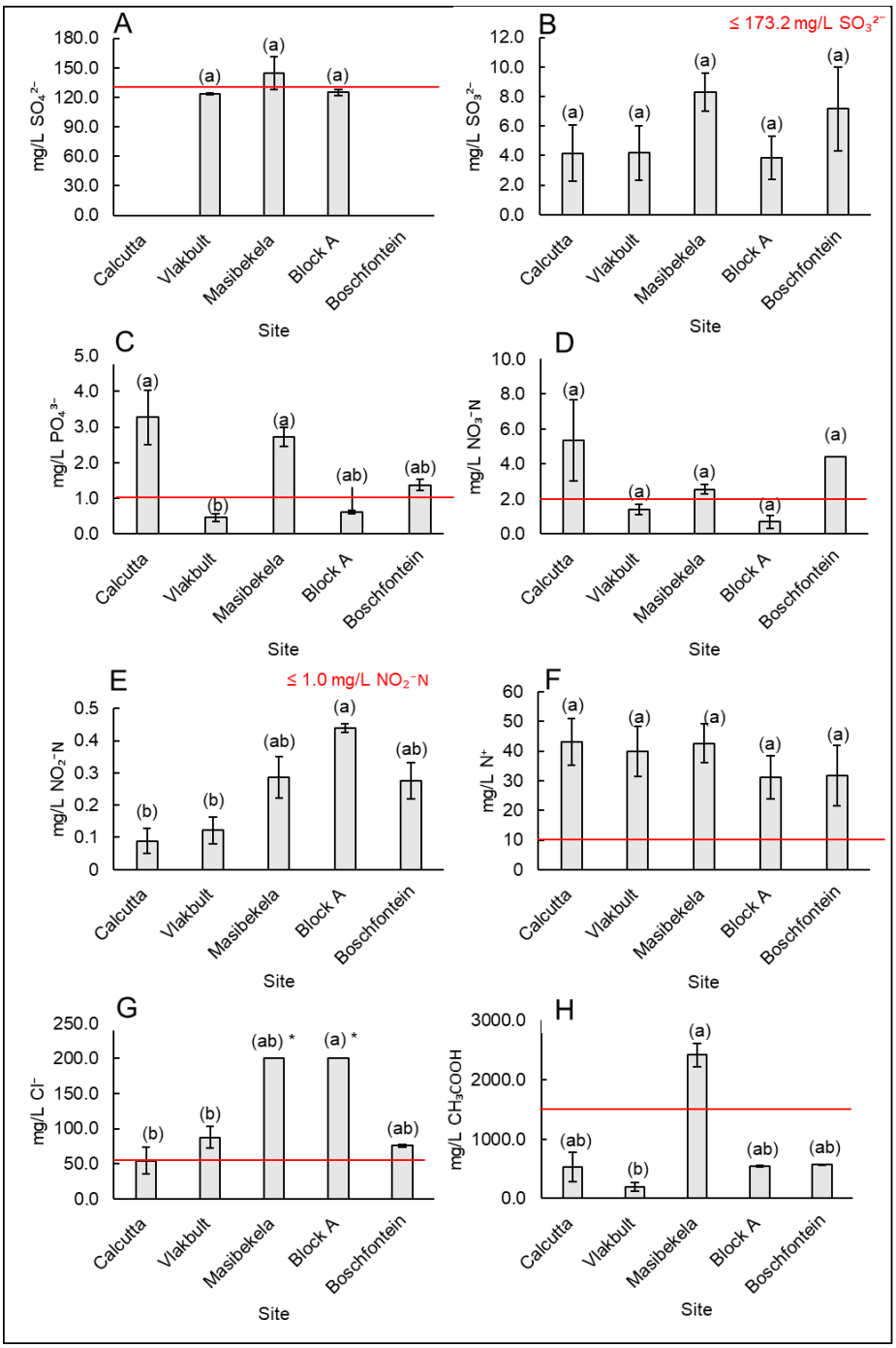


Figure 4.5. Concentration of agricultural pollutants in mg/ml present in water samples from *Anopheles gambiae s.l.* larval breeding sites in Ehlanzeni, Mpumalanga.

A) sulfate, B) sulfite, C) phosphate, D) nitrate, E) nitrite, F) nitrogen, G) chloride and H) organic acids measured as acetic acid. Letters indicate significant differences between sites. Asterisks (*) indicate a site had a pollutant present higher than the detectable amount, and the value therefore represents the minimum amount of pollutant present for the site. The red line or red text indicates the maximum allowable amount of each respective pollutant for freshwater ecosystems/invertebrates. Error bars represent the standard error of the mean.

4.3.1.3 Surfactants

Surfactants were present in water samples from all sites (Fig. 4.6). All sites, except Block A, had substantial anionic surfactant contamination, above the recommended level of 1.80 mg/L for aquatic invertebrates, as per (Lewis and Suprenant, 1983) (Fig. 4.6 A). The sites at Masibekela and Boschfontein had anionic surfactant levels above the detectable amount, therefore for these sites a minimum of 4.00 mg/L was present (Fig. 4.6 A). Substantial contamination with cationic surfactants were present in water samples from all sites, above the recommended level of 0.025 mg/L for freshwater invertebrates, as per (Lewis and Suprenant, 1983) (Fig. 4.6 B). Contamination with nonionic surfactants was present at all sites, but especially the site at Boschfontein (Fig. 4.6 C). All sites had nonionic surfactant levels above the recommended level for freshwater invertebrates (0.29 mg/L), as per (Lewis and Suprenant, 1983).

The chemical oxygen demand for all sites except Boschfontein were above 200.00 mg/L, indicating substantial pollution of the water bodies (Chapman, 1996) (Fig. 4.6 D). The site at Block A was particularly polluted (981.67 mg/L O_2).

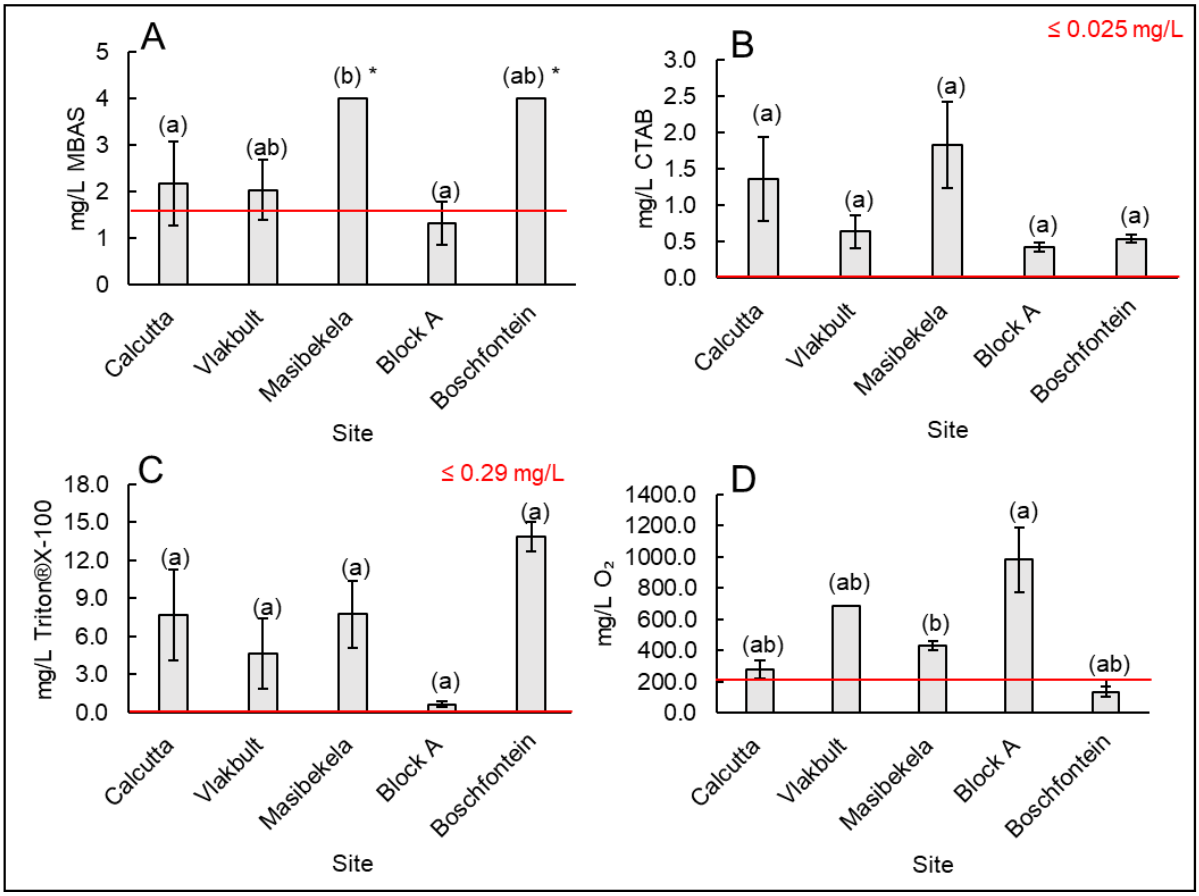


Figure 4.6. Concentration of surfactants and chemical oxygen demand in mg/ml present in water samples from *An. gambiae s.l.* larval breeding sites in Ehlanzeni, Mpumalanga.

A) anionic surfactants, B) cationic surfactants, C) non-ionic surfactants, D) chemical oxygen demand. Letters indicate significant differences between sites. Asterisks (*) indicate a site had a pollutant present higher than the detectable amount, and the value therefore represents the minimum amount of pollutant present for the site. The red line or red text indicates the maximum allowable amount of each respective pollutant for freshwater ecosystems/invertebrates. Error bars represent the standard error of the mean.

4.3.2 Bacterial composition

4.3.2.1 Larval habitats

A total of 21 different species of culturable bacteria were isolated from larval habitats in Vlakbult, Calcutta, Masibekela, Acornhoek, Saselan, Boschfontein and Block A. Vlakbult had the highest species richness (n = 15), followed by Calcutta (n = 9),

Masibekela (n = 9), Acornhoek (n = 9) , Saselan (n = 5), Boschfontein (n = 3) and Block A (n = 3) (Fig. 4.7).

Pseudomonas and *Aeromonas* spp. were relatively ubiquitous, occurring in all habitats sampled. *Bacillus* spp. were present at all sites, except Boschfontein. *Lysinibacillus* spp. were the next most ubiquitous, present at Acornhoek, Calcutta, Vlakbult and Masibekela. *Paraclostridium*, *Acinetobacter*, *Exiguobacterium* and *Enterobacter* spp. were only present at three of the seven larval sampling sites. *Vibrio*, *Enterococcus*, *Klebsiella* and *Plesiomonas* spp. only occurred at two sites. The following species were rare and only occurred at one site: *Comomonas*, *Clostridium*, *Citrobacter*, *Alishewanella*, *Alcaligenes*, *Stenotrophomonas*, *Providencia*, *Morganella* and *Escherichia*.

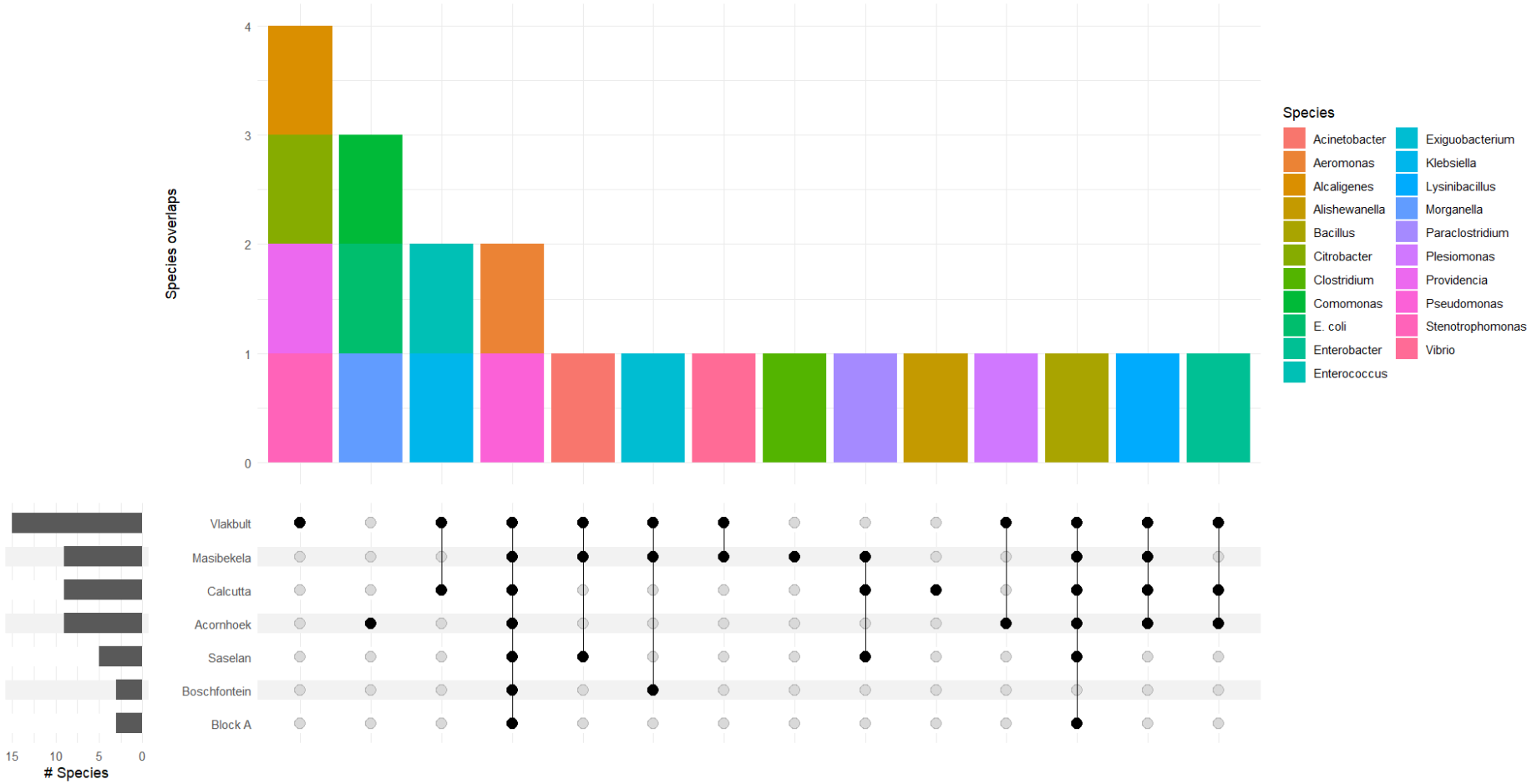


Figure 4.7. UpSet plot showing the overlap of 21 bacterial species isolated from water samples from *An. gambiae s.l.* larval breeding sites for each sampling site in Nkomazi and Bushbuckridge local municipalities.

Black dots with a connecting line represent the number of overlapping bacteria between sites, where single dots (without connecting line) represent species unique to a particular site.

4.3.2.2 Midguts

A total of 16 different bacterial species were isolated from *An. gambiae s.l.* larvae collected from Vlakbult, Masibekela and Block A. Masibekela had the highest species richness (n = 15), while Vlakbult and Block A had the lowest species richness (n = 4) (Fig. 4.8).

Bacillus was the most ubiquitous species, occurring at all three sites. *Exiguobacterium*, *Aeromonas*, *Staphylococcus*, and *Serratia* spp. only occurred at Block A and Vlakbult, while *Acinetobacter* only occurred at Block A and Masibekela. Other species such as *Paraclostridium*, *Micrococcus*, *Lysinibacillus*, *Klebsiella*, *Vibrio*, *Streptococcus*, *Shewanella*, *Pseudomonas* and *Enterobacter* were rare and only occurred at one site.

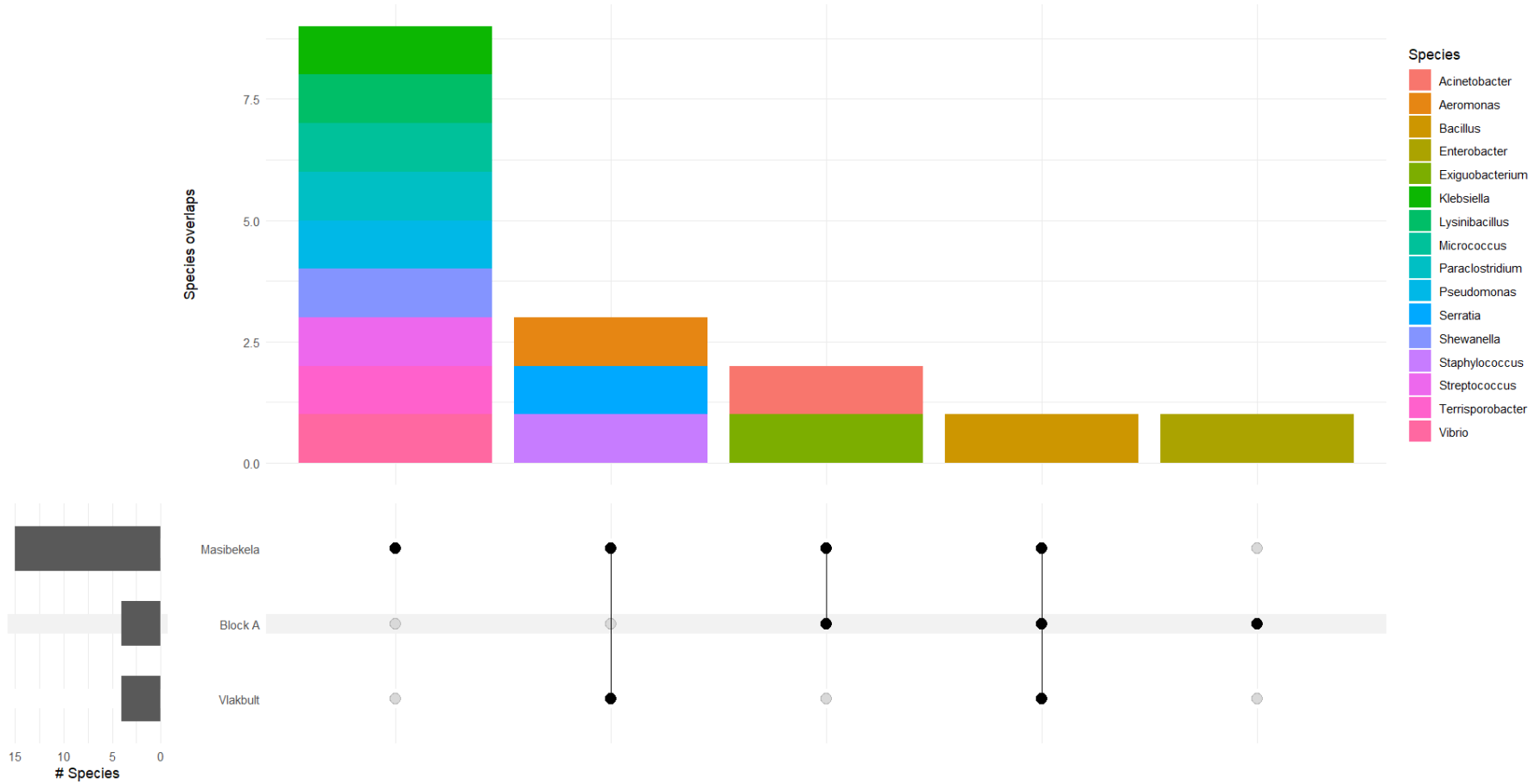


Figure 4.8. UpSet plot showing the overlap of 16 bacterial species isolated from *An. gambiae s.l.* larval midguts from sampling sites in Nkomazi and Bushbuckridge local municipalities.

Black dots with a connecting line represent the number of overlapping bacteria between sites, where single dots (without connecting line) represent species unique to a particular site.

4.3.2.3 Overlap of bacteria between larval habitats and larval midguts

Paraclostridium, *Vibrio*, *Lysinibacillus*, *Exiguobacterium*, *Klebsiella*, *Bacillus*, *Pseudomonas*, *Aeromonas*, *Acinetobacter* and *Enterobacter* spp. occurred in both the larval aquatic environment and within the larval midguts (Fig. 4.9).

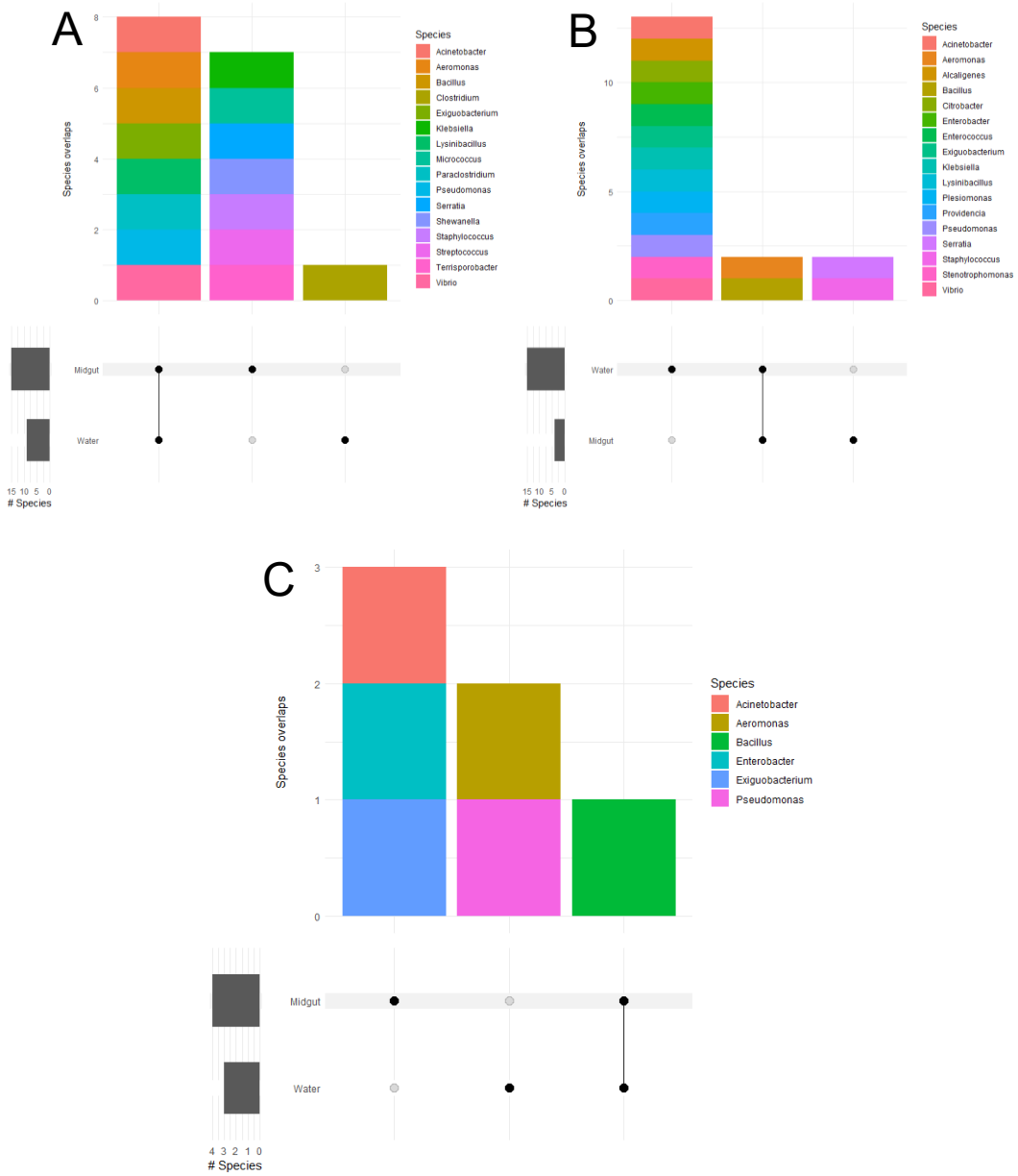


Figure 4.9. Overlap of bacterial species present in both the larval breeding sites and larval midguts from sampling sites in Nkomazi and Bushbuckridge local municipalities. A) Masibekela, B) Vlakbult, C) Block A. Black dots with a connecting line represent the number of overlapping bacteria between sites, where single dots (without connecting line) represent species unique to a particular site.

4.3.3 Metabolic detoxification enzyme profiles

4.3.3.1 Cytochrome P450

Larvae

Anopheles gambiae s.l. larvae collected from the sampling sites at Block A, Boschfontein and Xanthia had a 3.84, 2.48 and 3.38-fold increase, respectively, of cytochrome P450 equivalent, compared to an insecticide-susceptible laboratory strain of *An. arabiensis* (0.0040 mg/ml) (KGB) (Fig. 4.10). The data were not normally distributed (W = 0.96, p = 0.0027). There was a significant difference in mean cytochrome-C equivalent in larvae between sites (χ^2(2, n = 108) = 13.15, p = 0.0014). Larvae from Block A had significantly higher cytochrome P450 equivalent levels than those from Boschfontein (p = 0.00097), whilst no significant differences were observed between Block A and Xanthia (p = 0.22) or Xanthia and Boschfontein (p = 0.17).

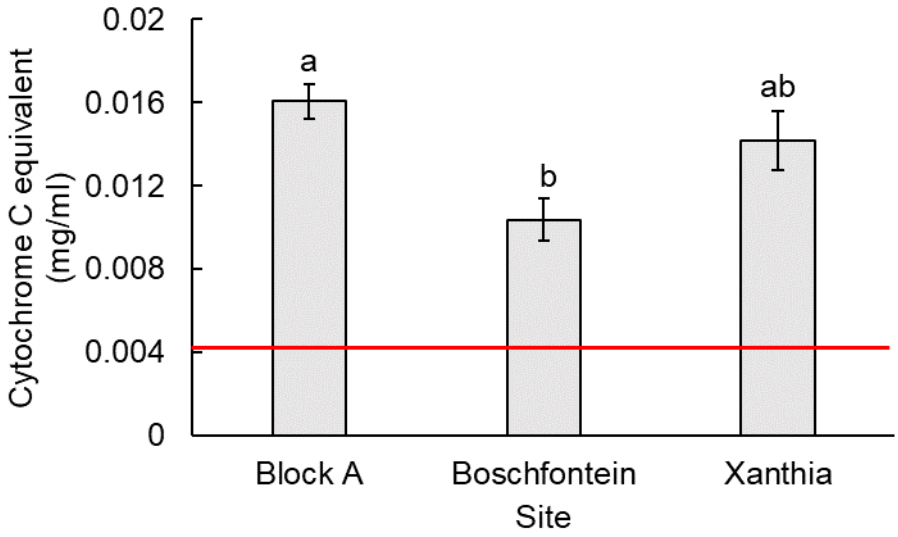


Figure 4.10. Mean cytochrome-C equivalents in mg/ml for *An. gambiae s.l.* larvae collected from Block A, Boschfontein and Xanthia.
Letters indicate significant differences between sites. The red line represents the mean cytochrome-C equivalent in mg/ml for larvae of an insecticide-susceptible laboratory strain (KGB). Error bars represent the standard error of the mean.

Adults

Adult *An. gambiae s.l.* collected from the sampling sites at Block A and Boschfontein had a 0.32 and 0.18-fold decrease, respectively, of cytochrome P450 equivalent, compared to a susceptible laboratory strain (0.0016 mg/ml) (KGB) (Fig. 4.11). The data were not normally distributed (W = 0.96, p = 0.030). There was a significant difference in mean cytochrome-C equivalent in adults between sites (χ^2(1, n = 72) = 16.30, p = 5.40 x 10^{-5}). Adults from Block A had significantly higher cytochrome P450 equivalent levels than those from Boschfontein (p = 5.40 x 10^{-5}).

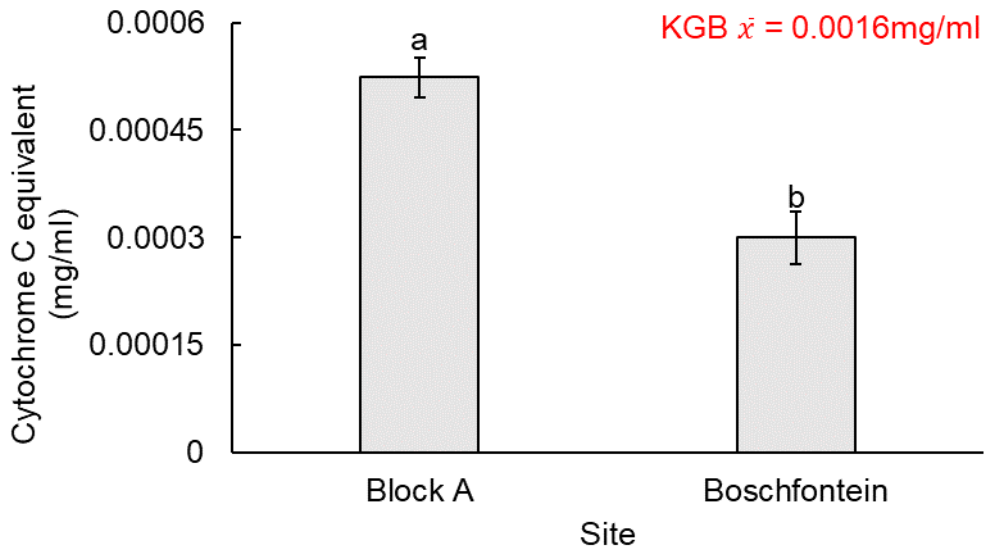


Figure 4.11. Mean cytochrome-C equivalent in mg/ml for *An. gambiae s.l.* adults collected from Block A and Boschfontein.
Letters indicate significant differences between sites. The red text indicates the mean cytochrome-C equivalent in mg/ml for adults of an insecticide-susceptible laboratory strain (KGB). Error bars represent the standard error of the mean.

4.3.3.2 Alpha esterases

Larvae

Anopheles gambiae s.l. larvae collected from the sampling site at Block A had a 1.11-fold increase of α-esterase activity, compared to a susceptible laboratory strain

(0.0038 mg/ml) (KGB) (Fig. 4.12). Larvae from the Boschfontein and Xanthia sampling sites had a 0.85 and 0.15-fold decrease, respectively, of α-esterase activity, compared to the susceptible laboratory strain (Fig. 4.12). The data were not normally distributed (W = 0.90, p = 0.00024). There was a significant difference in mean α-esterase activity in larvae between sites (χ^2(2, n = 54) = 16.68, p = 0.00024). Larvae from Block A had significantly higher α-esterase activity levels than those from Boschfontein (p = 0.029) and Xanthia (p = 0.00029), whilst no significant differences were observed between Boschfontein and Xanthia (p = 1.00).

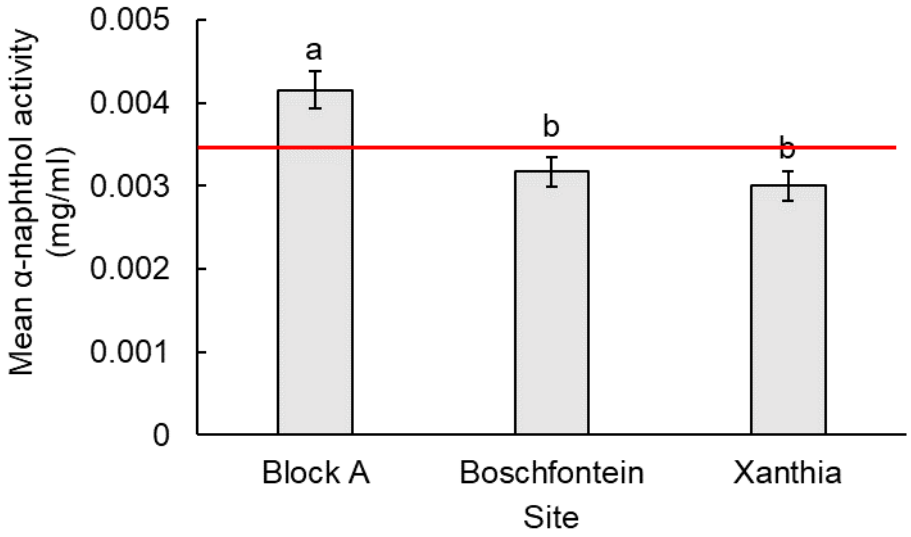


Figure 4.12. Mean α-esterase activity in mg/ml for *An. gambiae s.l.* larvae collected from Block A, Boschfontein and Xanthia.
Letters indicate significant differences between sites. The red line represents the mean α-esterase activity in mg/ml for larvae of an insecticide-susceptible laboratory strain (KGB). Error bars represent the standard error of the mean.

Adults

The α-esterase activity of *An. gambiae s.l.* adults collected from Block A and Boschfontein had a 0.080 and 0.15-fold decrease, respectively, compared to a susceptible laboratory strain (0.0023 mg/ml) (KGB) (Fig. 4.13). The data were not normally distributed (W = 0.71, p = 1.07 x 10^{-10}). There was a significant difference in mean α-esterase activity in adults between sites (χ^2(1, n = 72) = 3.98, p = 0.046).

Adults from Boschfontein had significantly higher α-esterase activity levels compared to those from Block A (p = 0.046).

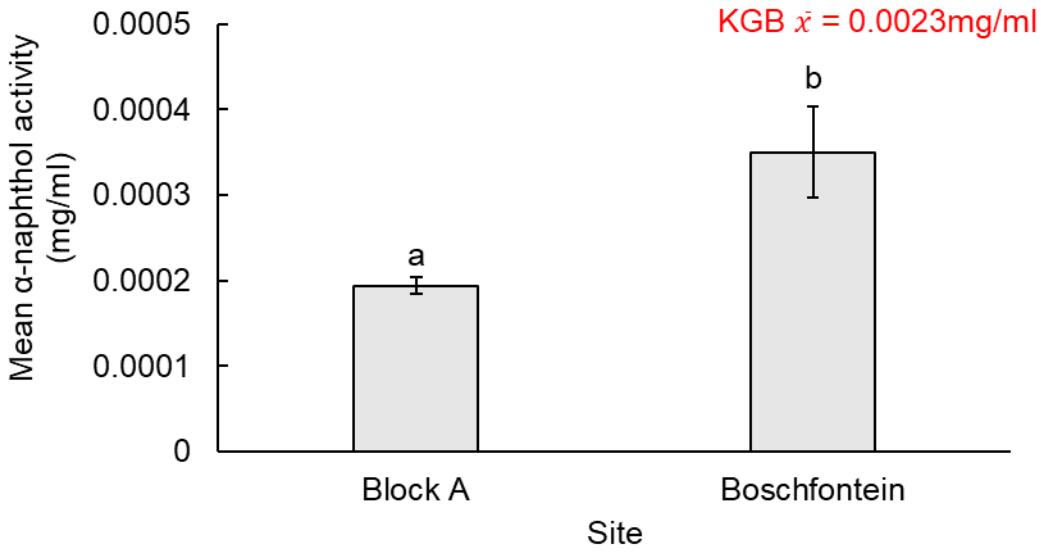


Figure 4.13. Mean α- esterase activity in mg/ml for *An. gambiae s.l.* adults collected from Block A and Boschfontein.
Letters indicate significant differences between sites. The mean α-esterase activity in mg/ml for adults of an insecticide-susceptible laboratory strain (KGB) is indicated in red text. Error bars represent the standard error of the mean.

4.3.3.3 Beta esterases

Larvae

Anopheles gambiae s.l. larvae collected from the sampling sites at Block A, Boschfontein and Xanthia had a 0.70, 0.66 and 0.82-fold decrease**,** respectively, of β-esterase activity, compared to a susceptible laboratory strain (0.012 mg/ml) (KGB) (Fig. 4.14). The data were not normally distributed (W = 0.73, p = 1.13 x 10^{-8}). No significant differences were observed in β-esterase activity of larvae between sites (χ^2(2, n = 54) = 0.80, p = 0.67).

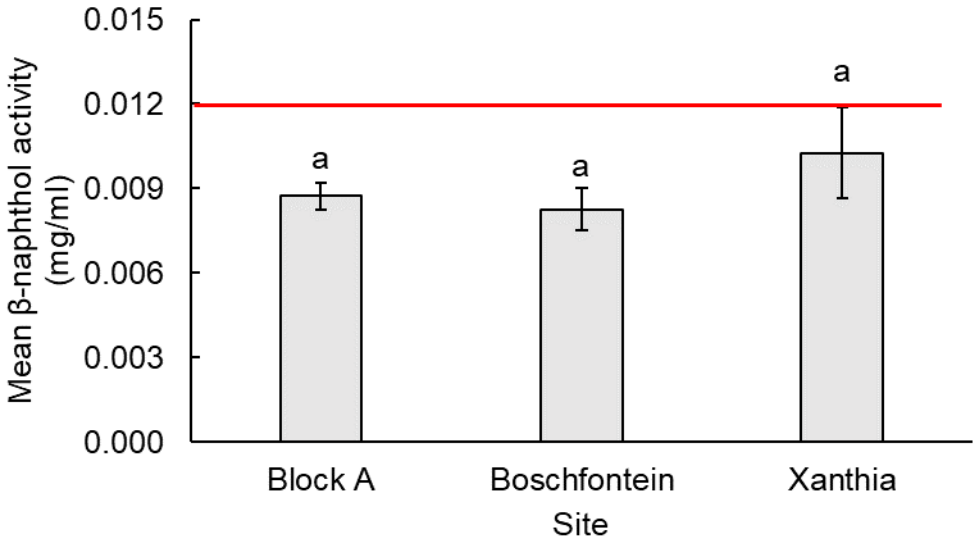


Figure 4.14. Mean β-esterase activity in mg/ml for *An. gambiae s.l.* larvae collected from Block A, Boschfontein and Xanthia.
Letters indicate significant differences between sites. The red line indicates the mean β-esterase activity in mg/ml for larvae of an insecticide-susceptible laboratory strain (KGB). Error bars represent the standard error of the mean.

Adults

Anopheles gambiae s.l. adults collected from Block A and Boschfontein had a 0.22 and 0.32-fold decrease, respectively, of β-esterase lowered compared to a susceptible laboratory strain (0.0049 mg/ml) (KGB) (Fig. 4.15). The data were not normally distributed ($W = 0.77$, $p = 3.26 \times 10^{-9}$). There was no significant difference in β-esterase activity of adults between sites ($\chi^2(1, n = 72) = 3.04$, $p = 0.081$).

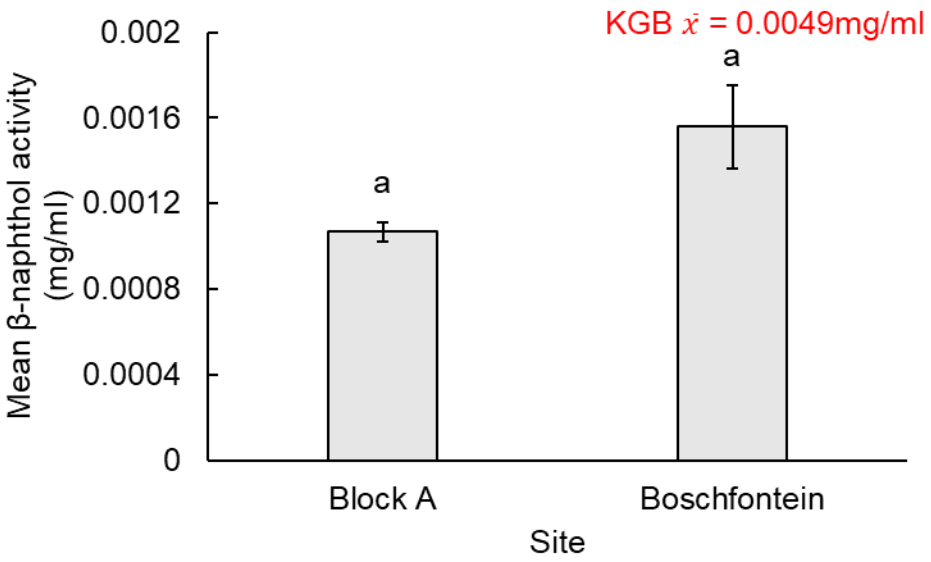


Figure 4.15. Mean β- esterase activity in mg/ml for *An. gambiae s.l.* adults collected from Block A and Boschfontein.
Letters indicate significant differences between sites. The mean β-esterase activity in mg/ml for adults of an insecticide-susceptible laboratory strain (KGB) is indicated in red text. Error bars indicate the standard error of the mean.

4.3.3.4 Glutathione-s-transferase

Larvae

Anopheles gambiae s.l. larvae collected from the sampling sites at Block A, Boschfontein and Xanthia had a 0.0058, 0.0044 and 0.0059-fold decrease, respectively, of GST activity, compared to a susceptible laboratory strain (4.13 x 10^{-6} mg/ml/min) (KGB) (Fig. 4.16). The data were not normally distributed (W = 0.88, p = 6.14 x 10^{-8}). No significant differences were observed in GST activity of larvae between sites (χ^2(2, n = 108) = 3.18, p = 0.20).

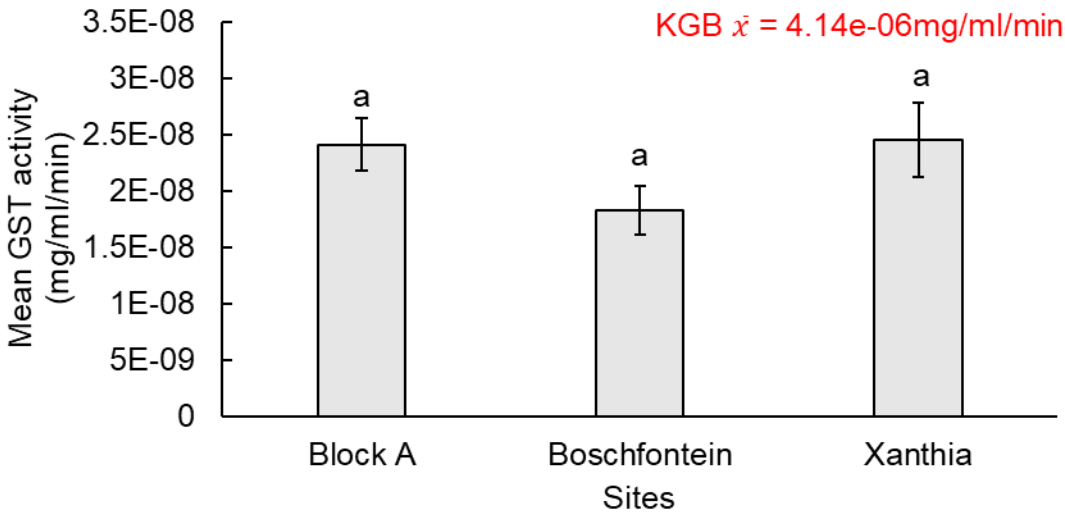


Figure 4.16. Mean GST activity measured in CNDB conjugation (mg/ml/min) for *An. gambiae s.l.* larvae collected from Block A, Boschfontein and Xanthia.
Letters indicate significant differences between sites. The mean GST activity for adults of an insecticide-susceptible laboratory strain (KGB) is indicated in red text. Error bars represent the standard error of the mean.

Adults

GST activity levels of *An. gambiae s.l.* adults collected from Block A and Boschfontein had a 0.0011 and 0.0015-fold decrease, respectively, compared to a susceptible laboratory strain (2.82 x10^{-6} mg/ml/min) (KGB) (Fig. 4.17). The data were not normally distributed (W = 0.93, p = 0.00087). There was no significant difference in GST activity levels between Boschfontein and Block A (χ^2(1, n = 72) = 2.88, p = 0.090).

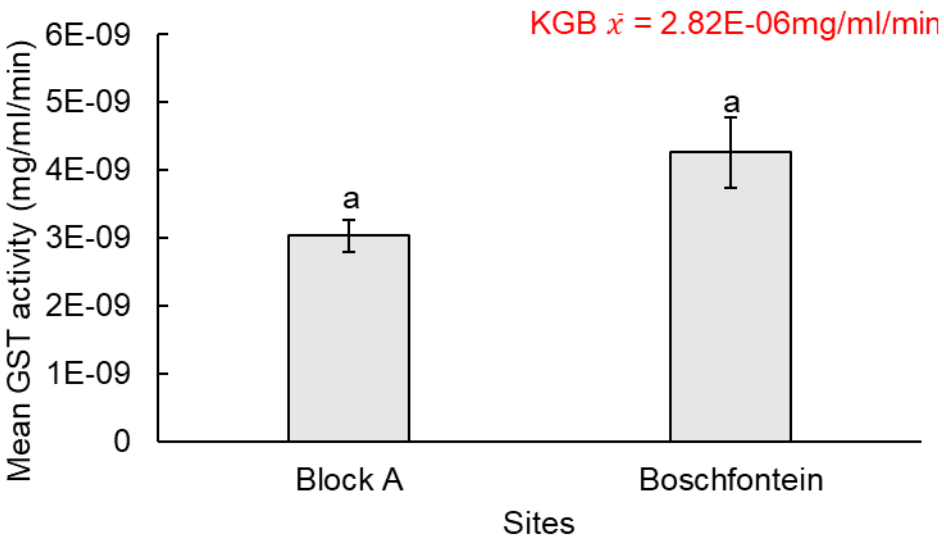


Figure 4.17. Mean GST measured in CNDB conjugation (mg/ml/min) for *An. gambiae s.l.* larvae collected from Block A, Boschfontein and Xanthia.
Letters indicate significant differences between sites. The mean GST activity levels in mg/ml/min for adults of an insecticide-susceptible laboratory strain (KGB) is indicated in red text. Error bars represent the standard error of the mean.

4.3.4 Insecticide susceptibility assays

4.3.4.1 Insecticide bioassays

A total of 326 *An. gambiae s.l.* adult mosquitoes collected from sampling sites at Block A and Vlakbult were exposed to deltamethrin (n = 114) and DDT (n = 120) (Table 4.3). From Vlakbult, 149 mosquitoes were collected and 177 mosquitoes were collected from Block A. For both insecticides, 100% mortality was observed after a one (1) hour exposure period to either insecticide. An insecticide susceptible strain (KGB) served as an environmental control (n = 20), and a total of 48 mosquitoes from either sampling site were exposed to a pyrethroid control (Table 4.3).

Table 4.3. Number of *An. gambiae s.l.* males and females exposed to deltamethrin or DDT from Vlakbult and Block A and percentage mortality of each treatment.

Area	Sex	Treatment	# Exposed	% Mortality
Vlakbult	♀	Deltamethrin	27	100
Vlakbult	♂	Deltamethrin	32	100
Vlakbult	♀	DDT	56	100
Vlakbult	♂	DDT	19	100
Block A	♀	Deltamethrin	55	100
Block A	♂	Deltamethrin	16	100
Block A	♀	DDT	36	100
Block A	♂	DDT	32	100
Vlakbult	♀/♂	PY Control	15	0
Block A	♂	PY Control	38	0
KGB	♀	Environmental Control	20	0

4.3.4.2 Species identification

Post-assay identification of samples using PCR showed that most samples were *An. merus* (n = 206), followed by *An. quadriannulatus* (n = 65) and then *An. arabiensis* (n = 11). Identification for 44 samples could not be resolved.

A total of 71.67% of adults exposed to DDT were identified as *An. merus*, while 27.50% were identified as *An. quadriannulatus* and 0.83% were identified as *An. arabiensis*. For deltamethrin exposures, 74.58% were identified as *An. merus*, 17.54% were identified as *An. quadriannulatus* and 7.89% were identified as *An. arabiensis.*

4.4 Discussion

This study has shown that rural water bodies in Mpumalanga have elevated levels of pollution, with pollution in most sampling sites exceeding the maximum safe level for aquatic organisms. Further, *Anopheles gambiae s.l.* larvae breeding in these rural water bodies showed elevated levels of cytochrome P450 enzyme levels, combined with several bacterial species occurring in both the larval environment and larval midguts that are associated with insecticide degradation. Despite this, adult *An. gambiae s.l.* were still fully susceptible when exposed to DDT and deltamethrin.

Results from this chapter show that different species of the *An. gambiae* complex are breeding in polluted water bodies. This is the first report of *An. merus* breeding in and adapting to polluted water bodies, based on historical species composition trends in Mpumalanga (Chapter 1). However, results of this study for the other species found breeding in polluted water were not new. Other members of the *An. gambiae* complex have also shown adaptation to polluted breeding environments, particularly *An. gambiae s.s* and *An. arabiensis* (Awolola *et al.*, 2007; Antonio-Nkondjio *et al.*, 2011; Jones, Toé, *et al.*, 2012). Additionally, other anopheline species outside the *An. gambiae* complex have also been shown to adapt to polluted environments, such as *An. culicifacies* and *An. stephensi* (Gunathilaka *et al.*, 2013; Fazeli-Dinan *et al.*, 2022). Notably, these species are all anthropophilic to a degree (Gillies and de Meillon, 1968; Prasad *et al.*, 2015; Balkew *et al.*, 2021). A study has shown that anthropogenic disturbance of *Anopheles gambiae s.s.* habitats has driven their adaptation to polluted breeding habitats (Kamdem *et al.*, 2012). The association of *An. merus* with polluted breeding sites may be driven by a similar mechanism.

The analysis of the water samples showed that *An. gambiae s.l.* breeding habitats in Mpumalanga are heavily polluted with heavy metals (Figure 4.4), Agri-pollutants (Figure 4.5) and surfactants (Figure 4.6). Heavy metals were especially prevalent at levels well above the maximum acceptable level for aquatic organisms. This is a significant finding, as exposure to heavy metals have been shown to increase cytochrome P450 activity (Poupardin *et al.*, 2008; Musasia *et al.*, 2013; Oliver and Brooke, 2018b), and, consequently, increased insecticide resistance in the

subsequent adult generation (Oliver and Brooke, 2018b). Exposure to heavy metals in the female adult has also been shown to impact susceptibility to infection by *Plasmodium* parasites (Maya-Maldonado *et al.*, 2021). Heavy metals present in the breeding habitats of *An. gambiae s.l.* in Mpumalanga are likely originating from fertilisers. Pollution from agri-pollutants were present in water samples to a lesser degree, despite the proximity of sampling sites to agricultural activities. A multigenerational study showed that exposure to a mix of agricultural pesticides increased insecticide resistance of *An. gambiae s.s.* (Nkya *et al.*, 2014). Agricultural pollutants in Mpumalanga likely originate from fertilisers and pesticides. A substantial level of surfactant pollution was present in breeding habitats of *An. gambiae s.l.* in Mpumalanga. The likely source for the high levels of surfactants were local communities using water bodies as washing areas and areas to dump refuse (pers. observation). Studies have shown that exposure to acids and detergents have also been shown to increase insecticide resistance (Antonio-Nkondjio *et al.*, 2014; Chen and Oliver, 2024). High levels of pollution in the breeding habitats of *An. gambiae s.l.* in Mpumalanga could potentially contribute to the increasing problem of insecticide resistant vector populations, which may pose challenges to South Africa's malaria elimination agenda.

Elevated levels of cytochrome P450 were present in larvae of wild *An. gambiae s.l.* in Mpumalanga, compared to a susceptible laboratory of *An. arabiensis* (Figure 4.10). Wild *An. gambiae s.l.* adults, however, showed lower metabolic detoxification enzyme levels compared to the susceptible laboratory strain (Figure 4.11). The disconnection between larval and adult enzyme activity levels have been observed elsewhere. Adult *An. gambiae s.s.* showed lower enzyme activity compared to larvae when bred in polluted water for 10 generations (King *et al.*, 2017). However, metabolic detoxification enzyme activity alone is not informative about insecticide resistance, since it is not a phenotypic bioassay. Rather, it only suggests a potential mechanism. These results suggest that selection is occurring at the larval stage. Phenotypic bioassays using the WHO tube bioassay method showed full susceptibility of adult *An. gambiae s.l.* to deltamethrin and DDT (Table 4.3), reinforcing the results from the enzyme activity of the adults. This suggests that either an insecticide resistant phenotype may not be present, or, alternatively, incipient resistance may be present in the adults at too low a level for the WHO bioassay to detect.

A limitation of the WHO tube bioassay is that this method uses a single discriminating dose to test for insecticide resistance (World Health Organization, 2016). This single dose may be too high to detect low-level resistance present in adults. A critical assessment of the WHO tube bioassay method and the Centre for Disease Control (CDC) bottle bioassay methods identified the single discriminating dose as a key drawback of the method, stating "… the recommended diagnostic doses were not appropriate for all species." (Praulins *et al.*, 2024). Changes in insecticide resistance at lower concentrations may not be detected by the WHO tube bioassay, but may be identified by the CDC bottle bioassay which allows for control over the dose (Brogdon and McAllister, 1998).

There have been cases where selection for insecticide resistance at the larval level were insufficient to generate a resistance phenotype in the subsequent adult generation (Wood *et al.*, 2014). This study focused on a single generation of *An. funestus*. However, a multigenerational study of *An. gambiae s.s.* bred in polluted water showed that elevated metabolic detoxification levels present in larvae eventually manifested in the subsequent adults across 20 generations (Nkya *et al.*, 2014). Selection for insecticide resistance may, however, not be occurring as rapidly as for laboratory strains under controlled conditions. Additionally, the previously mentioned studies investigated *An. funestus* (Wood *et al.*, 2014) and *An. arabiensis* (Nkya *et al.*, 2014). The predominant member of the *An. gambiae s.l.* complex in Mpumalanga is *An. merus* (Chapter 1). There may therefore not be resistance genes circulating in *An. merus* populations, as no record of resistance has been recorded for this species in South Africa. This, however, does not preclude continued selection pressure from polluted water sources, which may lead to increased insecticide resistance in adults in future.

Further evidence that the larval environment requires monitoring is evident from the bacterial composition of aquatic habitats of larvae and the midgut of the larvae. Water samples from larval breeding sites at Vlakbult had the highest species richness (Figure 4.7), while midguts of larvae from sampling sites at Masibekela had the highest species richness (Figure 4.8). Masibekela was the most polluted site sampled, with high levels of heavy metals, sulphides, sulphites, nitrogen, chloride, organic acids and

surfactants (Figures 4.4 – 4.6). Polluted breeding sites can potentially increase the gut bacterial species richness. This was observed for wild *An. arabiensis* collected from Mamfene, KwaZulu-Natal, where F_1 adults bred in metal-treated water resulted in an increase in gut bacterial species richness (Singh *et al.*, 2024). The bacterial species associated with this increase in species richness were specifically adapted to degrading metal pollutants (Singh *et al.*, 2024). There was also a substantial overlap between bacteria isolated from the water samples and bacteria isolated from the midguts of larvae (Figure 4.9). This has been observed before in *Aedes aegypti* (Zouache *et al.*, 2022) and *Anopheles darlingi* (Mosquera *et al.*, 2023). Previous research has shown that the bacterial consortium within the midguts of mosquito larvae or newly emerged mosquito adults were derived from the aquatic environment of larval breeding habitats (Guégan *et al.*, 2018; Saab *et al.*, 2020). The environmental acquisition of bacteria therefore plays an important role in the bacterial composition of the mosquito midgut (Coon *et al.*, 2014; Dickson *et al.*, 2017), which in turn plays a crucial role in adapting to breeding in polluted breeding sites.

The bacterial consortium present within the midguts of mosquito larvae play an important role in the life-history traits of the subsequent adult generation, such as fertility, fecundity, metabolism, vector competence, longevity, egg hatch rate, larval development and insecticide resistance (Bahia *et al.*, 2015; Ezemuoka *et al.*, 2020; Harrison *et al.*, 2023). The midgut microbiota have also been shown to contribute to the development of an insecticide-resistant phenotype (Dada *et al.*, 2018; Barnard *et al.*, 2019). Several bacteria identified from the midguts of larvae in this study have previously been shown to be associated with pesticide degradation. Organophosphates have been shown to be degraded by *Pseudomonas* spp. (Khalid *et al.*, 2016), *Bacillus* spp. (Rana *et al.*, 2015), *Aeromonas* spp. (Shartooh, Abood and Hassan, 2022), *Enterobacter* spp. (Singh *et al.*, 2004), *Klebsiella* spp. (Elshikh *et al.*, 2022), *Micrococcus* spp.(Guha *et al.*, 1997), *Serratia* spp. (Cycoń, Wójcik and Piotrowska-seget, 2009) and *Lysinibacillus* spp. (Marei *et al.*, 2019). Degradation of neonicotinoids have been shown for *Pseudomonas* spp. (Lu *et al.*, 2016), *Bacillus* spp. (Sharma, Singh and Gupta, 2014), *Exiguobacterium* spp. (Yandigeri *et al.*, 2024), *Stenotrophomonas* spp. (Dai *et al.*, 2006) and *Klebsiella* spp. (Phugare *et al.*, 2013). *Enterobacter* spp. have been implicated in carbamate degradation (Mustapha *et al.*, 2020). Several bacterial species have been shown to be involved in the degradation

of pyrethroids, such as *Lysinibacillus* spp. (Hu *et al.*, 2014), *Acinetobacter* spp. (Zhan *et al.*, 2018), *Micrococcus* sp. (Tallur, Megadi and Ninnekar, 2008), *Citrobacter* spp. (Zhou *et al.*, 2022), *Streptococcus* spp. (Omoke *et al.*, 2021) and *Aeromonas* spp. (Liu *et al.*, 2020). Organochlorines have been shown to be readily degraded by *Klebsiella* spp. (Kwon *et al.*, 2005), *Acinetobacter* spp., *Pseudomonas* spp., *Stenotrophomonas* spp., *Bacillus* spp. and *Citrobacter* spp. (Ozdal, Ozdal and Algur, 2016). The bacterial consortium present within the larval midgut is therefore associated with the degradation of all the most commonly used insecticide classes for vector control. This high diversity of insecticide degrading bacteria within the larval midgut lends credence to the theory that selection for insecticide resistance might be occurring at the larval stage.

Gut bacteria have also been shown to play an important role in immunity. Several of the bacterial species found within the larval midgut of wild caught larvae from Nkomazi have been shown to be associated with reduced infection with *Plasmodium* spp. These include *Pseudomonas*, *Serratia*, *Acinetobacter*, *Enterobacter* and *Bacillus* spp. (Pumpuni *et al.*, 1993, 1996; Gonzales-Ceron *et al.*, 2003; Cirimotich *et al.*, 2011; Ramirez *et al.*, 2014; Yu *et al.*, 2020). The presence of these bacteria might present critical epidemiological consequences for malaria transmission in Mpumalanga. Therefore, understanding the interactions between these bacterial species and *Plasmodium* spp. opens new avenues for vector control. Indeed, some anti-*Plasmodium* bacteria have already been incorporated into anti-malarial drugs (Wang and Jacobs-Lorena, 2017).

There were a few limitations to this body of work. Since South Africa is on the malaria elimination pathway, finding high densities of malaria vectors to have reasonably large sample sizes for a more robust analysis was challenging. For this reason, the sample sizes to determine the metabolic detoxification enzyme levels and the bacterial characterisation of the larval midguts were relatively small. Species identification of these samples was not possible through PCR and relied instead on historical species composition due to the limited number of samples available. There were not enough samples to characterise the bacterial composition of the adult midguts. Furthermore, since the age, feeding status and reproductive status of wild adults are unknown, larvae were collected for the insecticide bioassays and the F_1 generation was

exposed. Due to the time and infrastructure limitations, only a limited number of larvae could be collected for the bioassays. Finally, due to the limited number of samples, the samples for the insecticide bioassays and the samples for the metabolic detoxification enzyme profiles were from different geographical locations and therefore from different populations, which may be genetically different.

This study, however, serves as a foundation for future research and has identified gaps in our knowledge regarding the physio-chemical characteristics of the breeding sites and insecticide resistance status of *An. gambiae s.l.* in Mpumalanga. We recommend monitoring insecticide resistance of adult *An. gambiae s.l.* in Mpumalanga with CDC bottle bioassays, particularly with pyrethroids (where possible) to detect developing resistance. Despite the elevated cytochrome P450 levels in larvae collected from Ehlanzeni, it is not yet clear if the larvae are presenting a resistant phenotype. Further research should investigate whether a resistant phenotype is present within the larvae by conducting resistance bioassays. Additionally, the midgut microbiota of adult *An. gambiae s.l.* need to be characterised. Further investigation into the link between insecticide resistance and the midgut microbiome is essential to understand the effects of pollution on metabolic detoxification enzyme profiles and contribution of gut bacteria to a resistant phenotype.

4.5 Conclusion

This study is the first report on *An. merus* in Ehlanzeni adapting to polluted breeding habitats. All *An. gambiae s.l.* breeding sites investigated in Ehlanzeni were substantially polluted. Due to significant xenobiotic exposure, *An. gambiae s.l.* in Mpumalanga are showing early signs of insecticide resistance evolution within the larval stages, but this resistance is not reflected in adults yet. This suggests that the development of insecticide resistance due to exposure to agricultural pollutants is of concern and requires consideration. Our data also suggest that the adaptation to polluted environments may be having a pleiotropic effect that should be monitored by more sensitive assays, such as the CDC bottle bioassay. The presence of elevated cytochrome P450 metabolic enzymes may be an early indication of pyrethroid and organophosphate resistance evolution within wild *An. gambiae s.l.* from Mpumalanga. These insecticide classes should ideally be placed on a watchlist. This study is also

the first report on the diversity of bacterial microbiota within larval midguts of wild *An. gambiae s.l* from Mpumalanga. Bacteria have not been implicated as a potential driver of resistance in South Africa yet, however this is due to the lack of investigation to date. The impact of the mosquito's midgut microflora on insecticide resistance and susceptibility to infection by *Plasmodium* spp. should form an essential part of surveillance efforts, as these factors can have critical epidemiological consequences for malaria transmission. Lastly, the data from this study highlights the importance of interdisciplinary approaches in combating infectious diseases.

CHAPTER 5

THE HORMETIC EFFECTS OF LARVAL EXPOSURE TO AGRI-POLLUTANTS ON THE LIFE-HISTORY TRAITS OF LABORATORY REARED *AN. ARABIENSIS* GILES (DIPTERA: CULICIDAE)

5.1 Introduction

5.1.1 Brief background on toxicology

The dose-response relationship is a fundamental model in the study of toxicology (Tsatsakis *et al.*, 2018). The model characterises an organism's response after exposure to a stressor over a certain amount of time and is visualised by dose-response curves (Tsatsakis *et al.*, 2018). The dose-response relationship and its corresponding curve depends on numerous factors, such as the characteristics of the stressor involved, the biologically effective dose, the mode of action of the stressor and homeostasis (Ramaiah *et al.*, 2017).

The model illustrates the magnitude of an organism's response to a stressor over a specific period, represented visually through dose-response curves

The most common dose-response relationships are linear (no threshold), threshold, J-shaped, saturated, sigmoid, and continuous. A linear response is observed when the response of the organisms increases proportionally to an increase in the dose (Lutz, 1998) (Fig. 5.1 A). A threshold response is observed when the minimum amount of a stressor needs to be exceeded before any response is observed (Andersen *et al.*, 2002) (Fig. 5.1 B). A J-shaped dose-response occurs when a small dose of a stressor elicits a strong response, which increases rapidly (Ricci, Straja and Cox, 2012) (Fig 5.1 C). Saturated responses occur when the organism has a limited response to the stressor, and no further response is observed once the limit is reached (Gardner, 2000) (Fig. 5.1 D). Sigmoid responses are observed when small doses of a stressor elicit a small response and increase to a peak as the stressor increases, and then no further response is observed (Meddings, Scott and Fick, 1989) (Fig. 5.1 E). The U-shaped or continuous response is observed when there is an optimal response level to a stressor, after which the response decreases (Calabrese and Baldwin, 2003) (Fig. 5.1 F). This

is also known as the hormetic response. Hormetic responses are discussed in more detail below.

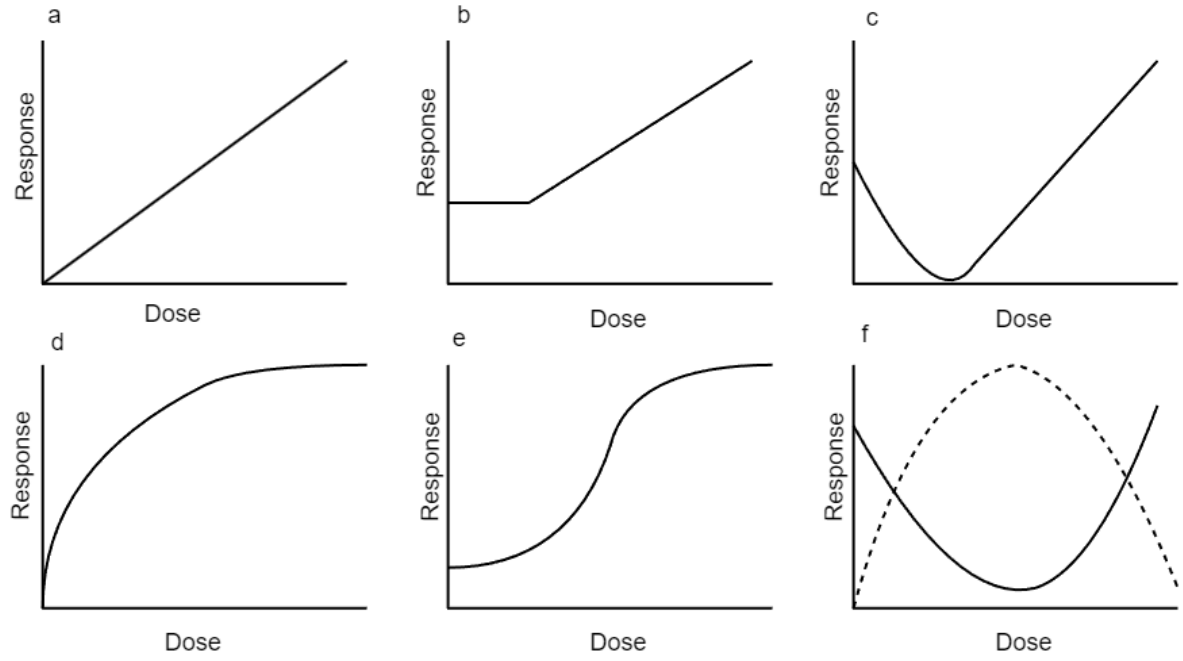


Figure 5.1. Most common dose-response curves in toxicology.
a) Linear dose-response (no threshold), b) threshold dose-response, c) J-shaped dose response, d) saturated dose response, e) sigmoid dose response and f) U-shaped dose-response. Adapted from Ramaiah *et al*. (2017).

5.1.2 Hormetic dose-relationship response

Hormesis is a physiological phenomenon where exposure to a toxicant or other stressor at low doses results in a stimulatory effect of one or more life-history traits, while at high doses an inhibitory effect is observed (Stebbing, 2000; Calabrese and Baldwin, 2002; Jager, Barsi and Ducrot, 2013) (Figure 5.2). Hormesis is a biologically widespread phenomenon (Calabrese and Baldwin, 2001), and the type of response elicited is unique to the particular endogenous and exogenous environments in which it occurs (Calabrese and Baldwin, 2002).

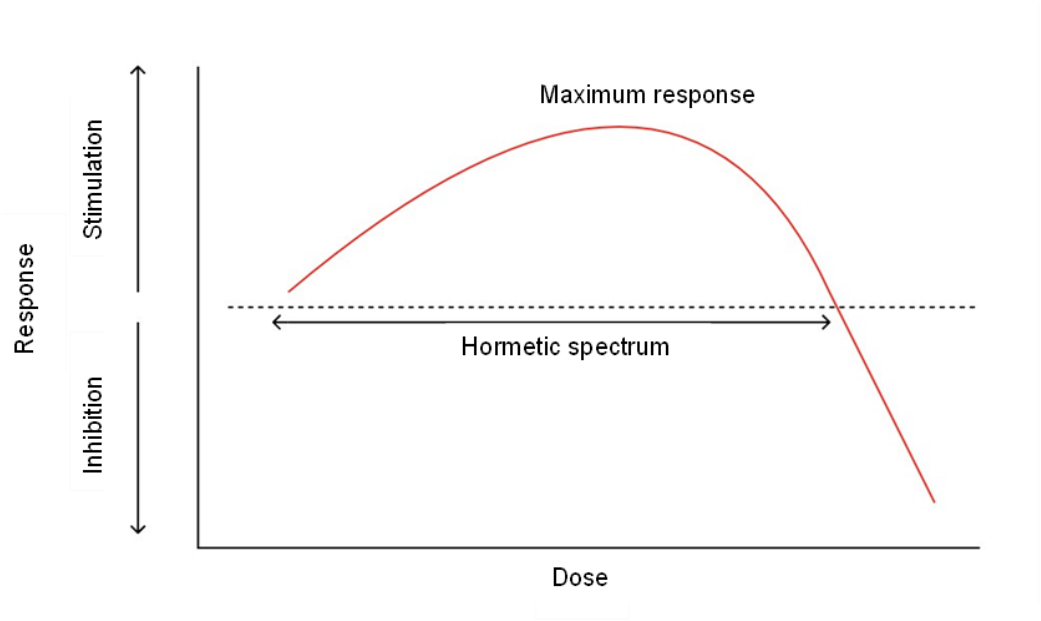


Figure 5.2. Schematic diagram of a hormetic response.
At lower doses a stimulatory effect is observed. As the dose increases and becomes more toxic, an inhibitory effect is observed. Adapted from Tsatsakis *et al*. (2018).

Hormesis is considered to be an adaptive response to the disruption of homeostasis, which either results from direct stimulation or is induced as a moderate overcompensation response of various metabolic processes in an attempt to restore homeostasis (Calabrese and Baldwin, 2002). No singular mechanism has been identified, but biological resource allocation is a common theme observed in most, if not all, hormetic responses (Calabrese and Baldwin, 2002). Resources are allocated to repair immediate damage of exposure to a toxicant or stressor, however excess resources are allocated than those needed to repair the damage (Calabrese and Baldwin, 2002). The excess resources are then used to optimise one life-history trait at the expense of another (Jager, Barsi and Ducrot, 2013). The process of hormesis is also capable of re-adapting the organism against any subsequent damage of a similar nature at bigger doses than the initial exposure dose for a limited period of time (Calabrese and Baldwin, 2002).

Hormetic effects are commonly induced by sub-lethal doses of chemical stressors such as insecticides, heavy metals and other agricultural pollutants (Guedes and Cutler, 2014). Tolerance to heavy metals and agricultural pollutants in insecticide-

resistant *Anopheles funestus s.s.* and *An. gambiae s.l.* larvae was associated with reduced fertility and longevity of the subsequent adult generation and increased larval developmental time, compared to susceptible counterparts (Mireji *et al.*, 2010; Nkahe *et al.*, 2020; Tchouakui *et al.*, 2020). Other traits involved in hormetic responses include, but are not limited to, survival, fecundity, metabolic and molecular processes and immune responses (Calabrese and Blain, 2005). Hormesis can also be induced by non-chemical stressors such as temperature stress (Gómez *et al.*, 2009), nutritional stress (Wang *et al.*, 2022), radiation (Shephard *et al.*, 2018) and reduced oxygen (De La Torre and López-Martínez, 2023).

5.1.3 Context and rationale

Hormesis has been suspected to contribute to the development of insecticide resistance, since hormetic responses may cause the induction of metabolic detoxification enzymes (Guedes and Cutler, 2014). This makes it an important aspect to consider in vector control programs. Sub-lethal doses of larviciding, for instance, may have consequences for the subsequent adult generation, which can impact vector control programs, especially since impacts on life-history traits have been shown to occur within one generation (Jeanrenaud, Brooke and Oliver, 2020). This is especially true if the subsequent adult generation shows increased insecticide resistance or increased longevity. Increased longevity of female *Anopheles* spp. increases their potential to transmit malaria due to the increased window of human-to-vector contact (Garrett-Jones and Shidrawi, 1969; Coosemans *et al.*, 1992).

Anopheles arabiensis is often associated with agricultural activities and is responsible for an increase in malaria transmission in agriculturally rich areas (Yadouleton *et al.*, 2009; Abuelmaali *et al.*, 2013). Mpumalanga is an agriculturally rich province, with 3% of the gross value added by region (GVA-R) attributed to agricultural activities (Young, 2022). Urban invasion of this vector has been observed, where *An. arabiensis* is adapting to urban water bodies that are often severely polluted with chemicals, albeit at too low concentrations to have a killing effect on aquatic organisms, such as mosquito larvae (Chapter 2). Since such sub-lethal doses of toxic xenobiotics can trigger hormetic effects, it could be possible that hormetic reactions are occurring in wild *An. arabiensis* populations from Mpumalanga. The impact of these hormetic

reactions on life-history traits and insecticide resistance status of malaria vectors is largely unknown. This work intends to fill this research gap.

This study therefore aims to investigate whether hormesis occurs in an insecticide-selected and insecticide-unselected laboratory strain of *An. arabiensis* (SENN and SENN DDT) when exposed to four common agricultural pollutants. The pollutants investigated include a heavy metal (manganese), a pesticide (glyphosate), a plastic component (styrene) and an electrolyte (chloride). Laboratory strains were used, since larvae for experiments were readily available compared to wild larvae, which are often difficult to obtain due to relatively low vector densities in Mpumalanga. Furthermore, despite *An. merus* being the more abundant member of the *An. gambiae complex* in Mpumalanga, *An. arabiensis* was used as it is the only member of the *An. gambiae* complex directly implicated as a vector in the South Africa (Dandalo *et al.*, 2017).

The specific objectives for this chapter are to expose both insecticide-selected and insecticide-unselected strains of *An. arabiensis* (SENN DDT and SENN) to sub-lethal doses of manganese, glyphosate, styrene and chloride and measure the impact on the percentage of larvae successfully pupating, time taken to pupation and adult longevity.

5.2 Materials and Methods

5.2.1 Impact of pollutants on larval development and adult longevity

5.2.1.1 Preparation of pollutant solutions

The choice of sample concentration was based on survivability of the larvae at the respective dose. This is due to a lack of information on the concentrations of these chemicals in mosquito breeding sites.

A 1 M solution of manganese was prepared. A low dose of 25 µl, a middle dose of 50 µl and a high dose of 100 µl were used for the treatments (Table 5.1). Each dose was added to 1 L of distilled water.

A solution of glyphosate was used from commercially available glyphosate (Roundup® Power 360SL, Efekto®). A low dose of 3.5 µl, a middle dose of 7 µl and a high dose of 14 µl were used for the treatments (Table 5.1). Each dose was added to 1 L of distilled water.

A stock solution of chloride (10 000 mg/L) was used directly for the treatments (Sigma Aldrich: 06740, lot number BCCJ4156). A low dose of 2 µl, a middle dose of 4 µl and a high dose of 8 µl were used for the treatments (Table 5.1). Each dose was added to 1 L of distilled water.

A stock solution of styrene (≥ 99%, liquid) was used directly for the treatments (Sigma Aldrich: S4972, CAS number 100-42-5). A low dose of 5 µl, a middle dose of 9.5 µl and a high dose of 19 µl were used for the treatments (Table 5.1). Each dose was added to 1 L of distilled water.

Table 5.1. Dosages in µl used per pollutant for exposure of an insecticide-selected and an insecticide-unselected strain of *An. arabiensis* larvae under controlled conditions, with final concentration (v/v%) in brackets.

	Control	Treatment 1	Treatment 2	Treatment 3
Manganese	0	25 (0.0025)	50 (0.0050)	100 (0.010)
Glyphosate	0	3.5 (0.00035)	7 (0.00070)	14 (0.00140)
Chloride	0	2 (0.00020)	4 (0.00040)	8 (0.00080)
Styrene	0	5 (0.00050)	9.5 (0.00095)	19 (0.0019)

Each treatment for each pollutant was replicated three times.

5.2.1.2 Development assays

A development assay was conducted using methodology as described in Chapter 2 General Materials and Methods: 2.3 Protocol for Development Assays of Laboratory Reared Mosquito Strains. One of the four rectangular containers per replication served as control and received no treatment. The three remaining containers received each either a low, medium or high dose of one of the four pollutant mixtures.

5.2.1.3 Measurements

The number of larvae that successfully pupated was recorded for each pollutant and each treatment. Time to pupation was recorded as the number of days between hatching and pupation and was done for each pollutant and each treatment. Finally, the longevity of adults from each pollutant and treatment was recorded as the number of days of survival.

5.2.2 Baseline characterisation of SENN and SENN DDT insecticide resistance

Baseline characterisation of insecticide resistance present in SENN and SENN DDT was done using methodology outlined in Chapter 2 General Materials and Methods-2.4: Bioassays for Characterisation of SENN and SENN DDT Insecticide Resistance.

5.2.3 Data analysis

The percentage of larvae successfully pupating was calculated for each treatment of each pollutant. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to test the normality of data. An analysis of variance (ANOVA) (Fischer, 1925) was used to determine significant differences in means between strains and between treatments for each pollutant. A post hoc analysis was conducted using a Welch two-sample t-test (Welch, 1938) to determine statistically significant differences between strains for each pollutant. A Tukey HSD test (Tukey, 1949) was used to determine statistically significant differences between treatments for each pollutant.

Time to pupation (used as a proxy for larval development) and adult longevity were analysed using a Kaplan-Meier estimator (Kaplan and Meier, 1958). Statistically significant differences between treatments and strains for each pollutant were determined using a log-rank test (Harrington and Fleming, 1982). All analyses were performed in R-studio (RStudio Team, 2023).

5.3 Results

5.3.1 Comparison of the effect of varying pollutant concentrations on pupation success- between strains

This analysis investigated whether significant differences existed in the percentage pupation between strains for each individual treatment.

5.3.1.1 Manganese

The mean percentage of pupation for the SENN control treatment was 52.00% ± 20.22% and 53.00% ± 10.82% for the SENN DDT control treatment (Fig. 5.3 A). No significant difference was evident between the two control cohorts ($t_{(3.06)}$ = -0.08, p = 0.94).

The mean percentage of pupation for the SENN 0.0025% treatment was 66.00% ± 26.00% and 66.00% ± 14.05% for the SENN DDT 0.0025% treatment (Fig. 5.3 B). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(3.08)}$) = -1.04, p = 0.38).

The mean percentage of pupation for the SENN 0.005% treatment was 37.67% ± 20.50% and 50.33% ± 7.09% for the SENN DDT 0.005% treatment (Fig. 5.3 C). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(2.47)}$ = -1.01, p = 0.40).

The mean percentage of pupation for the SENN 0.01% treatment was 30.67% ± 27.57% and 24.67% ± 20.31% for the SENN DDT 0.01% treatment (Fig. 5.3 D). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(3.68)}$ = 0.30, p = 0.78).

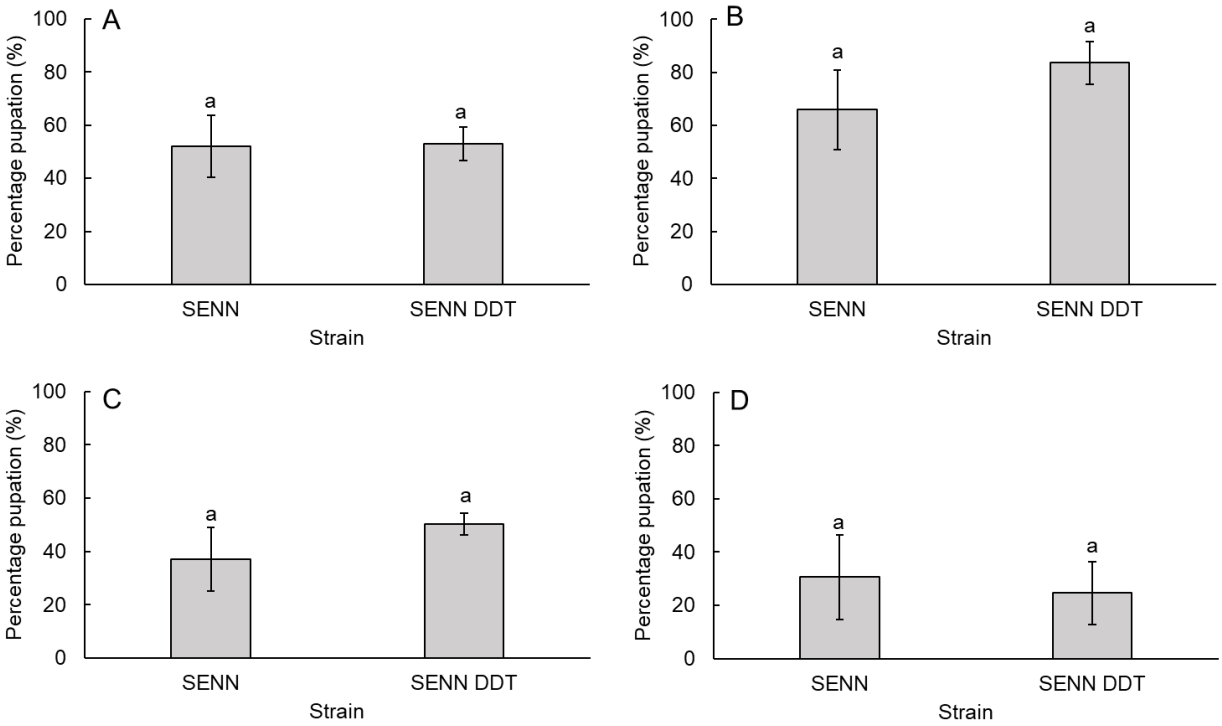


Figure 5.3. Mean percentage pupation per treatment for each strain of *An. arabiensis* larvae exposed to different concentrations of manganese.

A) control treatment; B) 0.0025% manganese; C) 0.005% manganese; D) 0.01% manganese. Letters indicate significant differences between strains for each treatment. Error bars represent the standard error of the mean.

5.3.1.2 Glyphosate

The mean percentage of pupation for the SENN and SENN DDT control treatments were 35.33% ± 16.80% and 86.33% ± 8.74%, respectively (Fig. 5.4 A). There was a statistically significant difference in mean percentage pupation between the two control cohorts ($t_{(3.01)}$ = -4.66, p = 0.02).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.00035% glyphosate was 78.67% ± 6.35% and 87.33% ± 18.15%, respectively (Fig. 5.4 B). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(2.48)}$ = -0.78, p = 0.50).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0007% glyphosate was 80.00% ± 13.45% and 74.33% ± 20.60%, respectively (Fig. 5.4 C). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(3.44)}$ = 0.40, p = 0.71).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0014% glyphosate was 75.33% ± 17.56% and 76.67% ± 32.72%, respectively (Fig. 5.4 D). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(3.06)}$ = -0.06, p = 0.95).

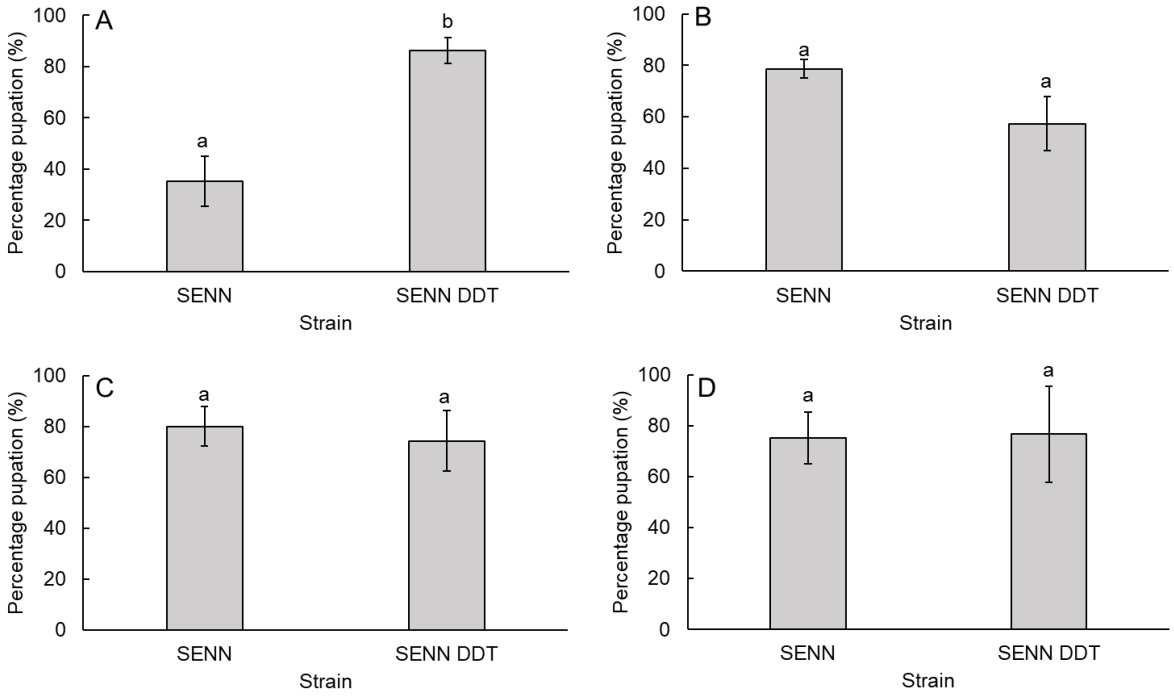


Figure 5.4. Mean percentage pupation per treatment for each strain of *An. arabiensis* larvae exposed to different concentrations of glyphosate.
A) control treatment; B) 0.00035% glyphosate; C) 0.0007% glyphosate; D) 0.0014% glyphosate. Letters indicate significant differences between strains for each treatment. Error bars represent the standard error of the mean.

5.3.1.3 Chloride

The mean percentage of pupation for the SENN and SENN DDT control treatments were 21.80% ± 16.07% and 52.00% ± 25.47%, respectively (Fig. 5.5 A). There was no statistically significant difference in mean percentage pupation between the two control cohorts ($t_{(6.75)}$ = -2.24, p = 0.060.)

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0002% chloride were 66.50% ± 37.39% and 66.75% ± 43.37%, respectively (Fig. 5.5 B). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(5.87)}$ = -0.010, p = 0.99).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0004% chloride were 64.75% ± 26.44% and 53.50% ± 30.03%, respectively (Fig. 5.5 C). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(5.91)}$ = 0.56, p = 0.59).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0008% chloride were 66.75% ± 31.85% and 71.25% ± 22.43%, respectively (Fig. 5.5 D). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(5.39)}$ = -0.23, p = 0.83).

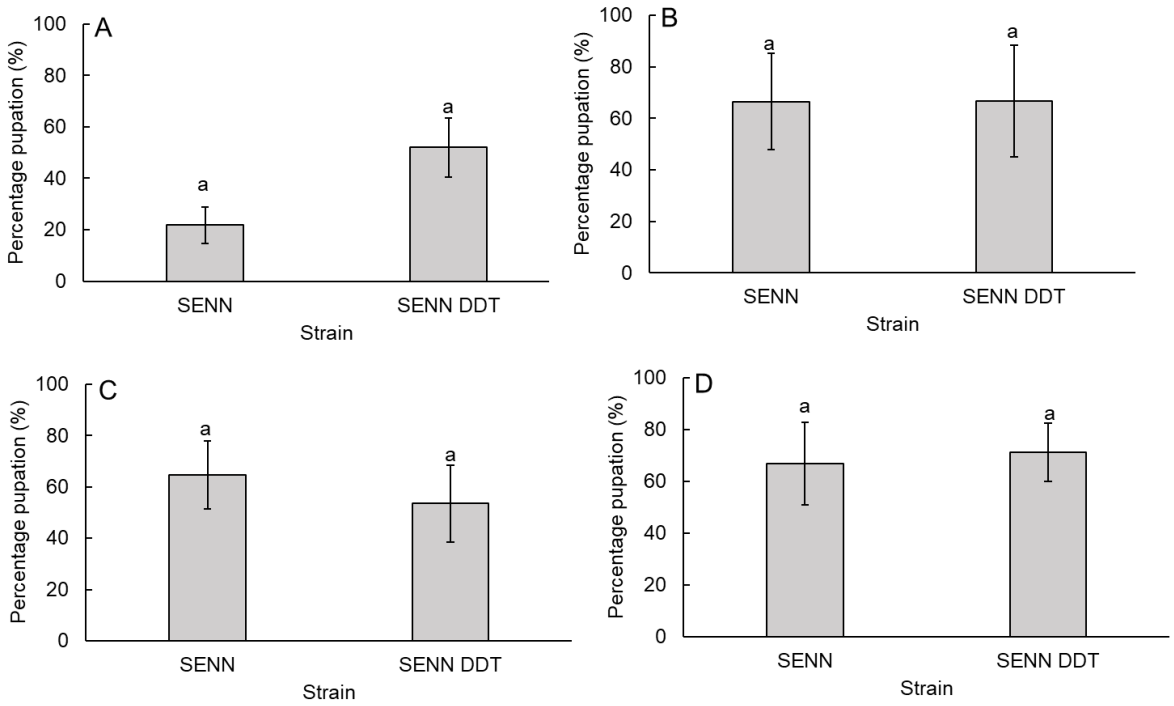


Figure 5.5. Mean percentage pupation per treatment for each strain of An. arabiensis larvae exposed to different concentrations of chloride.
A) control treatment; B) 0.0002% chloride; C) 0.0004% chloride; D) 0.0008% chloride. Letters indicate significant differences between strains for each treatment. Error bars represent the standard error of the mean.

5.1.3.4 Styrene

The mean percentage of pupation for the SENN and SENN DDT control treatments were 81.00% ± 5.66% and 44.00% ± 53.74%, respectively (Fig. 5.6 A). There was no statistically significant difference in mean percentage pupation between the two control cohorts ($t_{(1.02)}$ = 0.97, p = 0.51).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0005% styrene were 90.00% ± 2.83% and 37.00% ± 46.67%, respectively (Fig. 5.6 B). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(1.01)}$ = 1.60, p = 0.35).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.00095% styrene were 72.50% ± 4.95% and 44.50% ± 62.93%, respectively (Fig. 5.6 C). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(1.01)} = 0.63$, $p = 0.64$).

The mean percentage of pupation for the SENN and SENN DDT larvae exposed to 0.0019% styrene were 46.00% ± 8.49% and 44.50% ± 25.50%, respectively (Fig. 5.6 D). There was no statistically significant difference in mean percentage pupation between the two strains when larvae were exposed to this concentration ($t_{(1.23)} = 1.11$, $p = 0.44$).

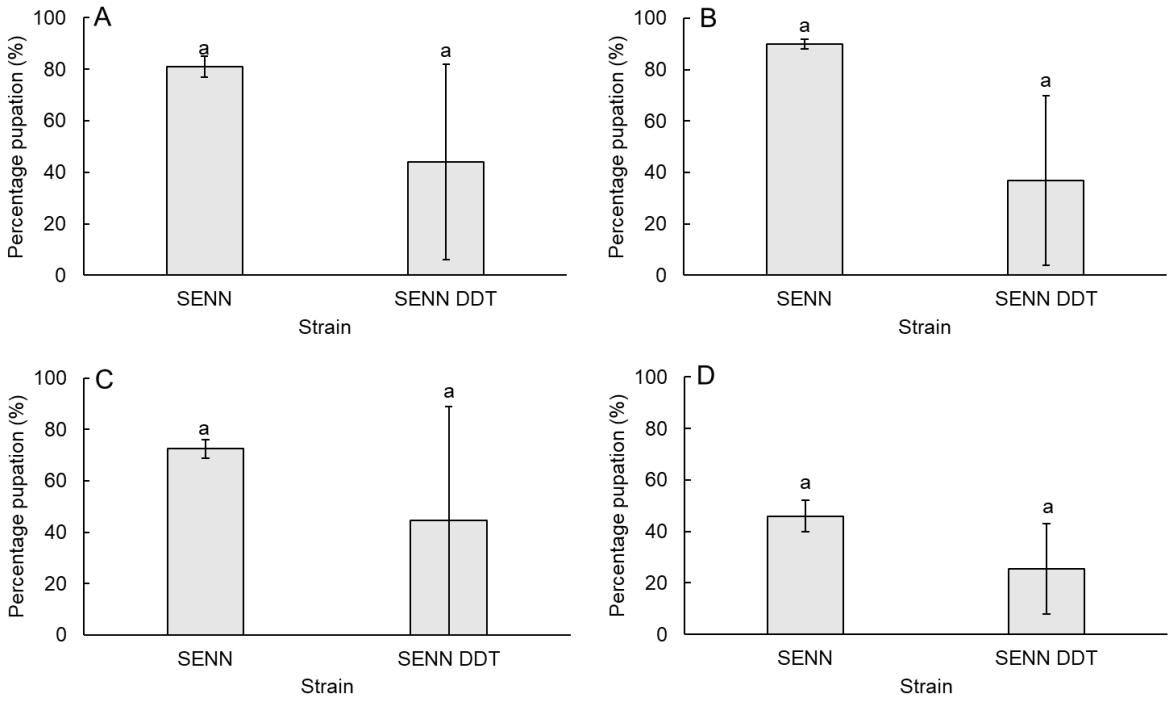


Figure 5.6. Mean percentage pupation per treatment for each strain of *An. arabiensis* larvae exposed to different concentrations of styrene.
A) control treatment; B) 0.0005% styrene; C) 0.00095% styrene; D) 0.0019% styrene. Letters indicate significant differences between strains for each treatment. Error bars represent the standard error of the mean.

5.3.2 Comparison of the effect of varying pollutant concentrations on pupation success- between treatments

This analysis investigated whether significant differences were present for the successful percentage of pupation between treatments separately for each strain.

5.3.2.1 Manganese

Data for both SENN and SENN DDT were normally distributed (W_{SENN} = 0.98, p = 0.97; W_{SDDT} = 0.96, p = 0.81). There was no significant difference in the mean percentage pupation between larval cohorts exposed to different manganese concentrations for SENN (ANOVA, $F_{(3,8)}$ = 1.31, p = 0.34) (Fig. 5.7 A). There were statistically significant differences in the mean percentage pupation observed between larval cohorts exposed to manganese treatments for SENN DDT (ANOVA, $F_{(3,8)}$ = 9.010, p = 0.0061) (Fig. 5.7 B). A Tukey HSD *post hoc* test showed that the mean percentage pupation for SENN DDT was significantly higher for larvae exposed to 0.0025% manganese compared to larvae exposed to 0.01% manganese (p = 0.0037)

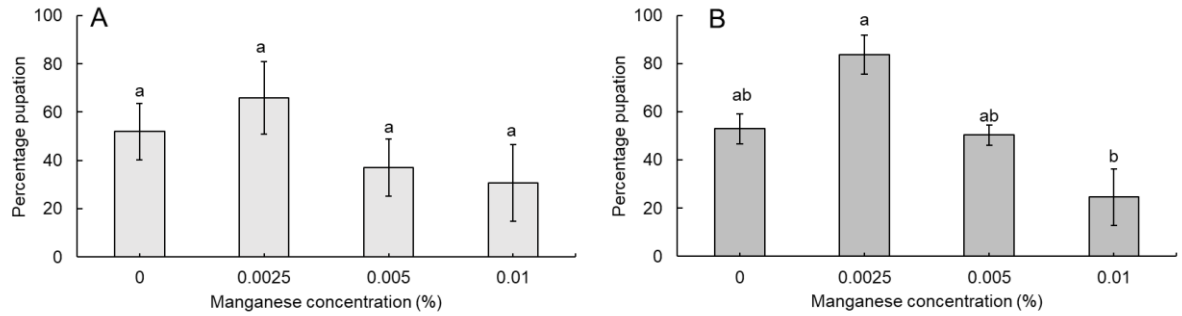


Figure 5.7. Mean percentage pupation of *An. arabiensis* larvae exposed to different concentrations of manganese.
A) Mean percentage pupation in SENN; B) Mean percentage pupation in SENN DDT. Letters indicate significant differences between treatments. Error bars represent the standard error of the mean.

5.3.2.2 Glyphosate

Data for both SENN and SENN DDT were normally distributed (W_{SENN} = 0.91, p = 0.19; W_{SDDT} = 0.88, p = 0.10). There was a statistically significant difference in the mean percentage pupation between the control treatment and all other treatments for SENN, while there was no statistically significant difference in the mean percentage pupation between treatments for SENN ($F_{(3,8)}$ = 6.78, p = 0.014). A Tukey HSD *post hoc* test showed that the control treatment for SENN had significantly lower mean percentage pupation compared to the 0.00035% treatment (p = 0.024), the 0.0007% treatment (p= 0.021) and the 0.0014% treatment (p = 0.036) (Figure 5.8 A). For SENN DDT, there were no statistically significant differences in the mean percentage pupation between all treatments ($F_{(3,8)}$ = 0.28, p = 0.84) (Figure 5.8 B).

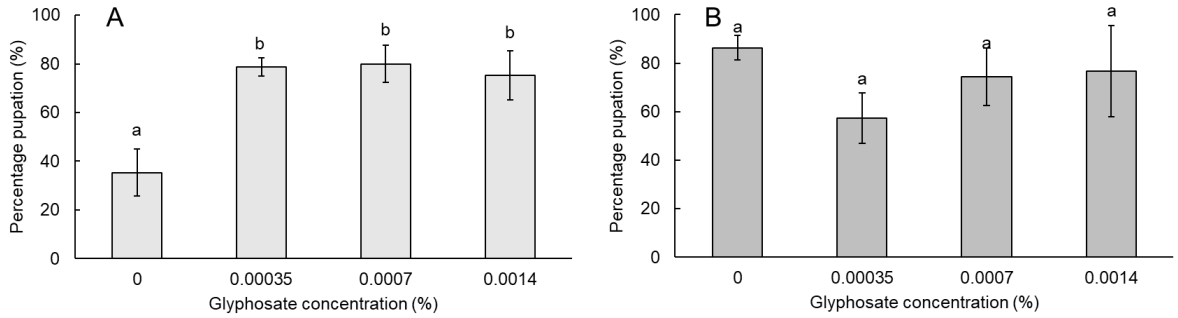


Figure 5.8. Mean percentage pupation of *An. arabiensis* larvae exposed to different concentrations of glyphosate.
A) Mean percentage pupation in SENN; B) Mean percentage pupation in SENN DDT. Letters indicate significant differences between treatments. Error bars represent the standard error of the mean.

5.3.2.3 Chloride

Data for SENN and SENN DDT were not normally distributed (W_{SENN} = 0.85, p = 0.010; W_{SDDT} = 0.89, p = 0.040). A Kruskal-Wallis test showed that there were no significant differences in the mean percentage pupation between treatments for both SENN (χ^2(13, n = 17) = 12.31, p = 0.50) (Fig. 5.9 A) and SENN DDT (χ^2(14, n = 17) = 11.40, p = 0.65) (Fig. 5.9 B).

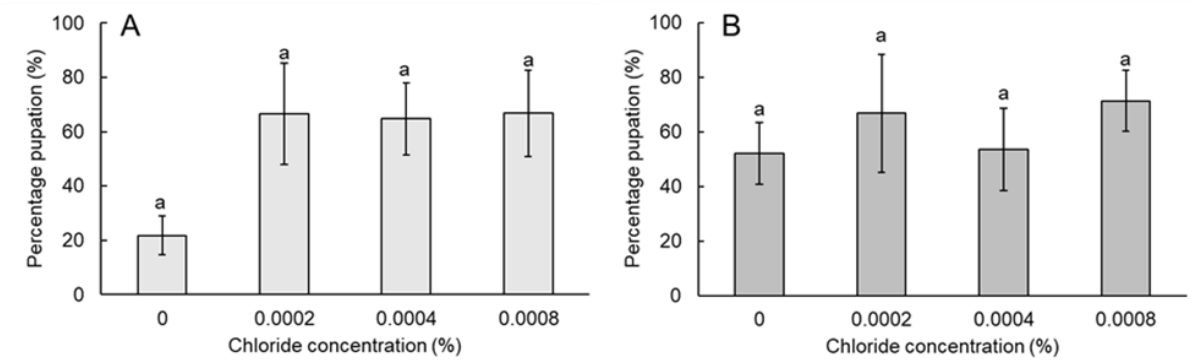


Figure 5.9. Mean percentage pupation of An. arabiensis larvae exposed to different concentrations of chloride.

A) Mean percentage pupation in SENN; B) Mean percentage pupation in SENN DDT. Letters indicate significant differences between treatments. Error bars represent the standard error of the mean.

5.3.2.4 Styrene

Data for both SENN and SENN DDT were normally distributed (W_{SENN} = 0.91, p = 0.35; W_{SDDT} = 0.83, p = 0.060). An ANOVA showed that there were significant differences in the mean percentage pupation between treatments for SENN ($F_{(3,4)}$ = 21.11, p = 0.010) (Fig. 5.10 A). A Tukey HSD *post hoc* test showed that the 0.0019% styrene treatment for SENN had a significantly lower mean percentage pupation compared to the control treatment (p = 0.01), the 0.0005% treatment (p = 0.01), and the 0.0019% treatment (p = 0.04). For SENN DDT, there was no significant differences in the mean percentage pupation between all treatments ($F_{(3,4)}$ = 0.070, p = 0.98) (Fig. 5.10 B).

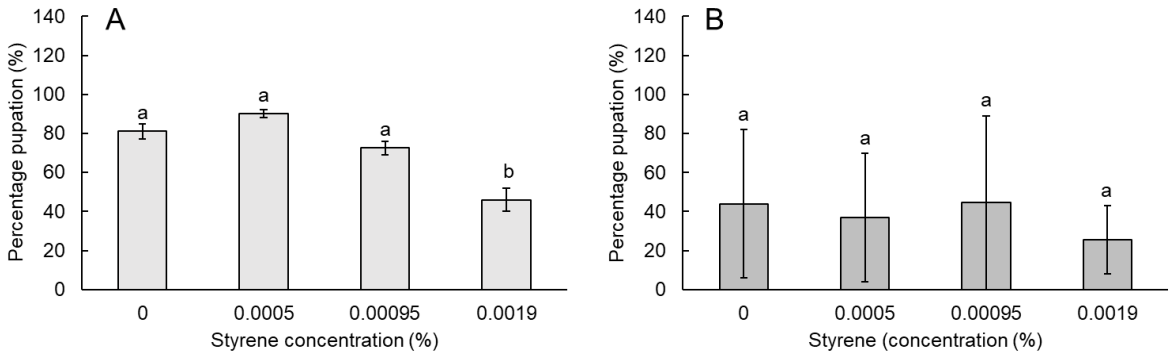


Figure 5.10. Mean percentage pupation of *An. arabiensis* larvae exposed to different concentrations of styrene.

A) Mean percentage pupation in SENN; B) Mean percentage pupation in SENN DDT. Letters indicate significant differences between treatments. Error bars represent the standard error of the mean.

5.3.3 Time to pupation

5.3.3.1 Manganese

The median time to pupation for the SENN control treatment was 14.00 ± 3.00 days (Fig. 5.11). The median time to pupation for the SENN 0.0025% manganese treatment was 14.00 ± 2.00 days (Fig. 5.11). The median time to pupation for the SENN 0.005% manganese treatment was 14.00 ± 3.00 days (Fig. 5.11). The median time to pupation for the SENN 0.01% manganese treatment was 14.00 ± 2.00 days (Fig. 5.11).

The median time to pupation for the SENN DDT control treatment was 14.00 ± 2.00 days (Fig. 5.11). The median time to pupation for the SENN DDT 0.0025% manganese treatment was 14.00 ± 2.00 days (Fig. 5.11). The median time to pupation for the SENN DDT 0.005% manganese treatment was 14.00 ± 2.00 days (Fig. 5.11). The median time to pupation for the SENN DDT 0.01% manganese treatment was 14.00 ± 1.00 days (Fig. 5.11).

The SENN 0.005% manganese treatment developed significantly slower than the control treatment (log-rank test: $\chi^2_{(3)} = 10.70$, $p = 0.010$), while for SENN DDT there was no significant difference between treatments (log-rank test: $\chi^2_{(3)} = 2.20$, $p = 0.50$).

There was no significant difference between strains per treatment (log-rank: $\chi^2_{(7)}$ = 14.40, p = 0.040).

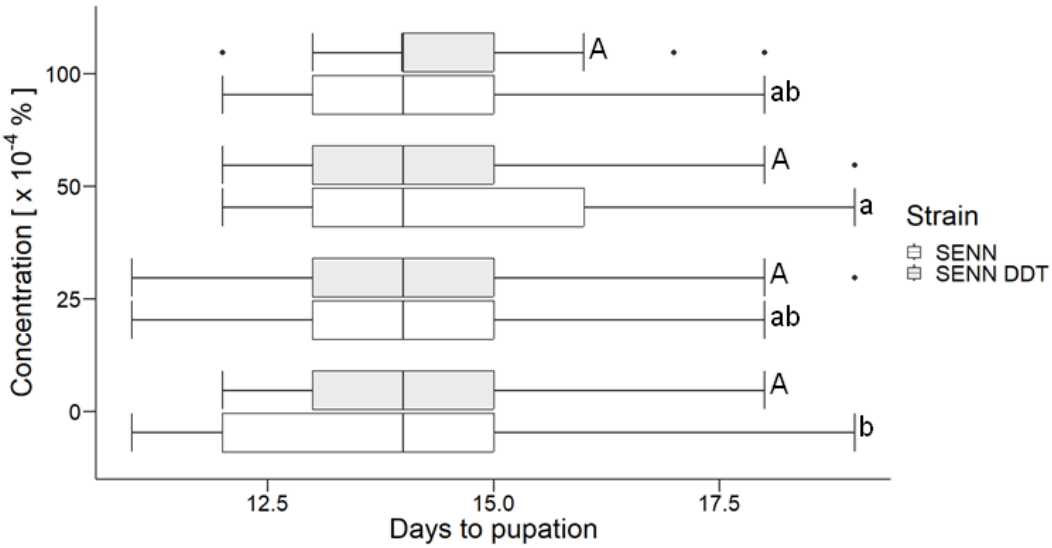


Figure 5.11. Time to pupation in days for SENN (white shading) and SENN DDT (grey shading) larvae exposed to different concentrations of manganese.
Lower case letters indicate significant differences between treatments for SENN, while upper case letters indicate significant differences between treatments for SENN DDT.

5.3.3.2 Glyphosate

The median time to pupation for the SENN control treatment was 13.50 ± 3.00 days (Fig. 5.12). The median time to pupation for the SENN 0.00035% glyphosate treatment was 13.00 ± 4.00 days (Fig. 5.12). The median time to pupation for the SENN 0.007% glyphosate treatment was 12.00 ± 3.00 days (Fig. 5.12). The median time to pupation for the SENN 0.0014% glyphosate treatment was 12.00 ± 2.00 days (Fig. 5.12).

The median time to pupation for the SENN DDT control treatment was 12.00 ± 2.00 days (Fig. 5.12). The median time to pupation for the SENN DDT 0.00035% glyphosate treatment was 11.00 ± 2.00 days (Fig. 5.12). The median time to pupation for the SENN DDT 0.0007% glyphosate treatment was 12.00 ± 3.00 days (Fig. 5.12). The median time to pupation for the SENN DDT 0.0014% glyphosate treatment was 12.00 ± 2.00 days (Fig. 5.12).

All SENN treatments pupated significantly faster than the control treatment (log-rank test: $\chi^2_{(3)}$ = 78.00, p = < 2.00 x 10^{-16}). For SENN DDT, the 0.00035% glyphosate treatment pupated significantly faster than the control and other two treatments, and the 0.0007% glyphosate treatment pupated significantly faster than the 0.0014% glyphosate treatment (log-rank test: $\chi^2_{(3)}$ = 26.90, p = 6.00 x 10^{-6}). SENN DDT pupated faster than SENN for the control treatment, while SENN pupated faster than SENN DDT for the 0.00035% and 0.0014% glyphosate treatments (log-rank: $\chi^2_{(7)}$ = 118.00, p = < 2.00 x 10^{-16}).

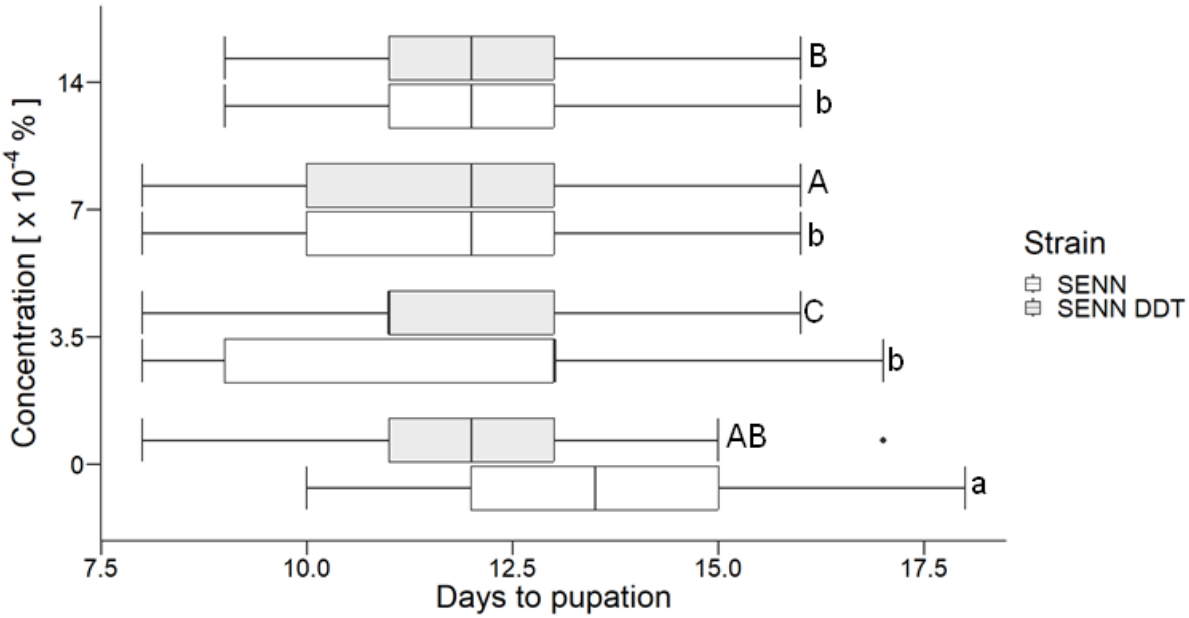


Figure 5.12. Time to pupation in days for SENN (white shading) and SENN DDT (grey shading) larvae exposed to different concentrations of glyphosate.
Lower case letters indicate significant differences between treatments for SENN, while upper case letters indicate significant differences between SENN DDT.

5.3.3.3 Chloride

The median time to pupation for the SENN control treatment was 14.00 ± 3.00 days (Fig. 5.13). The median time to pupation for the SENN 0.0002% chloride treatment was 13.00 ± 1.00 days (Fig. 5.13). The median time to pupation for the SENN 0.0004% chloride treatment was 14.00 ± 2.00 days (Fig. 5.13). The median time to pupation for the SENN 0.0008% chloride treatment was 13.00 ± 1.00 days (Fig. 5.13).

The median time to pupation for the SENN DDT control treatment was 14.00 ± 2.00 days (Fig. 5.13). The median time to pupation for the SENN DDT 0.0002% chloride treatment was 13.00 ± 1.00 days (Fig. 5.13). The median time to pupation for the SENN DDT 0.0004% chloride treatment was 14.00 ± 1.00 days (Fig. 5.13). The median time to pupation for the SENN DDT 0.0014% chloride treatment was 13.00 ± 2.00 days (Fig. 5.13).

All SENN treatments pupated significantly faster than the control treatment, while the 0.0002% and 0.0008% chloride treatments pupated significantly faster than the 0.0004% chloride treatment (log-rank test: $\chi^2_{(3)} = 39.40$, $p = 1.00 \times 10^{-8}$). For SENN DDT all treatments pupated significantly faster than the control treatment, with the 0.0008% chloride treatment pupating the fastest, followed by the 0.0004% and the 0.0002% chloride treatments (log-rank test: $\chi^2_{(3)} = 120.00$, $p = < 2.00 \times 10^{-16}$).

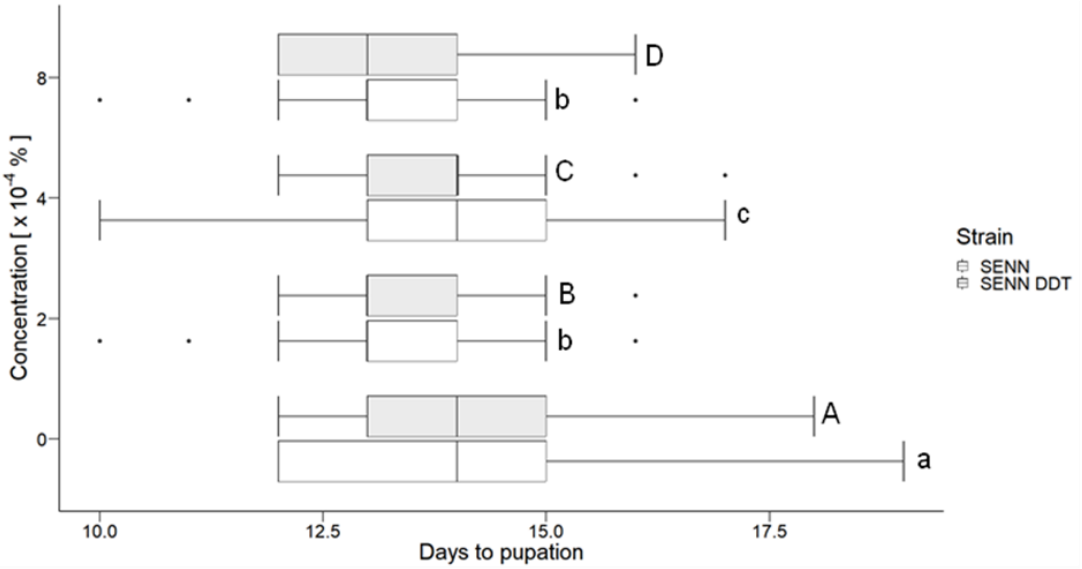


Figure 5.13. Time to pupation in days for SENN (white shading) and SENN DDT (grey shading) larvae exposed to different chloride concentrations.
Lower case letters indicate significant differences between treatments for SENN, while upper case letters indicate significant differences between SENN DDT.

5.3.3.4 Styrene

The median time to pupation for the SENN control treatment was 9.00 ± 2.00 days (Fig. 5.14). The median time to pupation for the SENN 0.0005% styrene treatment was

8.00 ± 1.00 days (Fig. 5.14). The median time to pupation for the SENN 0.00095% styrene treatment was 9.00 ± 2.00 days (Fig. 5.14). The median time to pupation for the SENN 0.0019% styrene treatment was 9.00 ± 1.25 days (Fig. 5.14).

The median time to pupation for the SENN DDT control treatment was 9.00 ± 2.00 days (Fig. 5.14). The median time to pupation for the SENN DDT 0.0005% styrene treatment was 9.00 ± 2.00 days (Fig. 5.14). The median time to pupation for the SENN DDT 0.00095% styrene treatment was 9.00 ± 1.00 days (Fig. 5.14). The median time to pupation for the SENN DDT 0.0019% styrene treatment was 9.00 ± 1.00 days (Fig. 5.14).

The SENN 0.0095% styrene treatment pupated faster than all the other treatments, followed by the 0.0005% styrene treatment (log-rank test: $\chi^2_{(3)} = 49.90$, $p = 8.00 \times 10^{-11}$). For SENN DDT, only the 0.00095% styrene treatment pupated faster than the control and the 0.0019% styrene treatments (log-rank test: $\chi^2_{(3)} = 12.20$, $p = 0.010$). The SENN 0.0005% styrene treatment pupated significantly faster than the SENN DDT 0.0005% styrene treatment (log-rank: $\chi^2_{(7)} = 69.30$, $p = 2.00 \times 10^{-12}$).

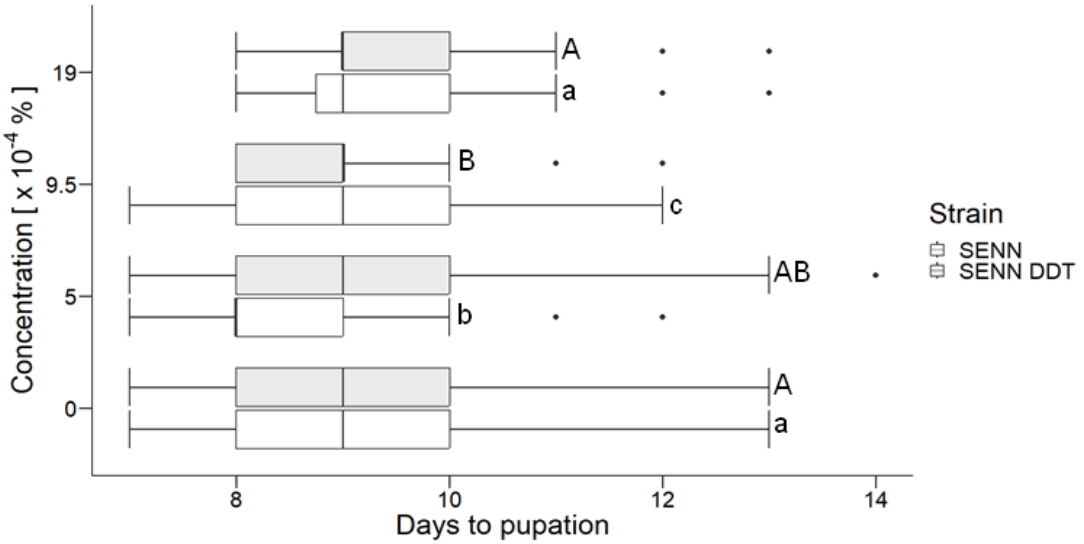


Figure 5.14. Time to pupation in days for SENN (white shading) and SENN DDT (grey shading) larvae exposed to different concentrations of styrene.
Lower case letters indicate significant differences between treatments for SENN, while upper case letters indicate significant differences between treatments for SENN DDT.

5.3.4 Longevity

5.3.4.1 Manganese

The median lifespan for adults of the SENN control treatment was 26.00 ± 17.50 days, with a maximum lifespan of 51.00 days. (Fig. 5.15). The median lifespan for the SENN 0.0025% manganese treatment was 24.00 ± 14.25 days, with a maximum lifespan of 38.00 days (Fig. 5.15). The median lifespan for the SENN 0.005% manganese treatment was 19.00 ± 13.75 days, with a maximum of 38.00 days (Fig. 5.15). The median lifespan for the SENN 0.01% manganese treatment was 15.00 ± 15 days, with a maximum lifespan of 32.00 days (Fig. 5.15).

Adults of the SENN control treatment had significantly longer lifespans compared to the other manganese treatments, and adults of the 0.01% manganese treatment had the shortest lifespan of all treatments (log rank test: $\chi^2_{(3)} = 31.60$, $p = 6.00 \times 10^{-7}$). There was no significant difference in adult lifespans between the 0.005% and 0.0025% manganese treatments (log rank test: $\chi^2_{(3)} = 31.60$, $p = 6.00 \times 10^{-7}$).

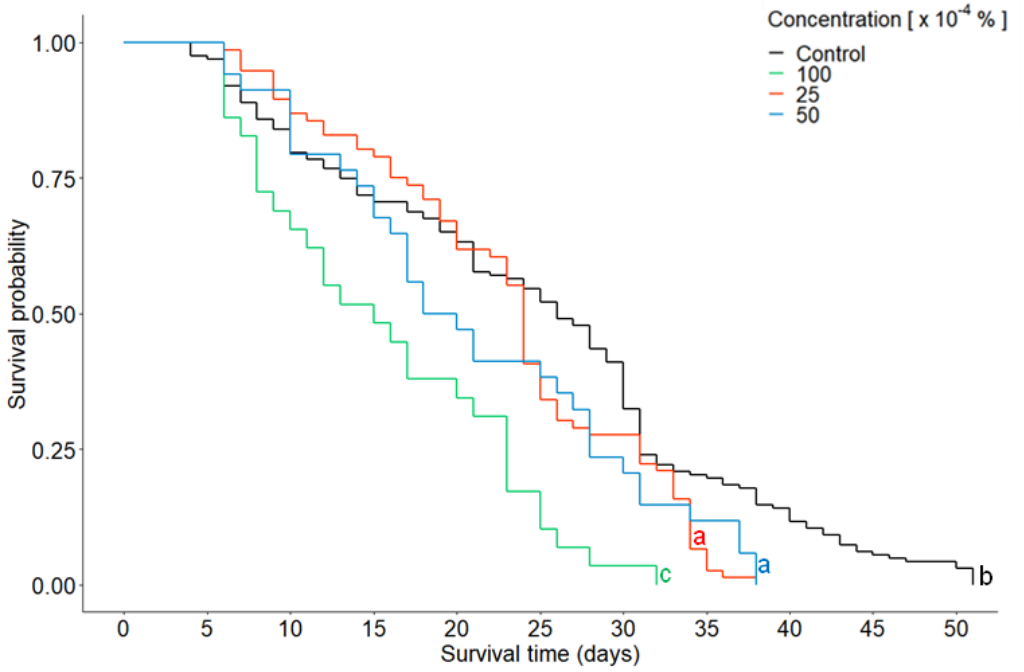


Figure 5.15. Survival probability of insecticide-unselected *An. arabiensis* (SENN) adults after larval exposure to different concentrations of manganese.
Letters indicate significant differences in longevity between treatments.

The median lifespan for adults of the SENN DDT control treatment was 26.00 ± 23.00 days, with a maximum lifespan of 52.00 days (Fig. 5.16). The median lifespan for the SENN DDT 0.0025% manganese treatment was 21.00 ± 18.00 days, with a maximum lifespan of 38.00 days (Fig. 5.16). The median lifespan for the SENN DDT 0.005% manganese treatment was 18.00 ± 16.25 days, with a maximum lifespan of 36.00 days (Fig. 5.16). The median lifespan for the SENN DDT 0.01% manganese treatment was 15.00 ± 21.00 days, with a maximum lifespan of 34.00 days (Fig. 5.16).

The adults of the SENN DDT control treatment had significantly longer lifespans compared to the other manganese treatments, while there were no differences in adult lifespan between the 0.0025%, 0.005% and 0.01% manganese treatments (log rank test: $\chi^2_{(3)} = 29.10$, $p = 2.00 \times 10^{-6}$).

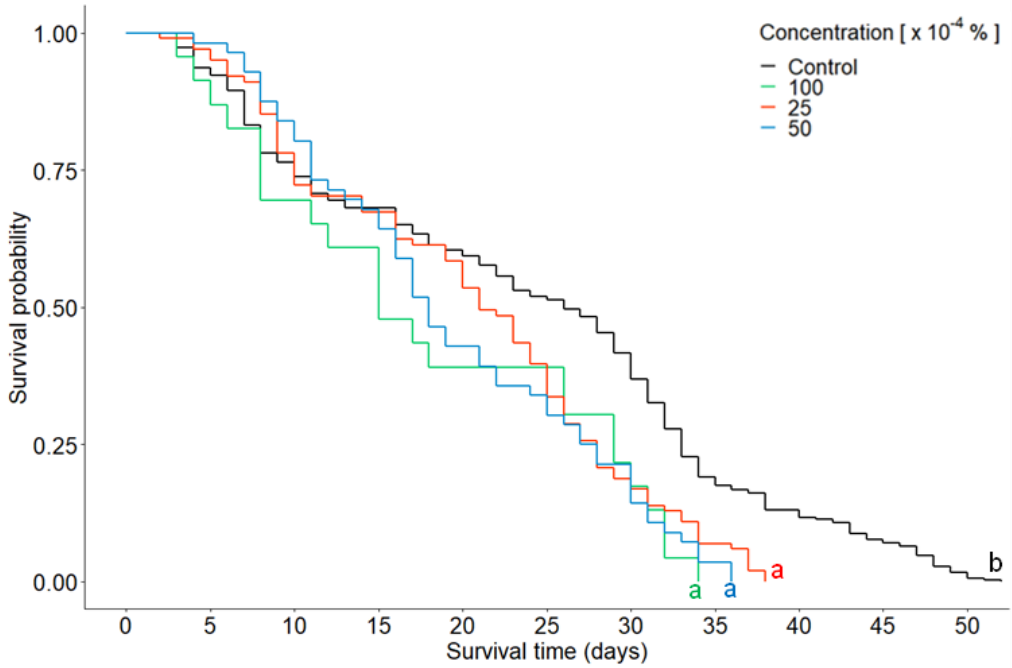


Figure 5.16. Survival probability of insecticide-selected *An. arabiensis* (SENN DDT) adults after larval exposure to different concentrations of manganese.
Letters indicate significant differences in longevity between treatments.

5.3.4.2 Glyphosate

The median lifespan for adults of the SENN control treatment was 26.00 ± 17.50 days, with a maximum lifespan of 51.00 days. (Fig. 5.17). The median lifespan for the SENN 0.00035% glyphosate treatment was 26.00 ± 15.75 days, with a maximum lifespan of

53.00 days (Fig. 5.17). The median lifespan for the SENN 0.0007% glyphosate treatment was 26.00 ± 5.00 days, with a maximum of 47.00 days (Fig. 5.17). The median lifespan for the SENN 0.0014% glyphosate treatment was 26.00 ± 6.25 days, with a maximum lifespan of 48.00 days (Fig. 5.17).

There was no significant difference in lifespan between the control and other glyphosate treatments for SENN adults, while adults of the 0.00035% treatment lived significantly longer than the 0.0007% and 0.0014% treatments (log rank: $\chi^2_{(3)} = 10.10$, $p = 0.02$).

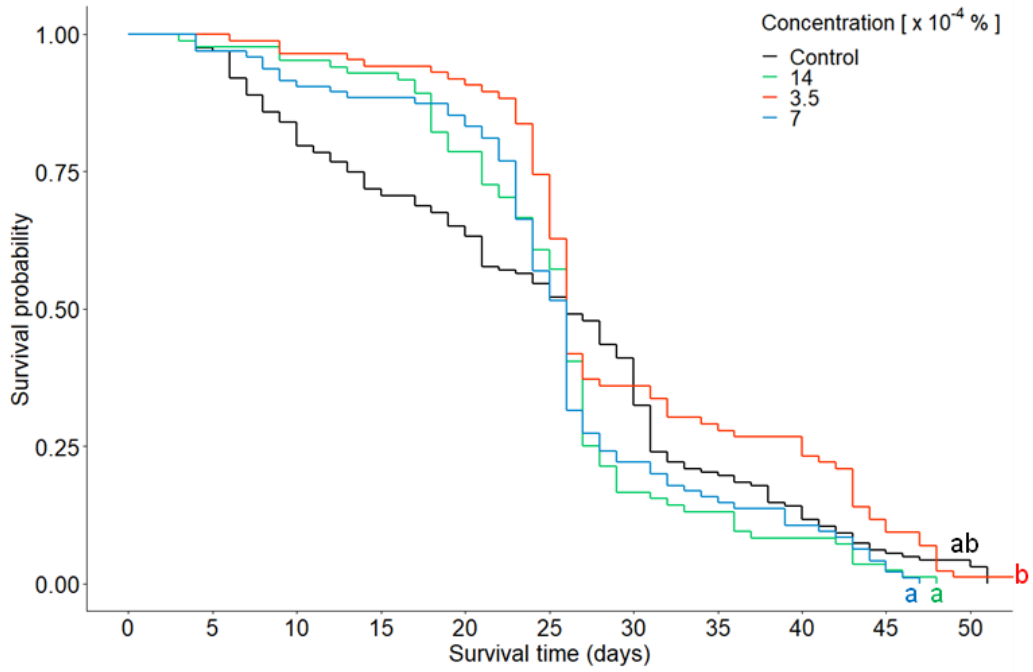


Figure 5.17. Survival probability of insecticide-unselected *An. arabiensis* (SENN) adults after larval exposure to different concentrations of glyphosate.
Letters indicate significant differences in longevity between treatments.

The median lifespan for adults of the SENN DDT control treatment was 26.00 ± 23.00 days, with a maximum lifespan of 52.00 days (Fig. 5.18). The median lifespan for the SENN DDT 0.00035% glyphosate treatment was 27.00 ± 5.00 days, with a maximum lifespan of 49.00 days (Fig. 5.18). The median lifespan for the SENN DDT 0.0007% glyphosate treatment was 28.00 ± 10.00 days, with a maximum lifespan of 51.00 days

(Fig. 5.18). The median lifespan for the SENN DDT 0.0014% glyphosate treatment was 27.00 ± 5.50 days, with a maximum lifespan of 48.00 days (Fig. 5.18).

There was no significant difference in adult lifespan between treatments for SENN DDT (log rank: $\chi^2_{(3)}$ = 3.20, p = 0.40).

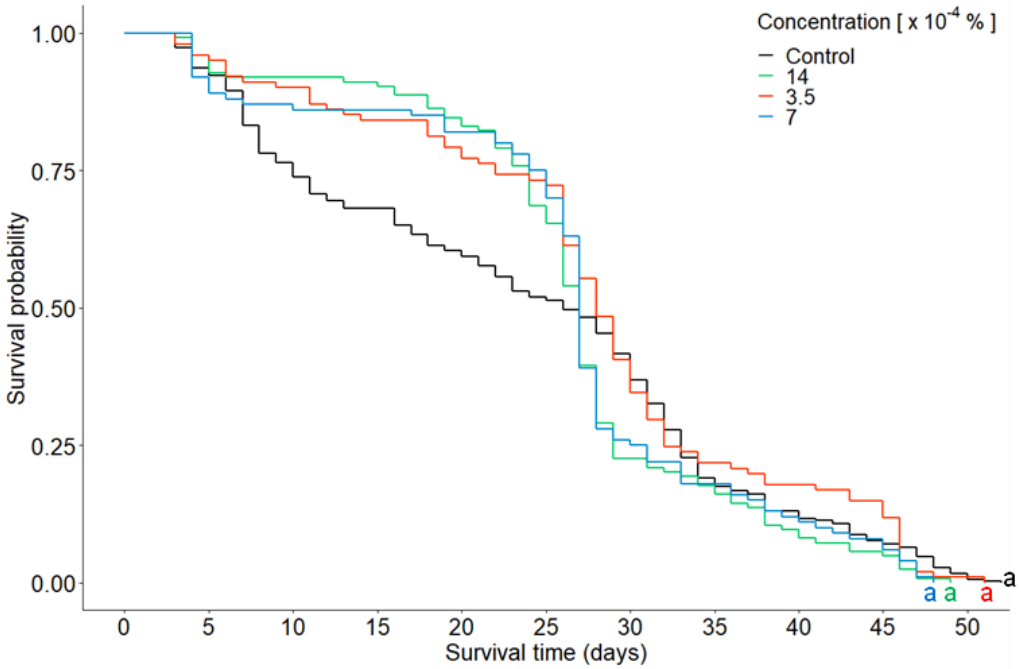


Figure 5.18. Survival probability of insecticide-selected *An. arabiensis* (SENN DDT) adults after larval exposure to different concentrations of glyphosate.
Letters indicate significant differences in longevity between treatments.

5.3.4.3 Chloride

The median lifespan for adults of the SENN control treatment was 26.00 ± 17.50 days, with a maximum lifespan of 51.00 days. (Fig. 5.19). The median lifespan for the SENN 0.0002% chloride treatment was 31.00 ± 12.00 days, with a maximum lifespan of 45.00 days (Fig. 5.19). The median lifespan for the SENN 0.0004% chloride treatment was 27.00 ± 16.00 days, with a maximum of 47.00 days (Fig. 5.19). The median lifespan for the SENN 0.0008% chloride treatment was 25.00 ± 18.50 days, with a maximum lifespan of 45.00 days (Fig. 5.19).

There was no significant difference in SENN adult lifespans between the control and other chloride treatments, and only adults of the 0.0002% treatment lived significantly longer than the 0.0004% and 0.0008% treatments (log rank: $\chi^2_{(3)}$ = 6.30, p = 0.10).

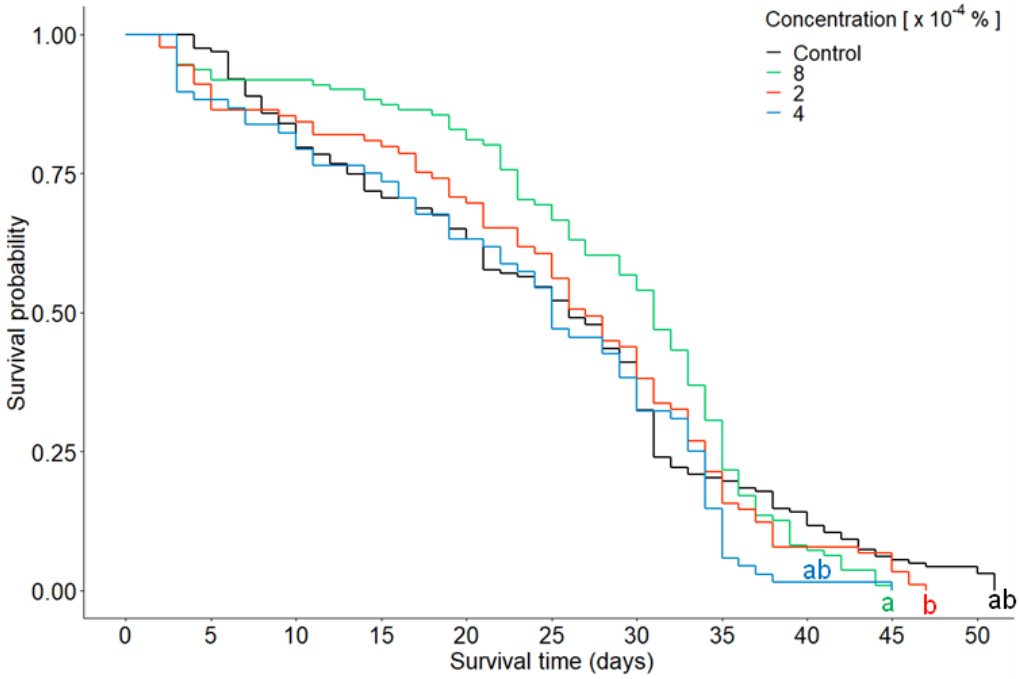


Figure 5.19. Survival probability of insecticide-unselected *An. arabiensis* (SENN) adults after larval exposure to different concentrations of chloride.
Letters indicate significant differences in longevity between treatments.

The median lifespan for adults of the SENN DDT control treatment was 26.00 ± 23.00 days, with a maximum lifespan of 52.00 days. (Fig. 5.20). The median lifespan for the SENN DDT 0.0002% chloride treatment was 27.00 ± 17.00 days, with a maximum lifespan of 46.00 days (Fig. 5.20). The median lifespan for the SENN DDT 0.0004% chloride treatment was 28.00 ± 18.00 days, with a maximum of 48.00 days (Fig. 5.20). The median lifespan for the SENN DDT 0.0008% chloride treatment was 26.00 ± 14.00 days, with a maximum lifespan of 46.00 days (Fig. 5.20).

There was no significant difference in SENN DDT adult lifespans between all chloride and control treatments (log rank: $\chi^2_{(3)}$ = 0.70=, p = 0.90).

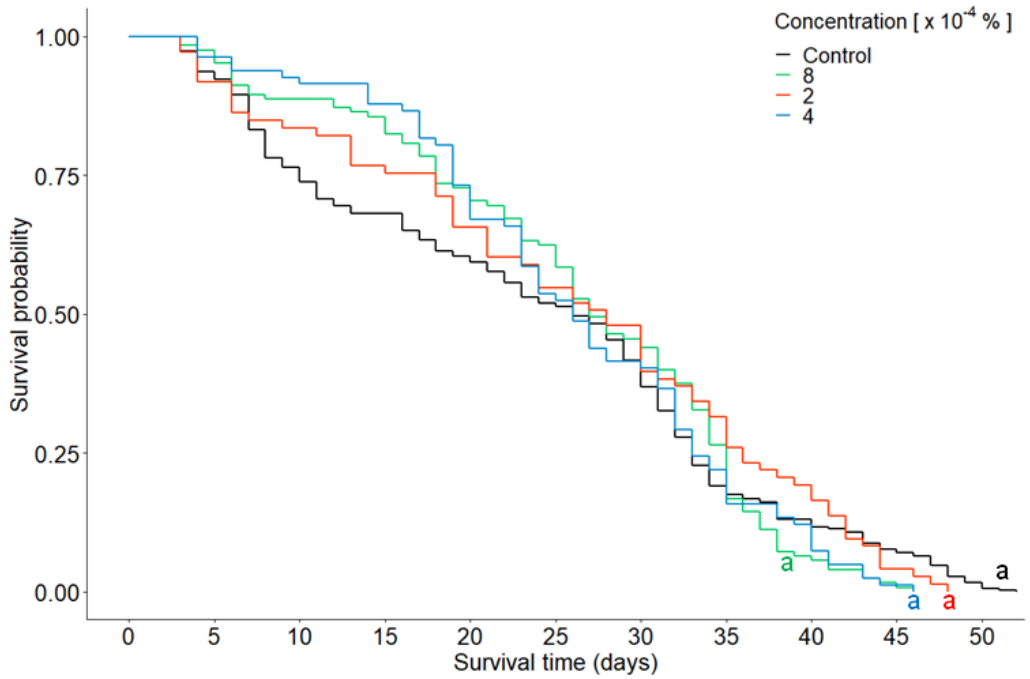


Figure 5.20. Survival probability of insecticide-selected *An. arabiensis* (SENN DDT) adults after larval exposure to different concentrations of chloride.
Letters indicate significant differences in longevity between treatments.

5.3.4.4 Styrene

The median lifespan for adults of the SENN control treatment was 26.00 ± 17.50 days, with a maximum lifespan of 51.00 days. (Fig. 5.21). The median lifespan for the SENN 0.0005% styrene treatment was 20.00 ± 7.25 days, with a maximum lifespan of 31.00 days (Fig. 5.21). The median lifespan for the SENN 0.00095% styrene treatment was 19.00 ± 14.00 days, with a maximum of 40.00 days (Fig. 5.21). The median lifespan for the SENN 0.0019% styrene treatment was 24.50 ± 7.50 days, with a maximum lifespan of 30.00 days (Fig. 5.21).

Adult lifespans of the SENN control treatment were significantly longer than the other styrene treatments and adults of the 0.0019% styrene treatment had significantly shorter lifespans than the 0.0005% treatment (log rank: $\chi^2_{(3)} = 45.80$, $p = 6.00 \times 10^{-10}$).

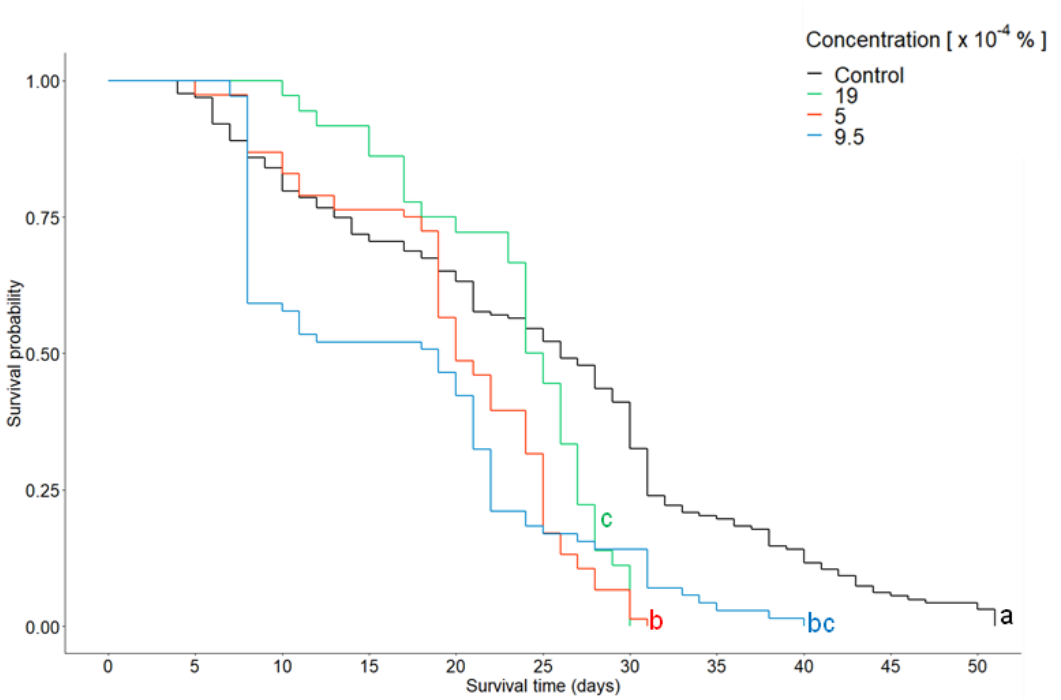


Figure 5.21. Survival probability of insecticide-unselected *An. arabiensis* (SENN) adults after larval exposure to different concentrations of styrene.
Letters indicate significant differences in longevity between treatments.

The median lifespan for adults of the SENN DDT control treatment was 26.00 ± 23.00 days, with a maximum lifespan of 52.00 days (Fig. 5.22). The median lifespan for the SENN DDT 0.0005% styrene treatment was 20.50 ± 9.00 days, with a maximum lifespan of 31.00 days (Fig. 5.22). The median lifespan for the SENN DDT 0.00095% styrene treatment was 8.00 ± 00.00 days, with a maximum of 9.00 days (Fig. 5.22). The median lifespan for the SENN DDT 0.0019% styrene treatment was 11.00 ± 21.00 days, with a maximum lifespan of 39.00 days (Fig. 5.22).

The adult lifespans of the SENN DDT control treatment were significantly longer than the other styrene treatments and adults of the 0.00095% styrene treatment had significantly shorter lifespans than the control and other styrene treatments (log rank: $\chi^2_{(3)} = 98.00$, $p = < 2.00 \times 10^{-16}$).

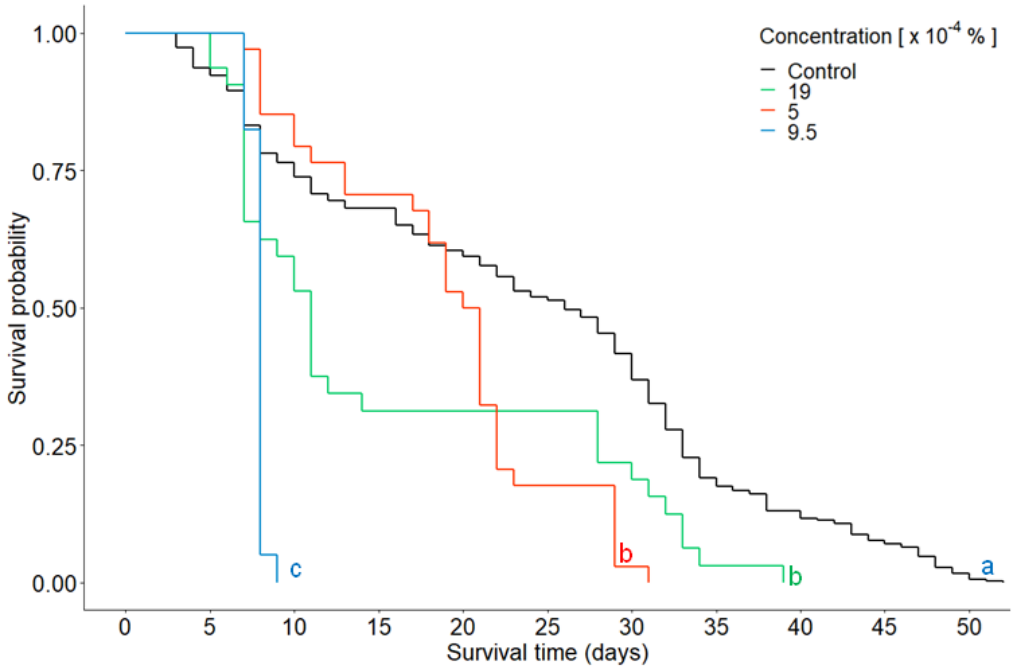


Figure 5.22. Survival probability of insecticide-selected *An. arabiensis* (SENN DDT) adults after larval exposure to different concentrations of styrene.
Letters indicate significant differences in longevity between treatments.

5.3.5 Baseline characterisation of SENN and SENN DDT insecticide resistance

Mean mortality of adult SENN females for 4% DDT was 85.86% (n = 114), 97.17% for 0.05% deltamethrin (n = 193), 88.74% for 0.75% permethrin (n = 117), 100.00% for 0.25% pirimiphos methyl (n = 111) and 95.21% for 5% malathion (n = 108) (Fig. 5.23).

Mean mortality of adult SENN DTT females for 4% DDT was 16.12% (n = 250), 58.78% for 0.05% deltamethrin (n = 236), 32.86% for 0.75% permethrin (n = 141), 97.73% for 0.25% pirimiphos methyl (n = 103) and 26.37% for 5% malathion (n = 162) (Fig. 5.23).

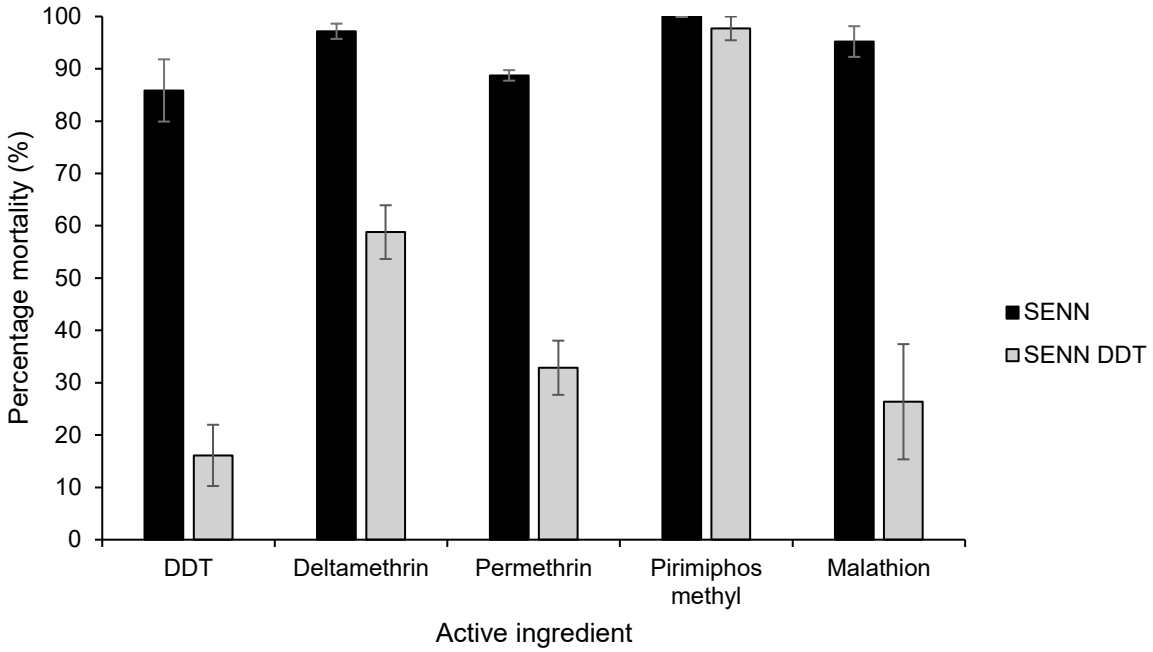


Figure 5.23. Percentage mortality of two laboratory strains of *An. arabiensis* after one hour exposure to different insecticidal active ingredients.

Black bars represent adult female SENN mosquitoes and grey bars represent adult female SENN DDT mosquitoes. Error bars represent the standard error of the mean.

5.4 Discussion

This study investigated the hormetic potential of four common agri-pollutants at different dosages in two laboratory strains of *An. arabiensis*. Previous studies that investigated the effects of pollutants on mosquito life-history traits focused on a single dose (Oliver and Brooke, 2018a; Jeanrenaud, Brooke and Oliver, 2019; Samuel, Brooke and Oliver, 2020). The results show that there is a possibility that common agri-pollutants may induce hormetic responses in *An. arabiensis* when reared under laboratory conditions. This may have implications for *An. gambiae s.l.* vector populations in field.

Hormesis plays a much more central role in toxicology than previously thought (Calabrese and Baldwin, 2001) and has been demonstrated for plants (Brito *et al.*, 2018; Erofeeva, 2022), aphids (Ayyanath *et al.*, 2013) and various other insect species (reviewed in Cutler *et al*. (2022)). Understanding hormesis and its effects on life-history traits is therefore important to optimise vector control programs.

The study of hormesis in vector populations is becoming increasingly relevant, since hormetic effects have been shown to have impacts on several life-history traits. These include increased reproduction, longevity, growth, insecticide resistance, and immunity (reviewed in Guedes and Cutler (2014) and Cutler *et al*. (2022)). Vectors with longer lifespans increase the risk of disease transmission, since the window of disease transmission becomes extended (Garrett-Jones and Shidrawi, 1969; Coosemans *et al.*, 1992). Shorter developmental periods likewise affect the risk of transmission, as this results in increased generational turnover, and consequently, an increased number of disease-carrying adults (Coosemans *et al.*, 1992; da-Silva Araújo, Gil and de Almeida e-Silva, 2012). Tolerance to insecticides have been shown to reduce the efficacy of vector control programs (Hargreaves *et al.*, 2000; Churcher *et al.*, 2016). Changes in life-history traits and insecticide resistance due to hormetic effects may therefore have critical epidemiological consequences.

The concentrations of pollutants used in this study that are relevant to mosquito breeding sites in natural environments were not known. References for the concentrations present in surface water, groundwater and drinking water exist, however, these concentrations do not necessarily accurately reflect the concentrations

present in mosquito breeding sites, as these sites are typically much smaller and more dilute. For example, styrene concentrations present in industrial effluent water ranges from < 0.010 mg/L to 0.97 mg/L (U.S. Agency for Toxic Substances and Disease Registry, 2010). Manganese concentrations in surface water ranges between 0.40 mg/L and 0.80 mg/L (World Health Organization, 2021), while glyphosate ranges between 0.21 mg/L (Álvarez Bayona *et al.*, 2022) and up to 6.10 mg/L in surface runoff (Lima *et al.*, 2023). Chloride concentrations in surface water has been found to range between 30.60 mg/L to 487.12 mg/L and 135.70 mg/L to 2 735.00 mg/L in wastewater (Hong *et al.*, 2023). These concentration ranges are unlikely to support the full lifecycle of the mosquito. We therefore used lower doses in this study to support the entire lifecycle of the mosquito.

From the WHO bioassay data, it is clear that SENN and SENN DDT differ in resistance phenotype (Figure 5.23). Since the two strains are genetically related, they are good candidates to investigate whether a pre-existing insecticide resistance phenotype influences the effects of pollution on the life-history traits of vectors. Clear differences were observed in the patterns of responses between SENN and SENN DDT when exposed to agri-pollutants. Dose-dependent responses were more pronounced in the insecticide-unselected strain SENN, compared to the insecticide-selected strain SENN DDT.

In general, SENN DDT was not adversely affected by exposure to Agri-pollutants. There were no marked effects on pupation success for any of the pollutants (Figures 5.7 – 5.10 B), and only chloride significantly decreased developmental time (Figure 5.13). There were dose-dependent responses on developmental time when exposed to glyphosate (Figure 5.12) and styrene (Figure 5.14), where the lower dose performed better than the higher dose. In terms of longevity, only manganese (Figure 5.16) and styrene (Figure 5.22) had a significant negative impact compared to the control treatment and dose-dependent responses were only evident for styrene.

By contrast, SENN had several variable effects as a result of exposure to Agri-pollutants. Manganese had no marked effects on pupation success (Figure 5.7 A) and developmental time (Figure 5.11). However, manganese exposure negatively impacted longevity (Figure 5.15), where adult longevity decreased as the dose increased. Glyphosate significantly increased pupation success (Figure 5.8 A) and decreased

developmental time (Figure 5.12). Exposure to glyphosate had dose-dependent effects on longevity (Figure 5.17), where the low dose performed significantly better than the other doses. Exposure to chloride had many effects. Treatment with chloride significantly increased pupation success (Figure 5.9 A) and reduced developmental time (Figure 5.13). There were dose-dependent responses in terms of longevity when exposed to chloride (Figure 5.19), where the highest dose had the greatest increase in longevity. Exposure to styrene had a high dose disadvantage on pupation success (Figure 5.10 A), as well as variable effects on development that were dependent on dose (Figure 5.14). Exposure to styrene also had variable dose-dependent effects on longevity (Figure 5.21). Longevity was generally significantly negatively impacted, and the highest dose had the greatest increase in longevity.

Previous studies have also shown a differential response between SENN and SENN DDT when exposed to heavy metal pollutants. Jeanrenaud, Brooke and Oliver (2022) showed that SENN was more sensitive to epigenetic changes in the environment compared to SENN DDT. With differential responses already evident in the laboratory strains of *An. arabiensis* when exposed with low dosages of agri-pollutants, it is plausible that higher doses that are present within the breeding habitats are likely having hormetic effects.

Studies have also shown that SENN DDT typically copes better with more toxic pollutants compared to SENN (Oliver and Brooke, 2018a; Jeanrenaud, Brooke and Oliver, 2019; Samuel, Brooke and Oliver, 2020). The most toxic pollutants used in this study were styrene and manganese (oral LD_{50} on mice 316 mg/kg for styrene and 275-450 mg/kg for manganese) (International Program on Chemical Safety (IPCS), 1981, 1996). Glyphosate and chloride are much less toxic (oral LD_{50} on mice > 2 000 mg/kg for glyphosate and 5 120 mg/kg for chloride) (Hoffmann *et al.*, 2010; Health Canada Pest Management Regulatory Agency, 2015). Results from this study corroborate this finding, as manganese and styrene generally did not affect SENN DDT, while SENN thrived in chloride and glyphosate.

It is difficult to effectively conclude whether the agri-pollutants used in this study cause hormesis due to the low dosages used. However, it is known that chloride (in the form of sodium chloride) is toxic to *An. arabiensis* at higher doses (LD_{50} 10.5 – 14.0 g/L NaCl; fourth instar larvae, acute exposure) and lethal at 17.5 g/L NaCl (LD_{99}, fourth

instar larvae, acute exposure) (Ould Lemrabott *et al.*, 2020). The advantages in longevity and development at low doses for SENN when exposed to chloride may indicate that chloride may induce hormesis. A study investigating the effects of glyphosate on life-history traits and insecticide resistance of SENN and SENN DDT showed evidence for hormesis at higher doses (Oliver and Brooke, 2018a). The doses used in this study may, therefore, have been too low to elicit a hormetic response in SENN DDT.

The general pattern observed in literature is that longer lifespans are typically associated with slower development, while faster development results in shorter lifespans (Hou, 2013; Lee, Monaghan and Metcalfe, 2013; Lind *et al.*, 2017). This trend may be related to increased nutrient accumulation, specifically proteins, as a result of increased developmental time, which leads to increased longevity, and vice versa (Reis, 2016). This has also been observed in *Anopheles* spp., where *An. funestus* develops significantly slower than *An. gambiae*, but has significantly increased longevity in comparison (Oliver, Lyons and Brooke, 2022). This trend was generally not observed in our results, except when both strains were exposed to styrene. This may be related to the low dosages that were used for the other three pollutants used in this study. As such, there is a likelihood that, although there were statistically significant developmental changes, the differences were not marked enough to affect longevity.

There were a few limitations in this study. Due to time and infrastructure constraints, this study only looked at three life-history traits: larval survival, larval developmental time and adult longevity. Laboratory strains of *An. arabiensis* were used as availability of field collected mosquitoes were limited, due to efficient vector control interventions. Although *An. merus* is the most abundant *An. gambiae* complex member in Mpumalanga, *An. arabiensis* was chosen for this study as it is the only member currently implicated as a vector in the province. The lower doses used in this study were chosen specifically to support the entire lifecycle of the mosquito. Future work should focus on investigating the effects of hormesis in *An. arabiensis* from Mpumalanga on insecticide resistance, as well as other life-history traits such as fertility and fecundity.

5.5 Conclusion

This study has shown that investigations into hormesis in *Anopheles* mosquitoes need to consider the toxicity of the compound, as well as the dose. Single-dose studies are limited in their scope. The most likely compounds that were investigated in this study to cause a hormetic response at the appropriate dose are glyphosate and chloride. Further research into the hormetic effects of pollutants is needed, as small changes in adult vector longevity, tolerance to insecticides, and immature developmental time may have critical epidemiological consequences.

CHAPTER 6

THE EFFECT OF SUB-LETHAL EXPOSURE OF TEMEPHOS ON THE LONGEVITY AND GUT MICROBIOTA OF THE MAJOR MALARIA VECTOR *ANOPHELES ARABIENSIS* PATTON (DIPTERA: CULICIDAE)

6.1 Introduction

6.1.1 Larval source management

The control of malaria vectors relies primarily on indoor-based strategies such as IRS programmes and LLINs (World Health Organization, 2023). These strategies have been effective for the control of indoor biting and/ or indoor resting malaria vectors, such as *Anopheles funestus* (Kouznetsov, 1977; Mabaso, Sharp and Lengeler, 2004). However, indoor-based control strategies are largely ineffective against outdoor-biting and/ or outdoor-resting vectors, such as *An. arabiensis* (Kitau *et al.*, 2012; Bayoh *et al.*, 2014). Indoor insecticide-based control strategies can also act as a selection pressure, where the vector populations might shift from predominantly indoor-resting and feeding to outdoor-resting and feeding. This shift may contribute to the residual transmission of malaria (Björkman *et al.*, 2019; Hii, Hustedt and Bangs, 2021). Therefore, there is a need to have a combination of vector control tools to mitigate these challenges.

Larval source management (LSM) is one of the methods that can address the problem of residual transmission that occurs as a result of outdoor-biting and resting vectors (Amajoh *et al.*, 2013). Key advantages of LSM are that it targets both indoor- and outdoor-biting and resting vectors and can target specific malaria hotspots (Amajoh *et al.*, 2013). Critically, LSM can help manage the development of insecticide resistance (Amajoh *et al.*, 2013).

The management of larval sources can be categorised into four distinct strategies. Environmental modification strategies include habitat modification and habitat manipulation (Amajoh *et al.*, 2013). Habitat modification involves the elimination of larval habitats by permanently altering the environment via landscaping or surface water drainage (Amajoh *et al.*, 2013). Habitat manipulation involves the temporary alteration of larval habitats via the elimination of water pooling, such as flushing or

drain clearance (Amajoh *et al.*, 2013). The third strategy involves biological control, which involves the use of natural enemies in larval habitats, such as predatory fish, invertebrates, parasites or pathogens (Amajoh *et al.*, 2013). Finally, larviciding involves the application of biological or chemical insecticides to larval habitats (Amajoh *et al.*, 2013). Successful LSM, however, requires a thorough understanding of larval breeding habitats (Gimnig *et al.*, 2001).

Anopheles gambiae s.l. typically prefer to breed in temporary, sunlit pools (Gillies and de Meillon, 1968). However, a large variety of different breeding habitats have been described for members of this species complex (Fillinger *et al.*, 2004). Increasingly, *An. gambiae s.l.* have been adapting to breeding in polluted breeding habitats (Awolola *et al.*, 2007; Antonio-Nkondjio *et al.*, 2011; Jones, Toé, *et al.*, 2012). *Anopheles funestus s.l.* prefers large, shaded, semi-permanent water bodies with aquatic vegetation and larvae often hide below the water surface for extended periods (Gillies and de Meillon, 1968). Effective LSM will therefore be dependent on species composition in areas where LSM can be considered as a vector control strategy.

Successful LSM have been implemented in the past. In 1929 modification of river boundaries, draining of swamps, and the application of oil to open water bodies significantly reduced the malaria burden within three to five years by 70 - 95% in Zambia (Utzinger, Tozan and Singer, 2001). A study in western Kenya that utilised a variety of LSM techniques found that *Anopheles* larvae were 55 times more likely to be present in habitats where no LSM interventions were implemented (Imbahale *et al.*, 2012), compared to areas where LSM were implemented. Successful LSM have also been reported from Israel, the United States and Italy (Kitron and Spielman, 1989).

Larviciding has been instrumental in vector control programs. During the 1930's and 1940's, larviciding with Paris Green (copper arsenate) was particularly effective in the complete eradication of the invasive *An. gambiae* from Brazil during a significant outbreak of malaria (Soper and Wilson, 1943). Egypt followed a similar eradication program in the 1940's and likewise eradicated *An. gambiae* from upper Egypt (Shousha, 1948). Larviciding was also a key strategy for the control of other disease vectors, such as the eradication of *Simulium damnosum* Theobald (Diptera: Simuliidae), the vector of onchocerciasis, in West Africa (Boatin, 2008; Traoré *et al.*, 2009).

A key advantage of larviciding is the diverse range of products that are available, compared to products for adult mosquito control. Chemical adulticides primarily stem from four classes of insecticides (Sternberg and Thomas, 2018), while larvicidal products include bacterial larvicides, organophosphates, benzoylureas, juvenile hormone mimics and spinosyns (Amajoh *et al.*, 2013). This diverse range of available products, particularly the diverse range of mechanisms available, help combat the development of insecticide resistance (Fillinger and Lindsay, 2011).

6.1.2 Impact of exposure of mosquitoes to sub-lethal doses of insecticide on life-history traits of mosquito vectors

Exposure of larvae to sub-lethal concentrations of insecticides have been shown to impact various life-history traits of mosquito vectors. Exposure of larvae to sub-lethal quantities of pyriproxyfen (growth hormone regulator) granules reduced fertility and fecundity in the subsequent adult generation of *An. gambiae s.l.* (Mbare, Lindsay and Fillinger, 2013). Sub-lethal exposure of fourth instar *Aedes aegypti* larvae to fenitrothion (organophosphate) decreased larval development time and adult emergence (Shaalan *et al.*, 2005). The adult lifespan of *Culex quinquefasciatus* has also been shown to be significantly reduced by sub-lethal exposure to cypermethrin (a pyrethroid) (Olorunfemi, Akinwande and Ashamo, 2016). Effects on these life-history traits as a result of pre-exposure to sub-lethal doses of insecticides can ultimately impact a vector's ability to develop and transmit pathogens, known as vector competence (Vantaux *et al.*, 2016). The sub-lethal exposure to insecticides does not only affect mosquito life-history parameters, but can also affect their midgut microbiota.

The mosquito midgut microbiota has been shown to be critical for normal larval development. The midgut microbiota strongly affects larval survival, adult size (Coon, Brown and Strand, 2016), fecundity, metabolism and longevity (Harrison *et al.*, 2023). The microbiome also assists with blood digestion and, consequently, increased fertility (Gaio *et al.*, 2011), and has been shown to influence vector competence (van Tol and Dimopoulos, 2016). In addition to affecting several life-history traits of both the immature and mature mosquito, the midgut microbiota also aid in the degradation of pesticides, which in turn contributes to the development of insecticide resistance (Dada *et al.*, 2018). Sub-lethal exposure of mosquitoes to insecticides have been shown to

alter their midgut bacterial composition (Juma *et al.*, 2020). Therefore, sub-lethal exposures can have cascading effects on gut bacterial composition, which in turn affects insecticide resistance, *Plasmodium* susceptibility and a range of life-history traits.

Temephos, an organophosphate, is one of the most common chemical larvicides and is recommended by the WHO for use in larviciding programs. It has been shown to be effective in both clean and polluted water bodies, whereas many other larvicides are not effective in polluted water (Amajoh *et al.*, 2013). Temephos gained popularity as a larvicide due to its stability and efficacy at low doses, and its low toxicity against non-target organisms (Amajoh *et al.*, 2013). Temephos has also been shown to work effectively in conjunction with other products, such as entomopathogenic fungi (Vivekanandhan *et al.*, 2018). Sub-lethal exposure to temephos specifically has been shown to impact various life-history traits such as larval survival, swimming behaviour, fertility, fecundity, sex ratio, and reproductive fitness (Sanil and Shetty, 2012; Alvarez Costa *et al.*, 2018). Critically, the lifespan of the adult female has also been shown to be affected by sub-lethal exposure to temephos (Reyes-Villaneuva, Juarez-Eguia and Flores-Leal, 1990; Sanil and Shetty, 2012). The widespread use of temephos may therefore have epidemiological consequences if applied at sub-lethal concentrations.

6.1.3 Context and rationale

South Africa's primary and secondary malaria vectors, *Anopheles parensis, An. vaneedeni* and *An. arabiensis,* are predominantly outdoor- biting and resting (Burke *et al.*, 2017, 2019; Dandalo *et al.*, 2017). Outdoor biting- and resting vectors are not effectively targeted by indoor vector control interventions such as IRS and LLINs. This makes larviciding an attractive option for vector control in South Africa, as larviciding targets both indoor- and outdoor-resting and feeding vectors. Larviciding may be particularly effective in Mpumalanga, where the dominant member of the *An. gambiae s.l.* complex is *An. merus* (this thesis), which primarily feeds and rests outdoors (Gillies and de Meillon, 1968). While *An. merus* has not been directly implicated as a vector of malaria in South Africa, it has been found to contribute to malaria transmission in Madagascar (Tsy *et al.*, 2003) and Mozambique (Cuamba and Mendis, 2009). It is

therefore likely that *An. merus* contributes, at least in part, to malaria transmission in Mpumalanga (Govere *et al.*, 2000; Mbokazi *et al.*, 2018).

Members of the *An. gambiae s.l.* complex are increasingly adapting to breeding in polluted habitats (Awolola *et al.*, 2007; Antonio-Nkondjio *et al.*, 2011; Gunathilaka *et al.*, 2013; King *et al.*, 2017; Azrag and Mohammed, 2018; Ossè *et al.*, 2019). This trend has been observed in Mpumalanga as well (this thesis). Increased temephos resistance in *An. arabiensis* was associated with breeding in polluted water in Sudan (Azrag and Mohammed, 2018). Compounding this issue is that sub-lethal exposure of pesticides have been shown to alter the gut microbiota of mosquitoes (Juma *et al.*, 2020), as well as the presence of an insecticide resistance phenotype. Gut microbiota have been shown to play an important role in insecticide resistance (Dada *et al.*, 2018), susceptibility to *Plasmodium* infection (Boissière *et al.*, 2012) and various life-history traits (Barnard *et al.*, 2019). Breeding in polluted water bodies and sub-lethal exposure to larvicides, such as temephos, may therefore undermine the efficacy of larval source control efforts.

The presence and effects of insecticide resistance on adult longevity and gut microbial composition as a result of sub-lethal exposure has not been characterised in South Africa. This information is important for optimising vector control to achieve malaria elimination goals. This study aimed to investigate the effects of sub-lethal larviciding with a common larvicide, temephos, on adult longevity and gut microbial composition of two laboratory strains of *An. arabiensis* that differ in their insecticide resistance profiles.

6.2. Materials and Methods

6.2.1. Mosquito strains

The mosquito strains used for this study are described in Chapter 2 General Materials and Methods: 2.1 Mosquito Strains Used in Laboratory Studies.

6.2.2 Sub-lethal exposure to temephos

A development assay was conducted using methodology as described in Chapter 2 General Materials and Methods: 2.3 Protocol for Development Assays of Laboratory Reared Mosquito Strains. One of the four rectangular containers per replication served as control and received no treatment. The three remaining containers received the same dose of temephos.

For the SENN DDT strain, a 1.00 x 10^{-4} M solution of temephos (PESTANAL®, analytical standard: cat. number: 31526) was used. For the SENN strain, the original dose was diluted to a final concentration of 1.00 x 10^{-5} M, as larvae did not survive the original dose.

6.2.3 Adult longevity

Sub-lethally exposed larvae were allowed to pupate, and the emerging adults were transferred to rearing cages on the day of emergence. Adults from SENN and SENN DDT were kept in separate rearing cages and provided with 10% sucrose solution *ad libitum*. A control treatment consisted of adults from SENN and SENN DDT that were not exposed to temephos in the larval stage. Longevity was recorded daily and carcasses were removed daily. Rearing cages consisted of 25 males and 25 females, which emerged on the same day. Males and females were allowed to mate, but females were not provided with any blood meals. Four replicate rearing cages were set up for each strain and treatment.

6.2.4 Tissue dissections and DNA extraction

Sub-lethally exposed larvae were allowed to pupate, and the emerging adults were transferred to rearing cages on the day of emergence. Adults from SENN and SENN DDT were kept in separate rearing cages and provided with a 10% sucrose solution *ad libitum*. On the third day post-emergence, female adults were collected and cold-killed at -70°C for two (2) minutes.

All equipment, surfaces, and specimens were sterilised with 70% ethanol and 3% hydrogen peroxide. The midguts of adult female mosquitoes were dissected using a dissection microscope (Olympus SZ61) with a magnification of 40x. Midguts were placed directly into tissue lysis buffer (ATL buffer) and proteinase K, available within the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). One replicate consisted of three midguts. For SENN DDT, there were nine replicates, while for SENN there were seven replicates. The number of replicates were uneven due to the limited number of larvae that pupated and adults that subsequently emerged. The total number of mosquito midguts dissected for SENN DDT were therefore 27 and for SENN 21. Five (5) control replicates were also prepared using SENN and SENN DDT adults originating from larvae that were not exposed to temephos. These served as untreated controls. A negative control consisted of PBS. A commercial microbial DNA extraction control (Zymobiomics microbial DNA community control, cat. number: D6306) was included as extraction positive control.

Total genomic DNA was extracted using the DNeasy® Blood & Tissue kit (Qiagen: 69506, Germany). The samples were incubated overnight at 56°C. The positive and negative controls were subjected to the same DNA extraction procedure.

6.2.5 DNA amplification and sequencing

Total genomic DNA of the midguts of both SENN and SENN DDT were amplified using PCR utilising universal bacterial primers targeting the V3 – V4 hypervariable region of the bacterial 16S rRNA gene (Klindworth *et al.*, 2013).

The forward primer sequence used was 5′ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3′, while the reverse primer was 5′ TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC

WGC AG 3′ (4 nmol Ultramer® DNA Oligo 55 bases: Integrated DNA Technologies, Coralville, IA, USA). Samples were amplified using Kappa HiFi hot start reaction mix (Roche, cat. number: 07958927001) according to the initial amplification protocol of the 16S Metagenomic Sequencing Library Preparation protocol (Illumina TM, San Diego, CA, USA).

A positive control consisted of known bacterial species (ZymoBIOMICS® Microbial Community Standard, D6300, Irvine, CA, USA). Negative controls consisted of a non-template and an extraction buffer control. Both negative and positive controls were also amplified by PCR.

The standard instructions of the 16S Metagenomic Sequencing Library Preparation protocol (Illumina TM, San Diego, CA, USA) were used to perform the library preparation. The libraries were subsequently sequenced using the MiSeq Reagent kit v3 (Illumina, USA) on the MiSeq platform.

Paired-end 2 × 300 base pair sequencing was performed at the Sequencing Core Facility housed at the NICD, Johannesburg.

6.2.6. Data analysis

6.2.6.1 Longevity

A Kaplan-Meier analysis (Kaplan and Meier, 1958) was used to test for significant differences between treatments for each strain and for differences between strains. A log-rank test was used to compare between treatments and controls (Harrington and Fleming, 1982).

6.2.6.2 Sequencing

The bioinformatic analyses performed followed methodology detailed in Chen *et al*. (2024), and is outlined below.

The package trimGalore (v0.6.5-1) was used to filter and pair-end trim sequences in order to remove the Nextera adapter sequences. The MultiQC (v1.6) package was

then used for quality control purposes in order to ensure that clean data were used for all downstream analyses, which were performed in R studio (RStudio Team, 2023). Downstream analyses included the classification, abundance estimations, statistical analysis, and visualization of the data. The DADA2 package (v1.24.0) (Callahan *et al.*, 2016) was used to pre-process the clean reads, which included quality inspection, filtering, trimming, dereplication, sample inference, merging paired-end reads and the removal of chimeric sequences. Training sequence sets based on the SILVA reference database (v138; https://zenodo.org/record/4587955#.Y9JGXnZBxPY, accessed 31 January 2024) was used to assign taxonomy to the obtained amplicon sequence variants (ASVs) and to determine abundance estimates. The phyloseq package (v1.40.0) (McMurdie and Holmes, 2013) was then used to construct the DADA2 outputs into phyloseq objects, and these phyloseq objects were used for further analyses.

6.2.6.3 Diversity of microbiota

Alpha diversity analyses compare bacterial diversity within a sample, while beta diversity compares bacterial diversity between different samples. Relative abundance refers to the proportion of each bacterial species present in the microbiome. Differential abundance analyses compare abundance of common bacterial taxa between two different samples. Finally, overlapping bacterial analyses show the number of bacterial species that are unique to each sample and the number of bacterial species shared between two samples.

Alpha diversity analyses included species richness and species diversity analyses. Species richness (the number of unique species) of the midgut of both SENN and SENN DDT was determined using the ACE diversity index (Chao and Lee, 1992). Species diversity (the number of species within a sample and their relative abundances) was determined using the Simpson diversity index (Simpson, 1949). A Wilcoxon rank-sum test (Wilcoxon, 1945) was used to determine if significant differences were present between treatments for each strain and between strains for each treatment.

A Principal Components Analysis (PCA) (Pearson, 1901) was used to visualise beta diversity. The adonis function contained within the vegan package in R-Studio (v2.6-4)

(Okansen *et al.*, 2024) was used to conduct a PERMANOVA (permutation test with pseudo-F ratios) (Mcardle and Anderson, 2001) to test whether significant differences were present between strains and treatments.

The relative abundance of the top ten most abundant bacterial genera for each strain and treatment were visualised using bar plots.

UpSet plots were used to visualise overlapping bacterial families, genera and species between strains and between treatments using the Venn Diagram (v1.7.3) and UpsetR (v1.4.0) packages in R-Studio (Conway, Lex and Gehlenborg, 2017).

Finally, the differential abundance of microbiota for each strain and treatment was analysed using the DESeq2 (v1.24.0) package in R-Studio (Love, Huber and Anders, 2014).

All plots besides the UpSet plots were generated using the ggplot2 package in R-Studio (v3.4.0) (Wickham, 2011).

6.3 Results

6.3.1 Longevity

SENN

The median survival time for temephos-treated SENN adults was 28 days and 26 days for untreated SENN adults (Fig. 6.1). There was no significant difference in longevity between temephos-treated and untreated SENN adults (log rank: $\chi^2_{(1)}$ = 0.60, p = 0.40.)

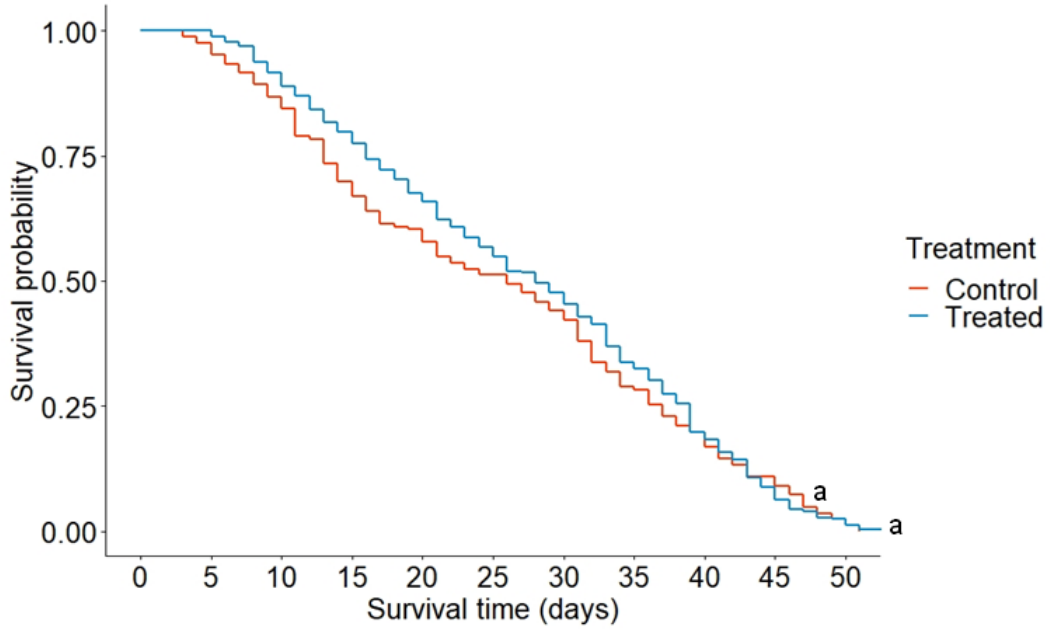


Figure 6.1. Survival probability of adults emerging from temephos-treated and untreated larvae of an insecticide-unselected strain (SENN) of *An. arabiensis*. Letters indicate significant differences between treatments.

SENN DDT

The median survival time of SENN DDT adults emerging from temephos-treated larvae was 32 days and 33 days for untreated SENN DDT adults (Fig. 6.2). SENN DDT adults from temephos-treated larvae had significantly decreased longevity compared to their untreated counterparts (log rank: $\chi^2_{(1)}$ = 26.90, p = 2.0 x 10^{-7}).

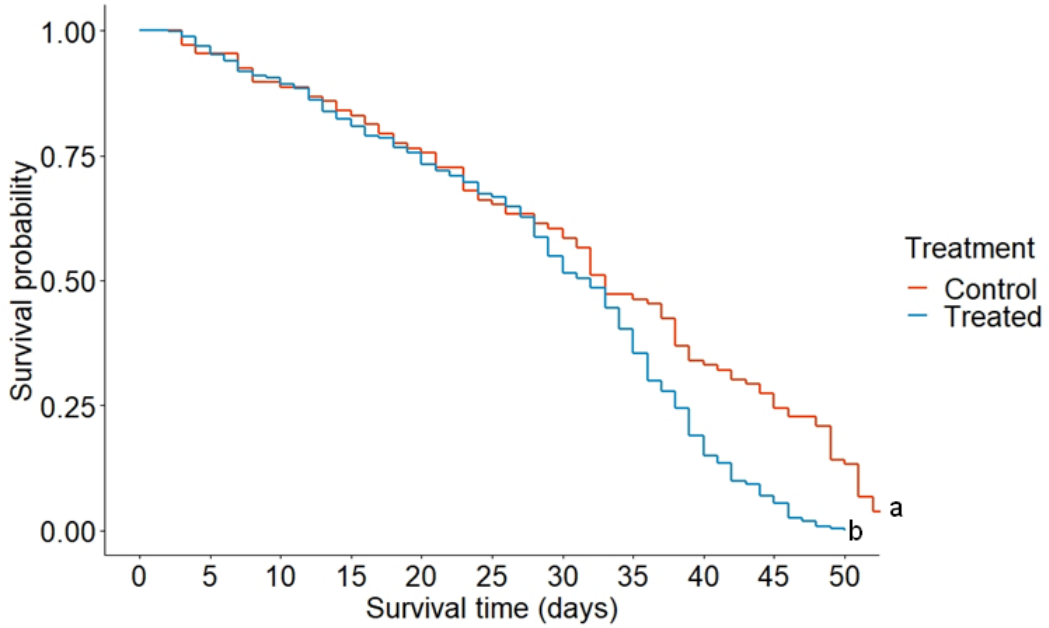


Figure 6.2. Survival probability of adults emerging from temephos-treated and untreated larval samples of an insecticide-selected strain (SENN DDT) of *An. arabiensis.*
Letters indicate significant differences in longevity between treatments.

6.3.2 Microbiota diversity

6.3.2.1 Alpha diversity

Alpha-diversity between treatments and control within strains- SENN

This analysis investigated differences in alpha diversity for the SENN strain between adults emerging from untreated larvae and adults emerging from treated larvae. There was no significant difference in species richness (ACE index; Wilcoxon rank-sum test, p = 0.34) or species diversity (Simpson Diversity index; Wilcoxon rank-sum test, p = 0.43) between adults emerging from treated and untreated SENN larvae (Fig. 6.3).

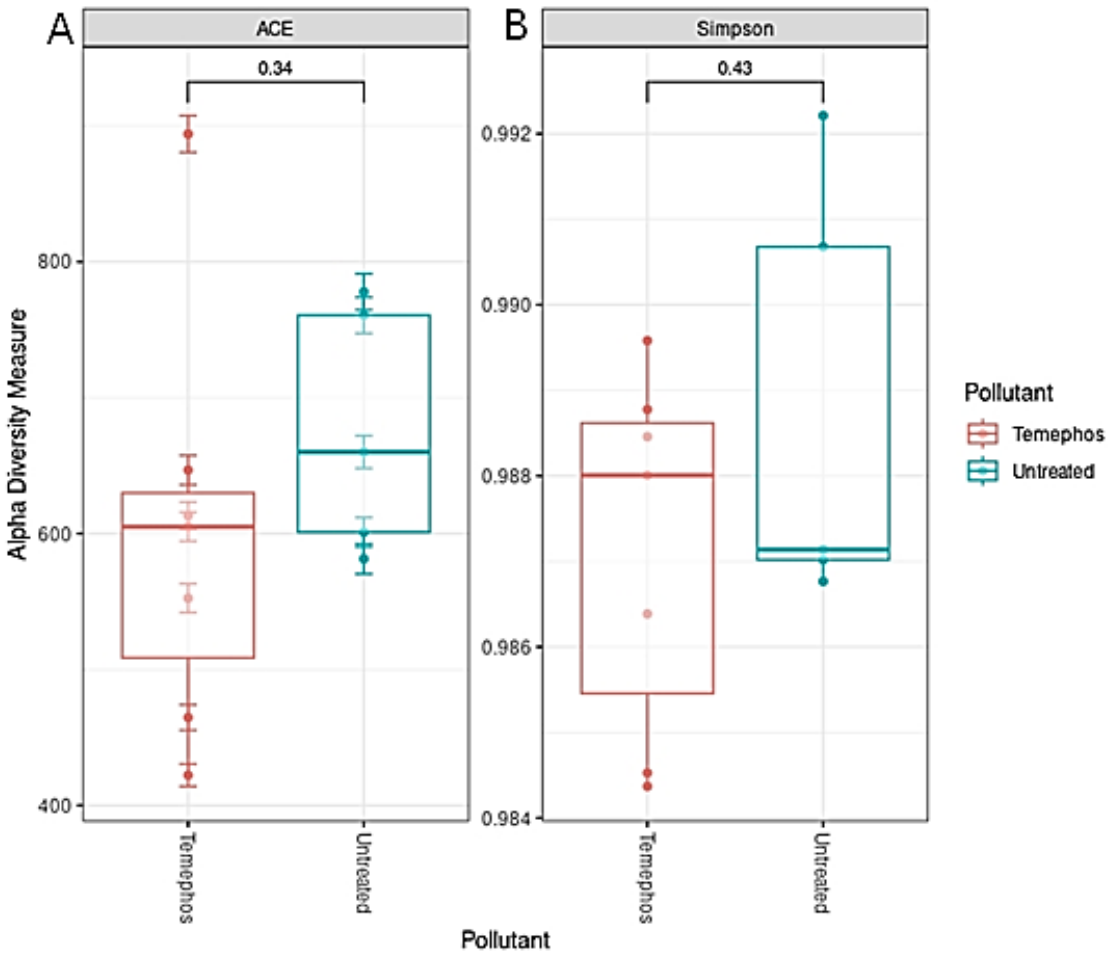


Figure 6.3. Comparison of alpha-diversity of midgut microbiota of adults emerging from temephos-treated and untreated larvae of an insecticide-unselected strain of *An. arabiensis* (SENN).

A) ACE diversity index, B) Simpson Diversity index.

Alpha-diversity between treatments and control within strains- SENN DDT

This analysis investigated differences in alpha diversity for the SENN DDT strain between adults emerging from untreated larvae and adults emerging from treated larvae. Species richness for adults emerging from untreated SENN DDT larvae were significantly higher compared to adults emerging from treated larvae (ACE index, Wilcoxon rank-sum test, p = 0.010 (Figure 6.4 A). There was no significant difference in species diversity between adults emerging from treated and untreated SENN DDT larvae (Simpson Diversity index, Wilcoxon rank-sum test, p = 0.11) (Fig. 6.4 B).

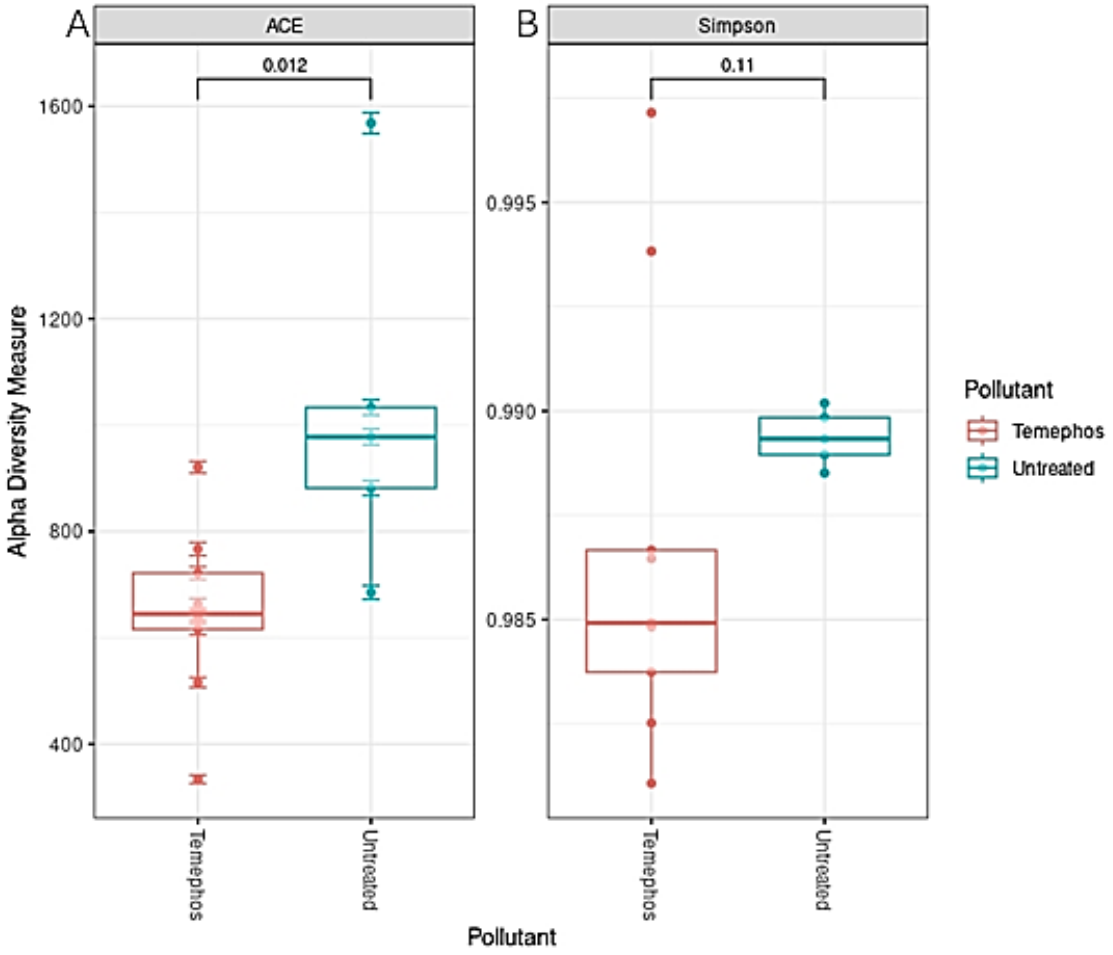


Figure 6.4. Comparison of alpha-diversity of midgut microbiota of adults emerging from temephos-treated and untreated larvae of an insecticide-selected strain of *An. arabiensis* (SENN DDT).

A) ACE diversity index, B) Simpson Diversity index.

Alpha-diversity between strains for each treatment- untreated larvae

This analysis investigated differences in alpha diversity for the adults emerging from untreated SENN larvae and adults emerging from untreated SENN DDT larvae. Species richness for adults emerging from untreated SENN DDT larvae were significantly higher compared to adults emerging from untreated SENN larvae (ACE index, Wilcoxon rank-sum test, p = 0.030) (Figure 6.5 A). There was no significant difference in species diversity between adults emerging from untreated SENN and SENN DDT larvae (Simpson Diversity index, Wilcoxon rank-sum test, p = 0.69) (Fig. 6.5 B).

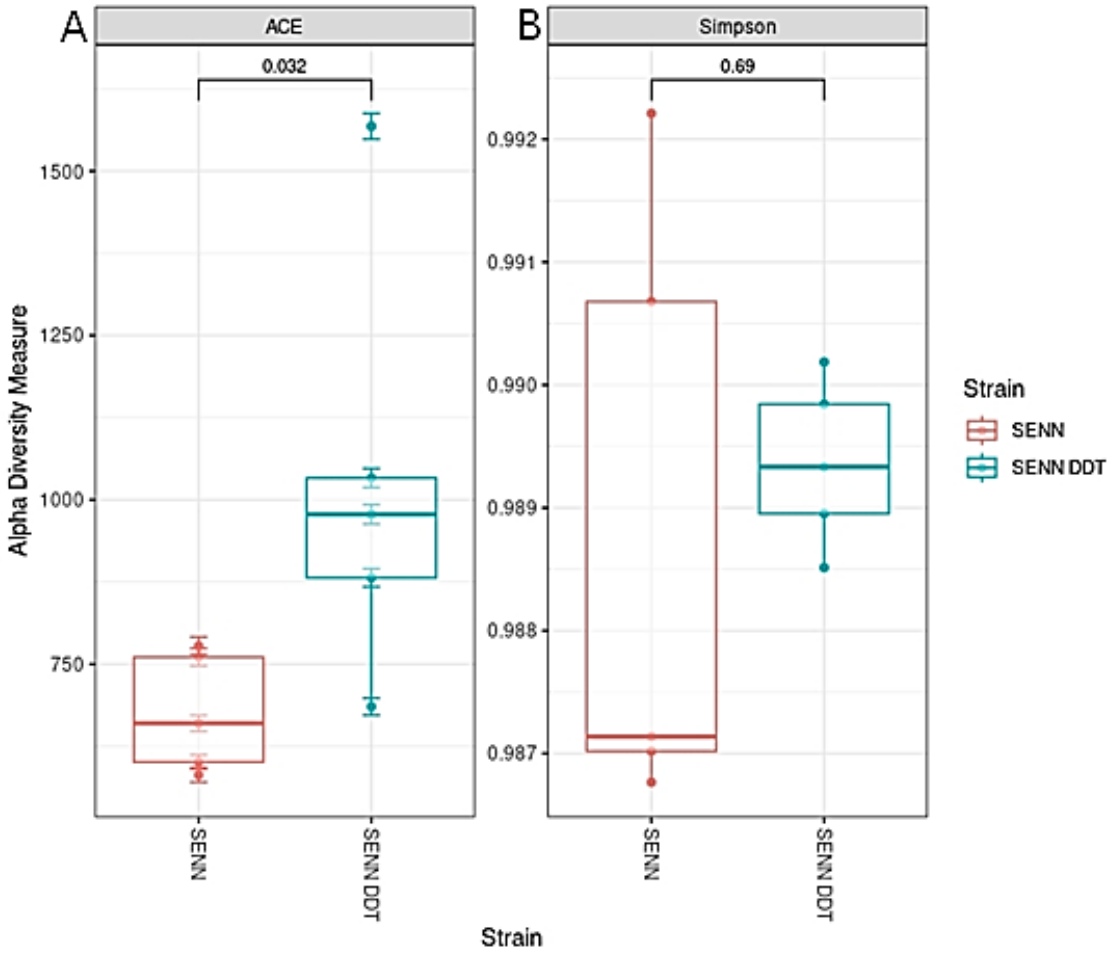


Figure 6.5. Comparison of alpha-diversity of midgut microbiota of adults emerging from untreated larvae between an insecticide-unselected (SENN) and an insecticide-selected (SENN DDT) strain of *An. arabiensis.*

A) ACE diversity index, B) Simpson Diversity index.

Alpha-diversity between strains for each treatment- treated larvae

This analysis investigated differences in alpha diversity for the adults emerging from treated SENN larvae and adults emerging from treated SENN DDT larvae. There was no significant difference in species richness (ACE index; Wilcoxon rank-sum test, p = 0.30) or species diversity (Simpson Diversity index; Wilcoxon rank-sum test, p = 0.47) between adults emerging from treated SENN and SENN DDT larvae (Fig. 6.6).

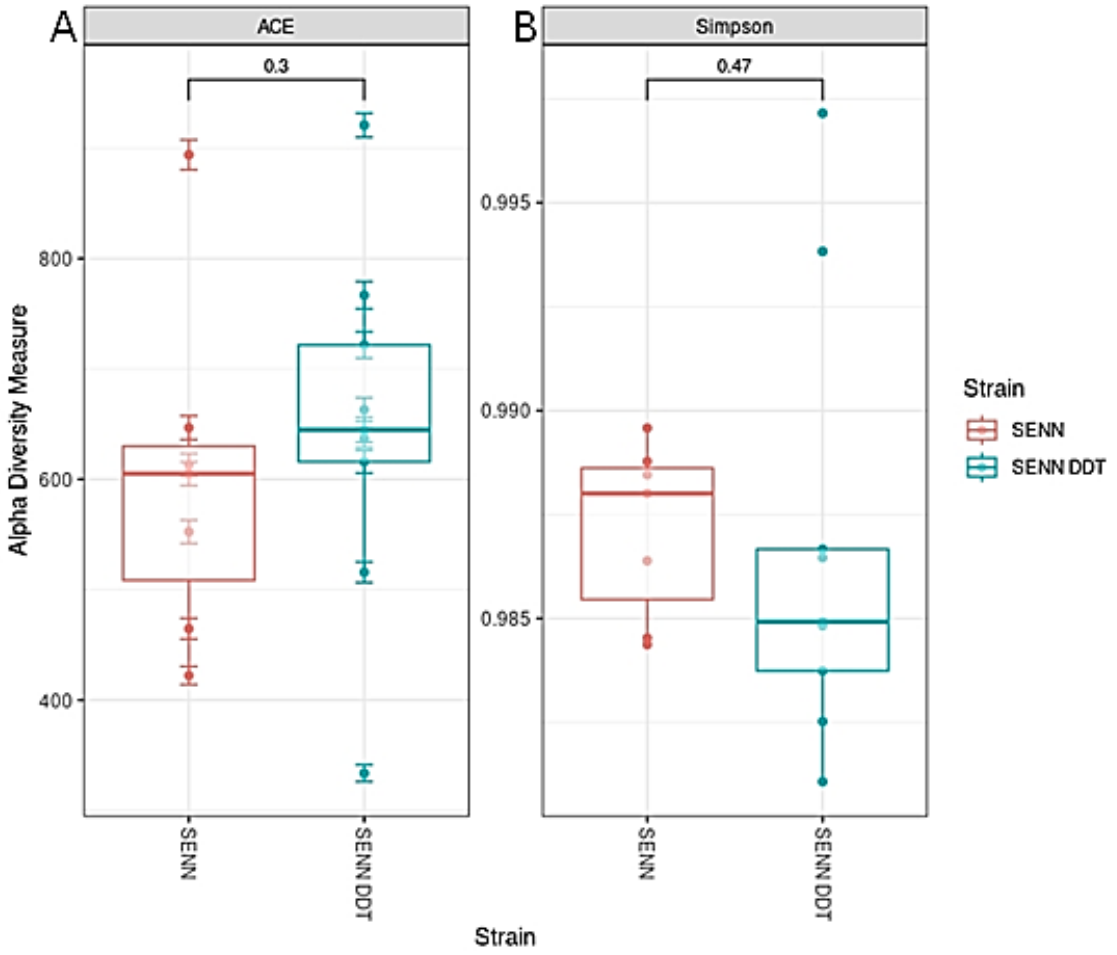


Figure 6.6. Comparison of alpha-diversity of midgut microbiota of adults emerging from temephos-treated larvae between an insecticide-unselected (SENN) and an insecticide-selected (SENN DDT) strain of *An. arabiensis.*
A) ACE diversity index, B) Simpson Diversity index.

6.3.2.2 Beta-diversity

Differences in bacterial diversity between samples were due to the effects of treatment of larvae with temephos (PERMANOVA, R^2 = 0.48 p = 0.0010) and not due to the effects of the different strains (PERMANOVA, R^2 = 0.039, p = 0.085) (Fig. 6.7). There was no significant effect from the interaction of strain and treatment with temephos on beta diversity (PERMANOVA, R^2 = 0.043, p = 0.078) (Fig. 6.7).

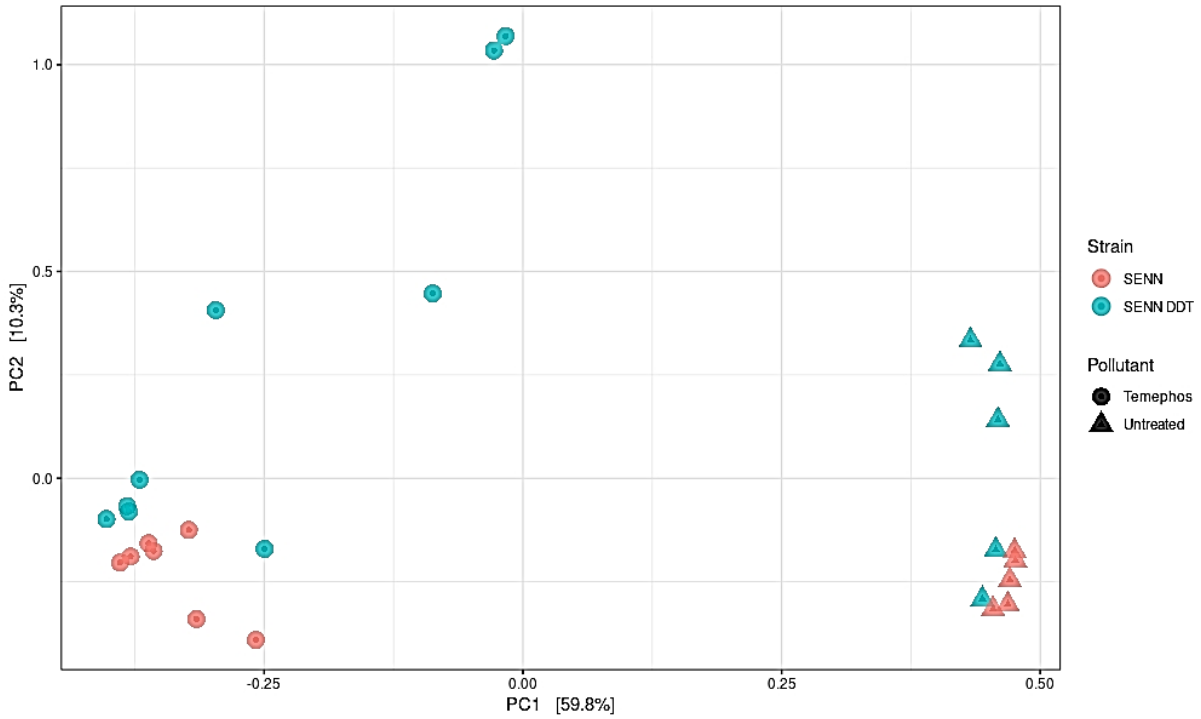


Figure 6.7. Principal Components Analysis (PCA) showing the beta diversity of the microbiota in the midguts of adults emerging from temephos-treated and untreated larvae of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) strain of *An. arabiensis.*

6.3.2.3 Relative abundance

Relative abundance refers to the proportion of each bacterial species present in the microbiome. Represented here are the top ten bacterial genera present for each treatment and each strain.

Comparison of relative abundance of gut bacteria between treatments for each strain - SENN

The most abundant genera for adults emerging from temephos-treated SENN larvae were *Asaia* and *Yersinia* (Fig. 6.8). Other bacterial genera that were relatively abundant in adults emerging from temephos-treated SENN larvae included *Cutibacterium*, *Pseudomonas* and *Rahnella* (Fig. 6.8). The most abundant genera for adults emerging from untreated SENN larvae were *Acinetobacter* and *Rahnella* (Fig. 6.8). Other relatively abundant bacterial genera for adults emerging from untreated SENN larvae

included *Chryseobacterium*, *Citrobacter*, *Elizabethkingia*, *Pseudomonas* and *Raoultella* (Fig. 6.8).

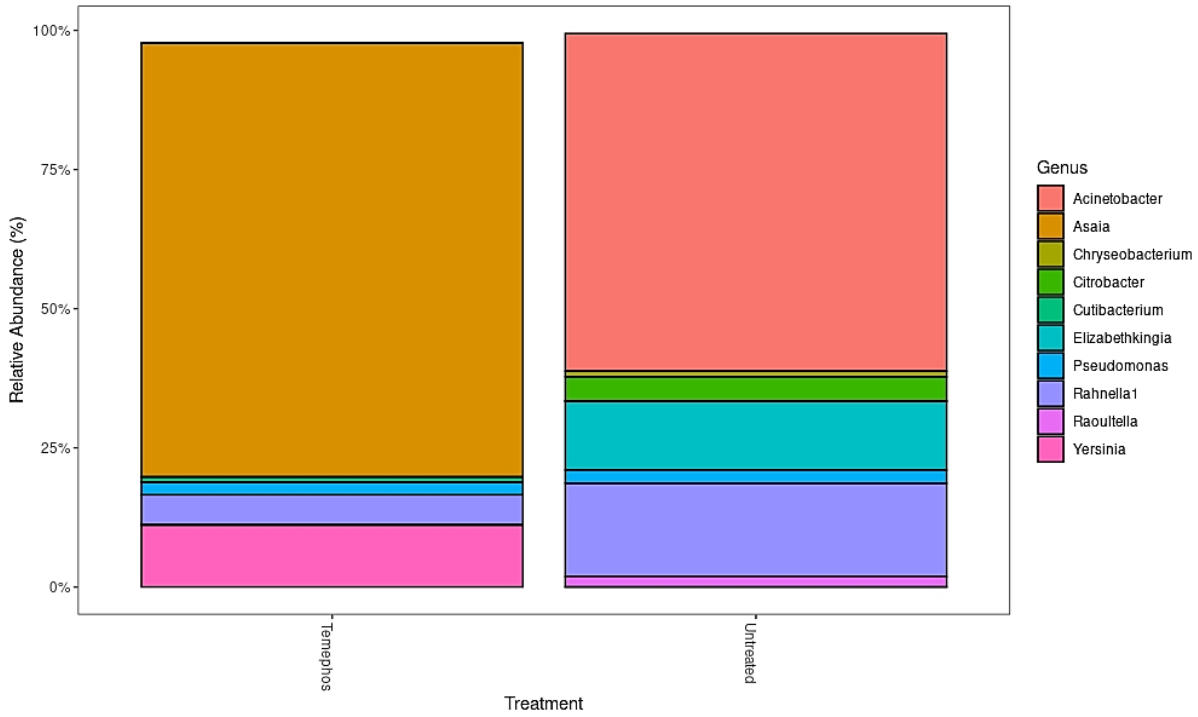


Figure 6.8. Relative abundance of midgut microbiota for adults emerging from temephos-treated and untreated larvae of an insecticide-unselected strain of *An. arabiensis* (SENN).

Comparison of relative abundance of gut bacteria between treatments for each strain – SENN DDT

The most abundant genera for adults emerging from temephos-treated SENN DDT larvae were *Asaia* and *Elizabethkingia* (Fig. 6.9). Other bacterial genera that were relatively abundant in adults emerging from temephos-treated SENN DDT larvae included *Cutibacterium*, *Escherichia-Shigella*, *Methylophilus* and *Rahnella* (Fig. 6.9). The most abundant genera for adults emerging from untreated SENN DDT larvae were *Acinetobacter* and *Elizabethkingia* (Fig. 6.9). Other relatively abundant bacteria for adults emerging from untreated SENN DDT larvae included *Cedecea*, *Citrobacter*, *Rahnella* and *Yersinia* (Fig. 6.9).

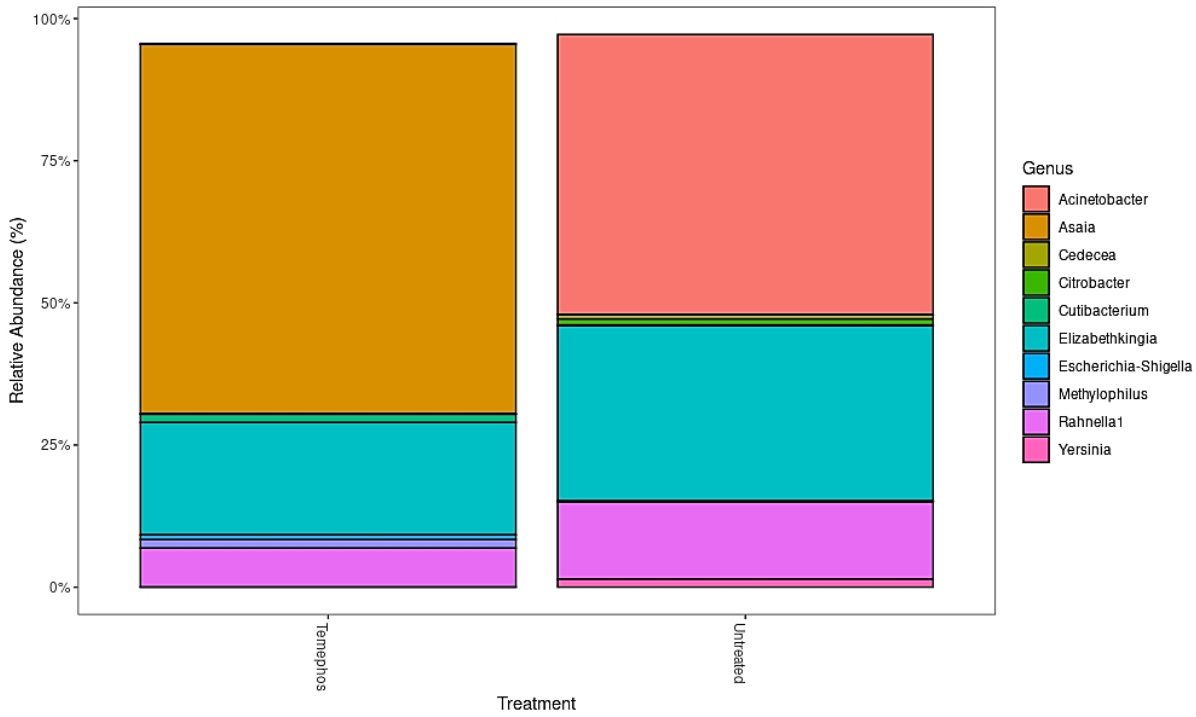


Figure 6.9. Relative abundance of midgut microbiota for adults emerging from temephos-treated and untreated larvae of an insecticide-selected strain of *An. arabiensis* (SENN DDT).

Comparison of relative abundance of gut bacterial species between strains for each treatment- untreated samples

The most abundant genera for adults emerging from untreated SENN larvae were *Acinetobacter* and *Yersinia* (Fig. 6.10). Other bacterial genera that were relatively abundant in adults emerging from untreated SENN larvae included *Chryseobacterium*, *Citrobacter*, *Elizabethkingia*, *Pseudomonas*, *Rahnella* and *Raoultella* (Fig. 6.10). The most abundant genera in adults emerging from untreated SENN DDT larvae were *Acinetobacter* and *Elizabethkingia* (Fig. 6.10). Other bacteria that were relatively abundant in adults emerging from untreated SENN DDT larvae included *Cedecea*, *Chryseobacterium*, *Citrobacter*, *Enterobacter*, *Rahnella*, *Raoultella* and *Yersinia* (Fig. 6.10).

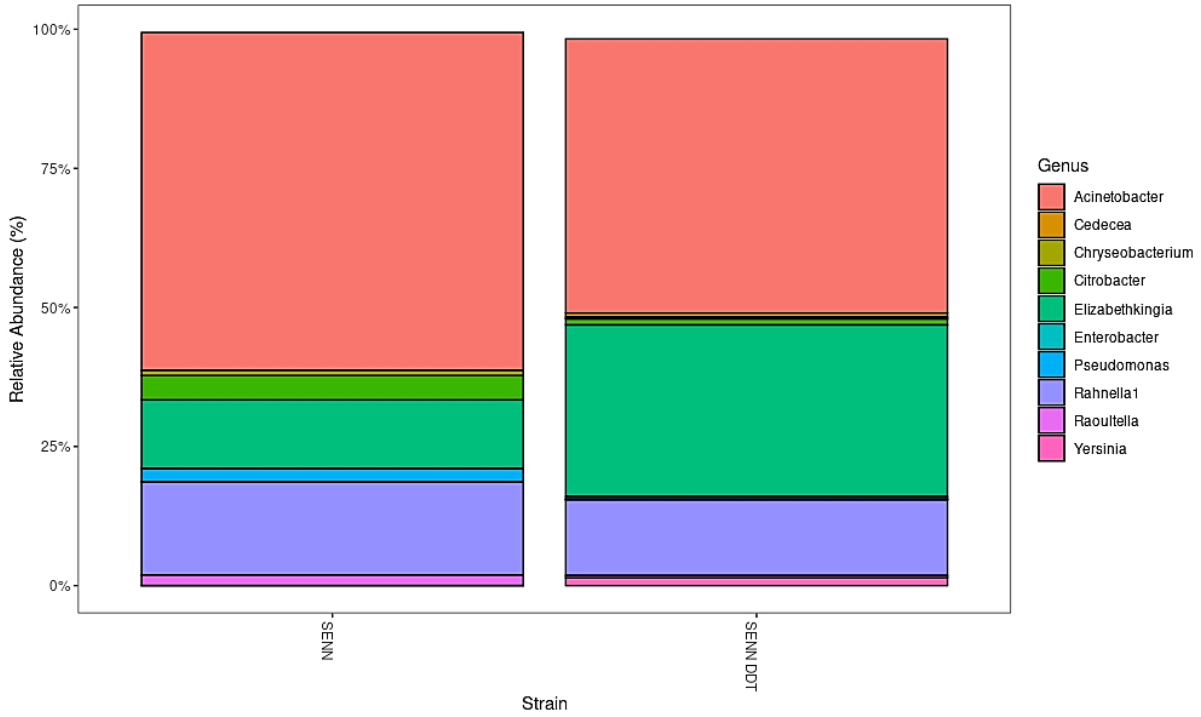


Figure 6.10. Relative abundance of midgut microbiota for adults emerging from untreated larvae of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) strain of *An. arabiensis*.

Comparison of relative abundance of gut bacterial species between strains for each treatment- treated samples

The most abundant bacterial genera for adults emerging from temephos-treated SENN larvae were *Asaia* and *Yersinia* (Fig. 6.11). Other bacterial genera that were relatively abundant in adults emerging from temephos-treated SENN larvae included *Cutibacterium*, *Escherichia-Shigella*, *Pseudomonas* and *Rahnella* (Fig. 6.11). The most abundant genera for adults emerging from temephos-treated SENN DDT larvae were *Asaia* and *Elizabethkingia* (Fig. 6.11). Other bacteria that were relatively abundant in adults emerging from temephos-treated SENN DDT larvae included *Burkholderia-Caballeronia-Paraburkholderia*, *Cutibacterium*, *Escherichia-Shigella*, *Methylophilus*, *Rahnella* and *Veilonella* (Fig. 6.11).

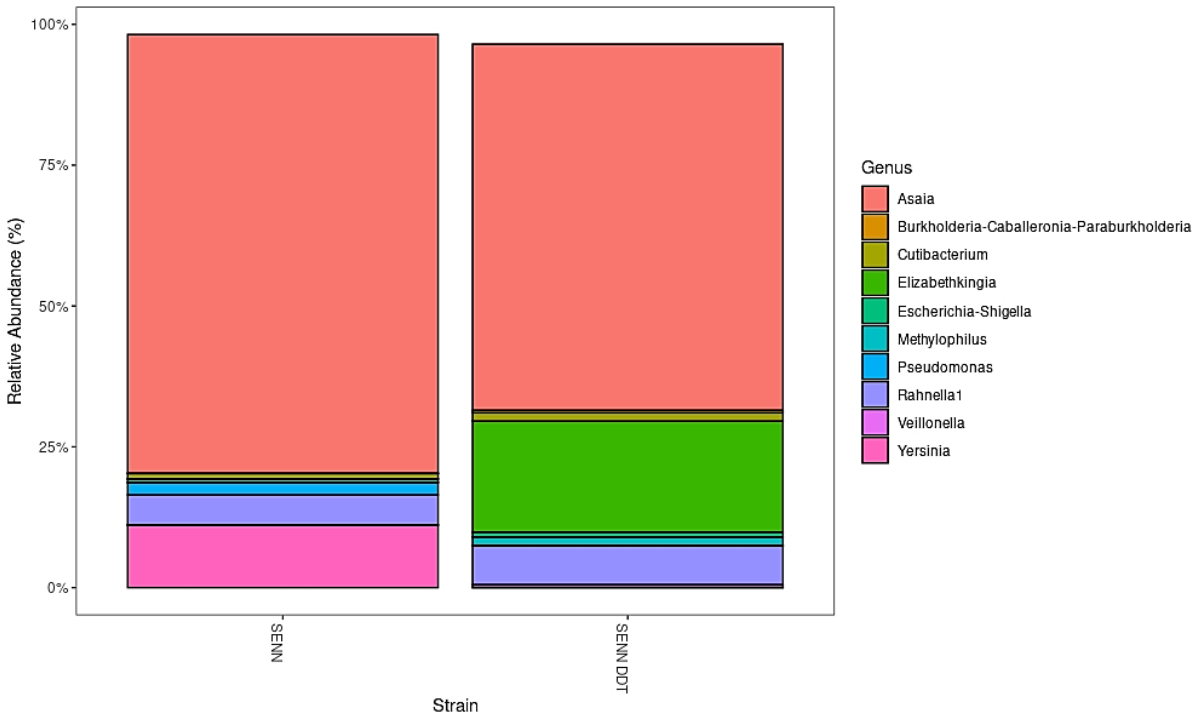


Figure 6.11. Relative abundance of midgut microbiota for adults emerging from temephos-treated larvae of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) strain of *An. arabiensis.*

6.3.2.4 Overlapping microbiota

Overlapping bacterial analyses show the number of bacterial species that are unique to each sample and the number of bacterial species shared between two samples.

Comparison of overlapping bacteria between treatments for each strain - SENN

Adults emerging from temephos-treated SENN larvae shared 52 bacterial genera with adults emerging from untreated SENN larvae, while 44 and 60 genera were unique to adults emerging from untreated and temephos-treated SENN larvae, respectively (Fig. 6.12 A). Adults emerging from temephos-treated SENN larvae shared 50 bacterial species with adults emerging from untreated SENN larvae, while 81 and 100 species were unique to adults emerging from untreated and temephos-treated SENN larvae, respectively (Fig. 6.12 B). The overlapping bacteria will be listed in Appendix C.

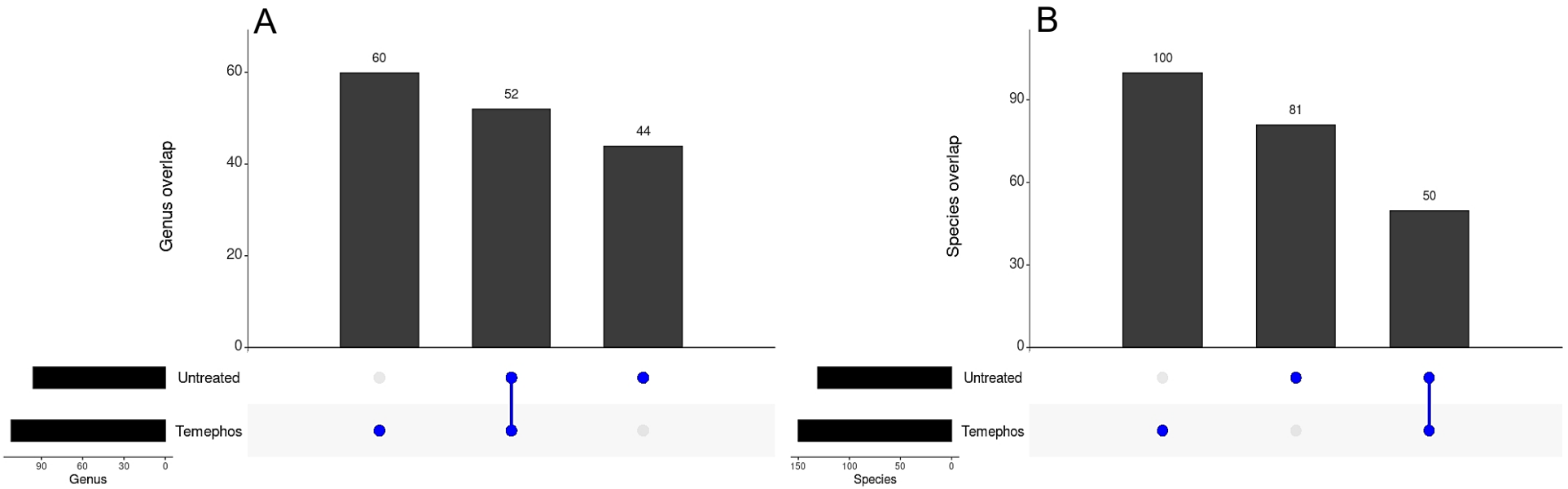


Figure 6.12. Number of shared and unique bacterial genera (A) and species (B) for adults emerging from temephos-treated and untreated larvae of an insecticide-unselected (SENN) strain of *An. arabiensis*.

Conjoined blue dots represent the shared bacteria between adults emerging from temephos-treated and untreated larvae, while singular blue dots represent the unique bacteria for each treatment. The numbers above each column indicate the number of bacterial genera (A) and species (B).

Comparison of overlapping bacteria between treatments for each strain – SENN DDT

Adults emerging from temephos-treated SENN DDT larvae shared 71 bacterial genera with adults emerging from untreated SENN DDT larvae, while 89 and 59 genera were unique to adults emerging from untreated and temephos-treated SENN DDT larvae, respectively (Fig. 6.13 A). Adults emerging from temephos-treated SENN DDT larvae shared 82 bacterial species with adults emerging from untreated SENN DDT larvae, while 139 and 109 species were unique to adults emerging from untreated and temephos-treated SENN DDT larvae, respectively (Fig. 6.13 B). The overlapping bacteria will be listed in Appendix C.

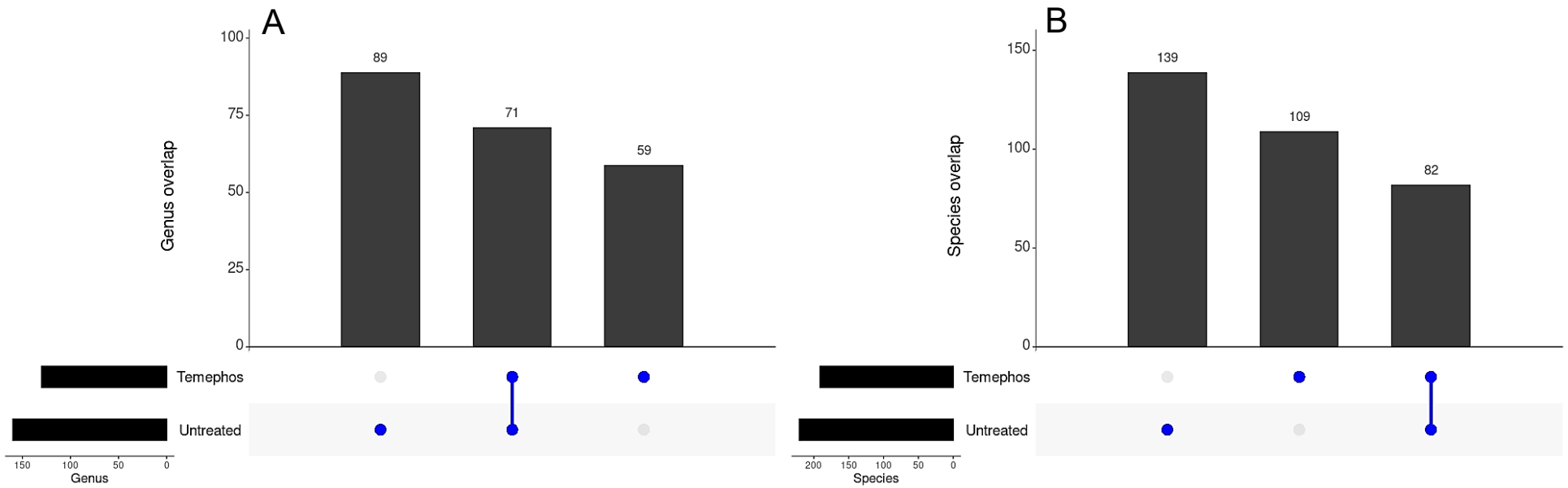


Figure 6.13. Number of shared and unique bacterial genera (A) and species (B) for adults emerging from temephos-treated and untreated larvae of an insecticide-selected (SENN DDT) strain of *An. arabiensis*.

Conjoined blue dots represent the shared bacteria between adults emerging from temephos-treated and untreated larvae, while singular blue dots represent the unique bacteria for each treatment. The numbers above each column indicate the number of bacterial genera (A) and species (B).

Comparison of overlapping bacteria between treatments for each strain - untreated samples

Adults emerging from untreated SENN and SENN DDT larvae shared 65 bacterial genera, while 31 and 95 genera were unique to adults emerging from untreated SENN and SENN DDT larvae, respectively (Fig. 6.14 A). Adults emerging from untreated SENN and SENN DDT larvae shared 76 bacterial species, while 55 and 145 species were unique to adults emerging from untreated SENN and SENN DDT larvae, respectively (Fig. 6.14 B). The overlapping bacteria will be listed in Appendix C.

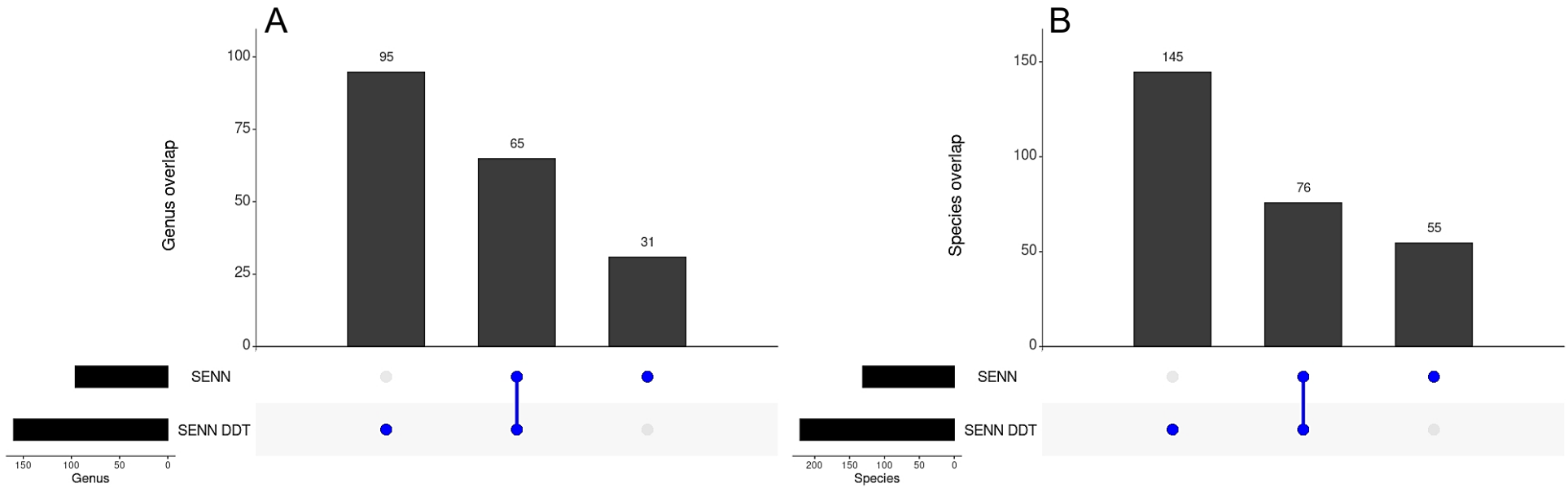


Figure 6.14. Number of shared and unique bacterial genera (A) and species (B) for adults emerging from untreated larvae of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) strain of *An. arabiensis*.

Conjoined blue dots represent the shared bacteria between adults emerging from untreated SENN and SENN DDT larvae, while singular blue dots represent the unique bacteria for each strain. The numbers above each column indicate the number of bacterial genera (A) and species (B).

Comparison of overlapping bacteria between treatments for each strain - treated samples

Adults emerging from temephos-treated SENN and SENN DDT larvae shared 58 bacterial genera, while 54 and 72 genera were unique to adults emerging from temephos-treated SENN and SENN DDT larvae, respectively (Fig. 6.15 A). Adults emerging from temephos-treated SENN and SENN DDT larvae shared 74 bacterial species, while 76 and 117 species were unique to adults emerging from temephos-treated SENN and SENN DDT larvae, respectively (Fig. 6.15 B). The overlapping bacteria will be listed in Appendix C.

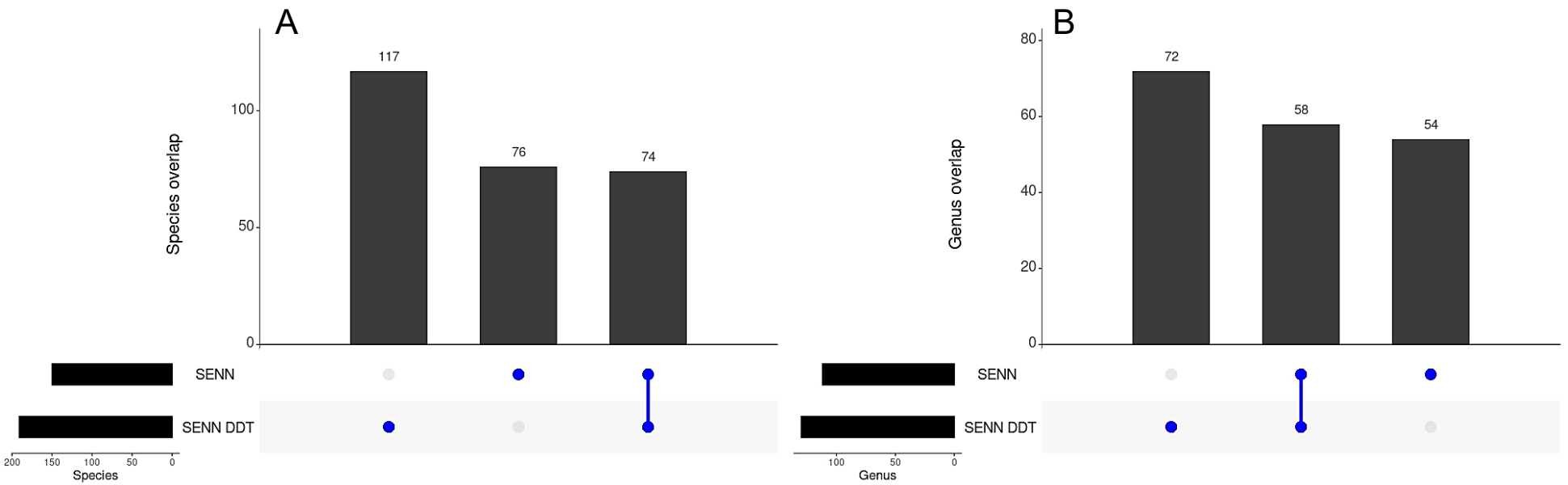


Figure 6.15. Number of shared and unique bacterial genera (A) and species (B) for adults emerging from temephos-treated larvae of an insecticide-unselected (SENN) and insecticide-selected (SENN DDT) strain of *An. arabiensis*.

Conjoined blue dots represent the shared bacteria between adults emerging from temephos-treated SENN and SENN DDT larvae, while singular blue dots represent the unique bacteria for each strain. The numbers above each column indicate the number of bacterial genera (A) and species (B).

6.3.2.5 Differential abundance

Differential abundance analyses compare abundance of common bacterial taxa between two different samples.

Comparison of differentially-abundant bacteria between treatments for each strain - SENN

Yersinia, *Asaia*, *Cutibacterium* and *Escherichia-Shigella* were significantly more abundant in adults emerging from temephos-treated SENN larvae compared to adults emerging from untreated SENN samples (Fig 6.16, left panel). *Rahnella*, *Raoultella*, *Citrobacter*, *Elizabethkingia*, *Moraxella*, *Pleomorphomonas*, *Actinomyces*, *Acinetobacter*, *Flavobacterium*, *Cloacibacterium*, *Chryseobacterium* and *Pseudomonas* were significantly more abundant in adults emerging from untreated SENN larvae compared to adults emerging from temephos-treated larvae (Fig. 6.16, right panel).

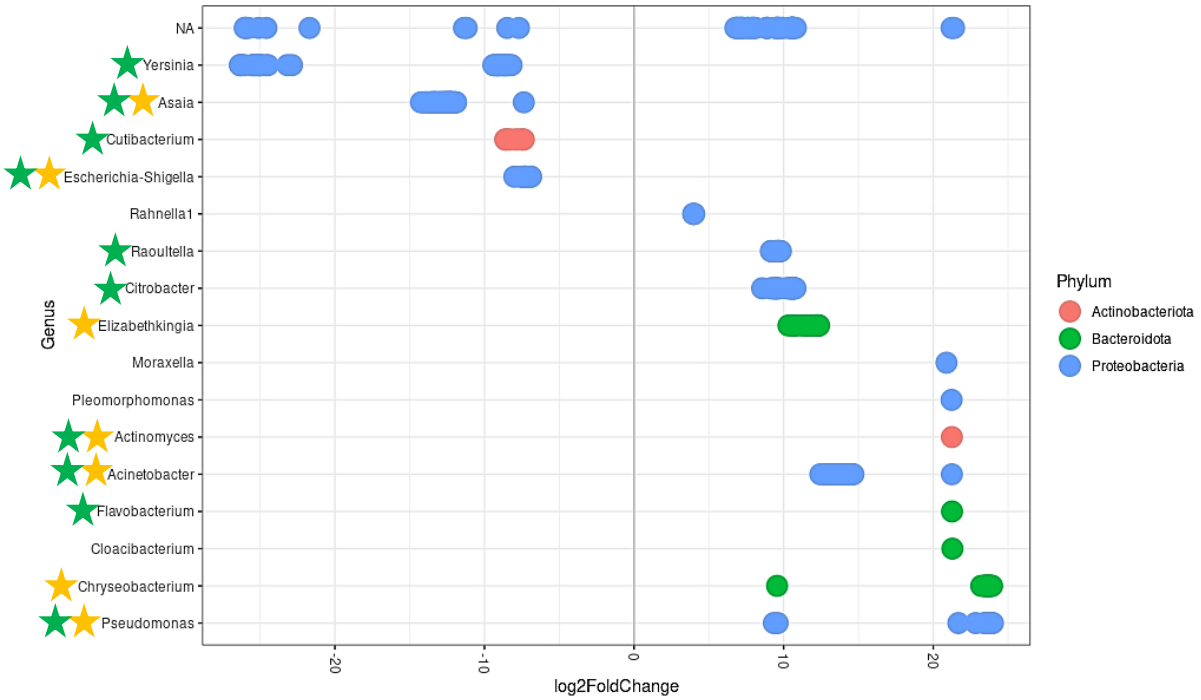


Figure 6.16. Pairwise comparisons of differentially-abundant bacteria of adults emerging from temephos-treated (left panel) and untreated (right panel) larvae of an insecticide-unselected (SENN) strain of *An. arabiensis*.

Numbers on the x-axis represent the log2fold change of bacterial genera that are significantly different at an alpha value of 0.010. Each dot represents a single genus. Green stars next to genera names on the y-axis indicate genera that have been associated with pesticide degradation, while gold stars indicate genera that have been associated with inhibition of *Plasmodium infection* in *Anopheles* spp.

Comparison of differentially-abundant bacteria between treatments for each strain - SENN DDT

Burkholderia-Caballeronia-Paraburkholderia, *Asaia*, *Methylophilus*, *Flectobacillus* and *Escherichia-Shigella* were significantly more abundant in adults emerging from temephos-treated SENN DDT larvae compared to adults emerging from untreated SENN DDT larvae (Fig. 6.17, left panel). *Rahnella*, *Yersinia*, *Raoultella*, *Citrobacter*, *Acinetobacter*, *Anoxybacillus*, *Sphingobium*, *Streptococcus*, *Rhodobacter*, *Corynebacterium*, *Staphylococcus*, *Zoogloea*, *Pedobacter*, *Leuconostoc*, *Methylobacterium-Methylorubrum*, *Dysgonomonas*, *Hydrocaboniphaga* and *Cedecea* were significantly more abundant in adults emerging from untreated SENN DDT larvae, compared to adults emerging from temephos-treated SENN DDT larvae (Fig. 6.17, right panel).

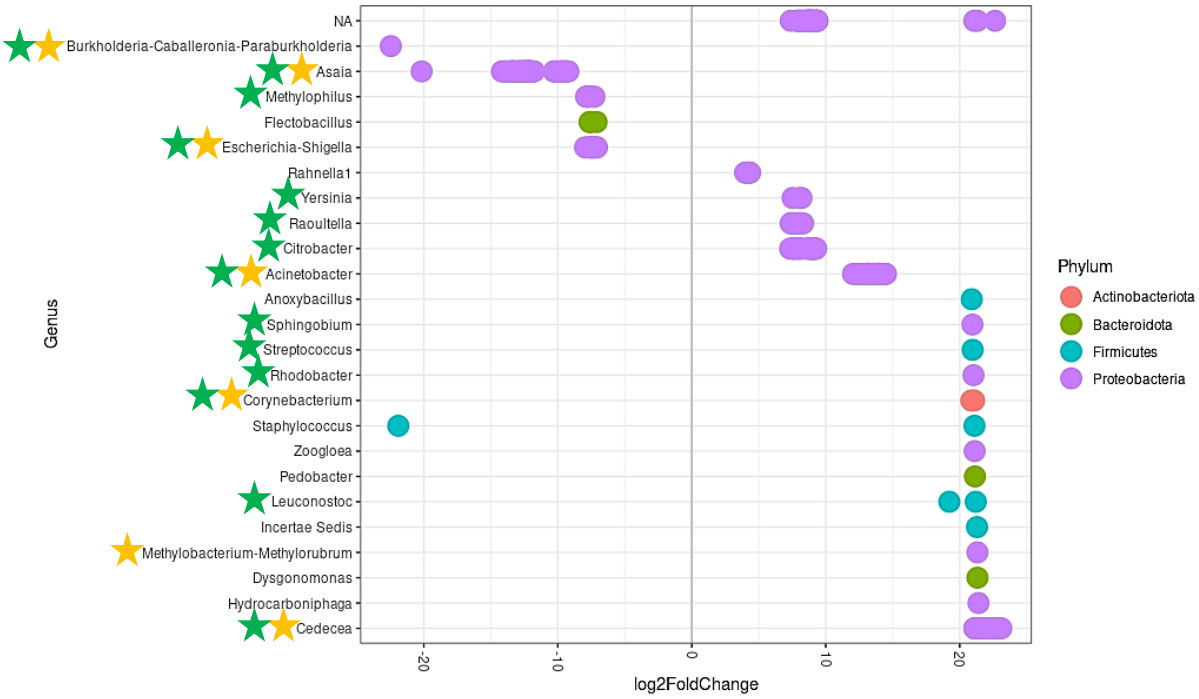


Figure 6.17. Pairwise comparisons of differentially-abundant bacteria of adults emerging from temephos-treated (left panel) and untreated (right panel) larvae of an insecticide-selected (SENN DDT) strain of *An. arabiensis*.

Numbers on the x-axis represent the log2fold change of bacterial genera that are significantly different at an alpha value of 0.010. Each dot represents a single genus. Green stars next to genera names on the y-axis indicate genera that have been associated with pesticide degradation, while gold stars indicate genera that have been associated with inhibition of *Plasmodium infection* in *Anopheles* spp.

Comparison of differentially-abundant bacteria between strains for each treatment - untreated samples

Cloacibacterium, *Acinetobacter*, *Pleomorphomonas*, *Moraxella*, *Flavobacterium* and *Pseudomonas* were significantly more abundant in adults emerging from untreated SENN larvae compared to adults emerging from untreated SENN DDT larvae (Fig. 6.18, left panel). *Yersinia*, *Cutibacterium*, *Methylobacterium-Methylorubrum*, *Hydrocarboniphaga*, *Dysgonomonas*, *Streptococcus* and *Cedecea* were significantly more abundant in adults emerging from untreated SENN DDT larvae compared to adults emerging from untreated SENN larvae (Fig 6.18, right panel).

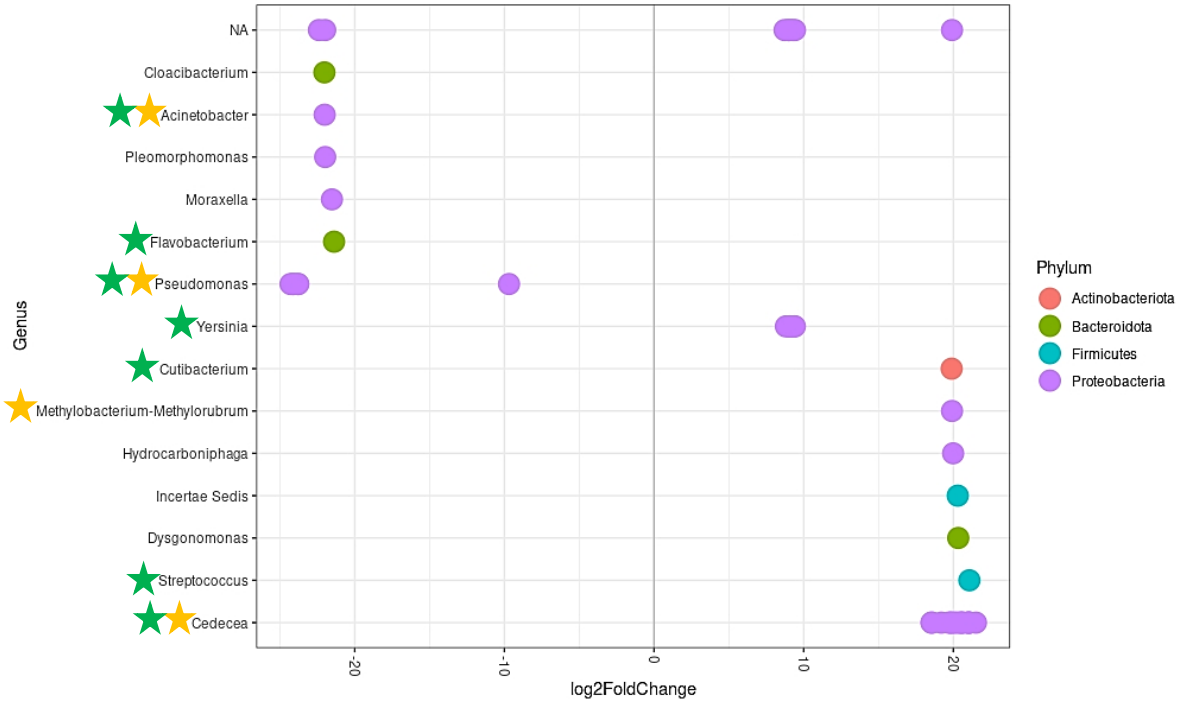


Figure 6.18. Pairwise comparisons of differentially-abundant bacteria of adults emerging from untreated larvae of an insecticide-unselected (SENN, left panel) and an insecticide-selected (SENN DDT, right panel) strain of *An. arabiensis*.
Numbers on the x-axis represent the log2fold change of bacterial genera that are significantly different at an alpha value of 0.010. Each dot represents a single genus. Green stars next to genera names on the y-axis indicate genera that have been associated with pesticide degradation, while gold stars indicate genera that have been associated with inhibition of *Plasmodium infection* in *Anopheles* spp.

Comparison of differentially-abundant bacteria between strains for each treatment - treated samples

Micrococcus and *Yersinia* were significantly more abundant in adults emerging from temephos-treated SENN larvae, compared to adults emerging from temephos-treated SENN DDT larvae (Fig. 6.19, left panel). *Flectobacillus*, *Methylophilus*, *Burkholderia-Caballeronia-Paraburkholderia* and *Elizabethkingia* were significantly more abundant in adults emerging from temephos-treated SENN DDT larvae compared to adults emerging from temephos-treated SENN larvae (Fig. 6.19, right panel).

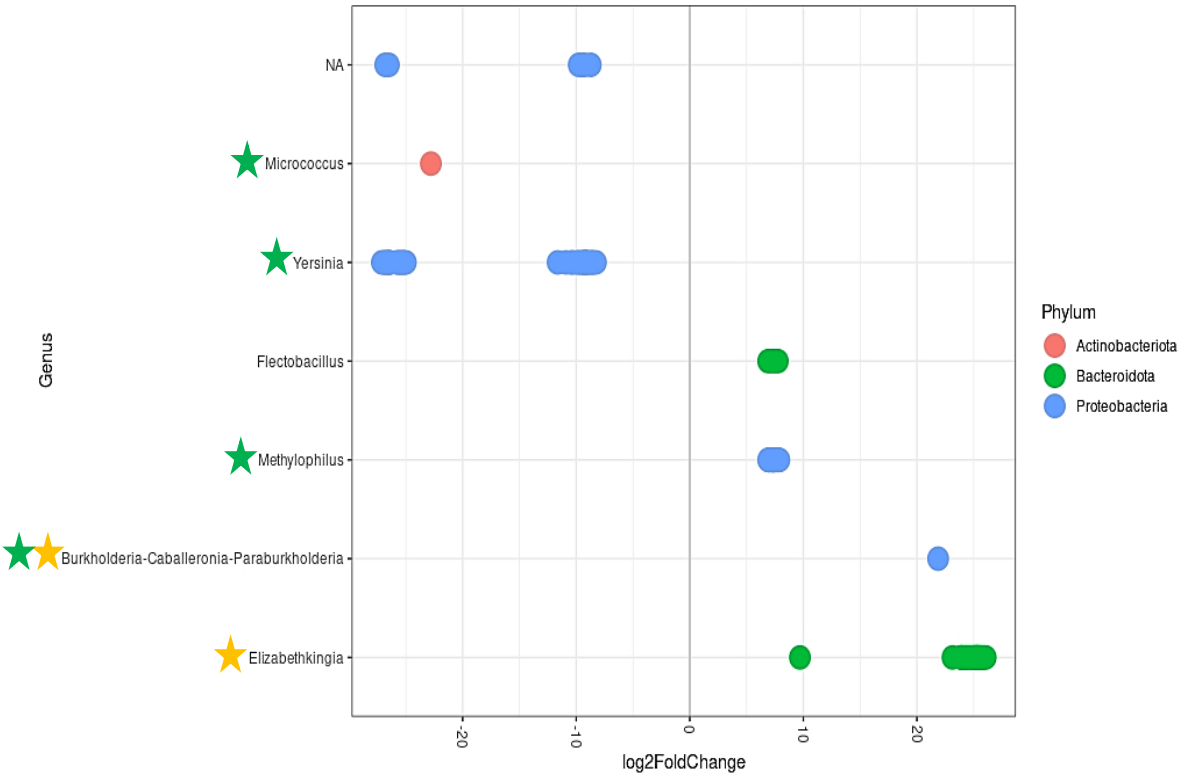


Figure 6.19. Pairwise comparisons of differentially-abundant bacteria of adults emerging from temephos-treated larvae of an insecticide-unselected (SENN, left panel) and an insecticide-selected (SENN DDT, right panel) strain of *An. arabiensis.* Numbers on the x-axis represent the log2fold change of bacterial genera that are significantly different at an alpha value of 0.010. Each dot represents a single genus**.** Green stars next to genera names on the y-axis indicate genera that have been associated with pesticide degradation, while gold stars indicate genera that have been associated with inhibition of *Plasmodium infection* in *Anopheles* spp.

6.4 Discussion

This study investigated the effects of the sub-lethal larval application of temephos on the adult longevity and gut microbiota in two strains of laboratory reared *An. arabiensis* that differ in insecticide resistance phenotype. Earlier studies have investigated the effects of sub-lethal exposure to temephos in *An. stephensi*, the Asian malaria mosquito, (Sanil and Shetty, 2012) and *Aedes aegypti*, the yellow fever mosquito (Reyes-Villaneuva, Juarez-Eguia and Flores-Leal, 1990; Alvarez Costa *et al.*, 2018). The effects of sub-lethal temephos exposure on the major African malaria vector *An. arabiensis*, especially the effects on the gut microbial composition and diversity, have to the best of our knowledge not been investigated yet. Our results show that sub-lethal exposure of larvae to temephos impacted longevity and gut microbial composition of two laboratory strains of *An. arabiensis*.

Treatment with temephos did not impact longevity of SENN adult females (Figure 6.1), while longevity for SENN DDT female adults were significantly reduced (Figure 6.2), compared to the control treatment. This may be due to the difference in insecticide susceptibility between the two strains. Resistance to insecticides have been shown to cause a strong fitness cost (Otali *et al.*, 2014), which may explain why SENN DDT adults suffered a reduction in longevity while SENN adults did not. Previous studies have shown that treatment with temephos generally increased longevity of a susceptible strain of *An. stephensi* (Sanil and Shetty, 2012). Similarly, Diniz *et al*. (2015) found a resistant strain of *Ae. aegypti* to have a significantly shorter lifespan than the susceptible strain. Our results and those from literature show that resistant and susceptible strains respond differently to sub-lethal exposure of temephos.

Sub-lethal exposure to temephos had no effect on the alpha diversity of midgut bacteria in both treated and untreated SENN samples (Figure 6.3). However, for SENN DDT treated samples, there was a significant reduction in species richness, while diversity remained unaffected (Figure 6.4). When comparing SENN and SENN DDT, no significant differences in bacterial diversity were found between untreated (Figure 6.5) and treated samples (Figure 6.6). This result is similar to a study by Arévalo-Cortés *et al*. (2020) on *Ae. aegypti* strains resistant to lambda-cyhalothrin (a pyrethroid), which also found no differences in gut microbial richness and diversity between resistant and susceptible strains. These findings, along with our results, are unique as generally

insecticide-resistant phenotypes show reduced bacterial diversity, compared to susceptible phenotypes (Dada *et al.*, 2018; Singh *et al.*, 2022).

By contrast, Muturi *et al*. (2021) found that a permethrin-resistant *Ae. aegypti* strain had increased bacterial diversity, compared to its susceptible counterpart. Notably, this study only assessed gut microbial diversity across insecticide-susceptible and resistant strains and did not consider the effects of sub-lethal exposures on bacterial diversity. A study by Juma *et al.* (2020), which investigated sub-lethal exposure of first instar larvae to malathion and permethrin, found that bacterial richness significantly increased in larvae of *Ae. albopictus* and *Culex pipiens* exposed to sub-lethal doses of malathion. These conflicting results suggest that mosquito midgut microbiomes respond to pesticide exposure in complex ways, likely influenced by factors such as pesticide type, dose, exposure duration, mosquito species, and resistance status. It is worth noting, however, that a lack of change in alpha diversity in treated and untreated samples does not indicate that treatment did not have an effect on bacterial dynamics. This can be determined by examining beta diversity.

Beta diversity represents differences in species diversity between two or more groups and would give an idea of whether the treatment had an effect. The bacterial diversity of treated cohorts was significantly different from that of their untreated counterparts (Figure 6.7). The differences in bacterial diversity between strains were due to treatment with temephos and not due to the effects of strain. There was a lack of strain-specific differences, despite the differences in the dose used for each strain. These results show that sub-lethal exposures to pesticides change the gut bacterial diversity, a finding corroborated by Juma *et al*. (2020). Our findings further suggest that the bacterial diversity changes regardless of the insecticide resistance phenotype present. This is notable as an insecticide-resistant phenotype is known to alter the gut bacterial composition (Dada *et al.*, 2018). It is worth noting, however, that the differences in diversity, particularly species richness, is not that marked in these two strains (SENN and SENN DDT), as noted by previous studies (Singh *et al.*, 2022).

When examining the relative abundance of the genera present, both SENN and SENN DDT were dominated by *Acinetobacter* in the untreated samples (Figures 6.8 – 6.11). The treated samples for both strains were dominated by *Asaia*. *Elizabethkingia* was also one of the primary genera in both the treated and untreated samples of SENN and

the treated samples of SENN DDT. These three genera are commonly found within the midguts of *An. gambiae* complex (Zoure *et al.*, 2020; Singh *et al.*, 2022).

The number of unique species as well as the number of species shared between the treatments were assessed. The number of unique bacterial species present in treated SENN samples were more than the number of unique bacterial species present in the untreated SENN samples (Figure 6.12 B). This pattern was reversed for SENN DDT, where the number of unique bacterial species present in the treated samples were less compared to the untreated samples (Figure 6.13 B).

Insecticide-resistant strains may have a built-in defence mechanism against larval toxicants if resistance is mediated by metabolic detoxification enzymes (Li, Schuler and Berenbaum, 2007). This is the case for SENN DDT, which showed higher survival rates than SENN when exposed to commercial herbicides and metal pollutants (Oliver and Brooke, 2018b, 2018a). Our results, which show that SENN DDT better survives exposure to temephos, corroborate this finding. The insecticide-unselected strain, SENN, does not exhibit the same elevated levels of metabolic detoxification enzymes (Oliver and Brooke, 2013).

The increase in bacterial diversity after exposure to temephos observed for SENN may therefore be a larval response to a stressor and suggests that SENN utilises midgut bacteria to assist with pesticide degradation. The hypothesis that the increased abundance of bacteria after treatment with temephos in SENN may be driven by an increased amount of pesticide-degrading bacteria is reflected by the differential abundance results. All four of the differentially-abundant bacterial genera in SENN adults pre-exposed to temephos at the larval stage (*Yersinia*, *Asaia*, *Cutibacterium* and *Escherichia-Shigella*) (Figure 6.16, left panel) have been shown to be involved in pesticide degradation (Lee *et al.*, 2004; Comandatore *et al.*, 2021; Muturi *et al.*, 2021; Fan *et al.*, 2024). By contrast, only half of the differentially-abundant bacterial genera in the untreated SENN samples (Figure 6.16, right panel) have been shown to be involved in pesticide degradation: *Raoultella* (Zhang *et al.*, 2019), *Citrobacter* (Chaussonnerie *et al.*, 2016), *Actinomyces* (Ataikiru, Okerentugba and Okpokwasili, 2020), *Acinetobacter* (Tang, Ji and Hou, 2017), *Flavobacterium* (Ning *et al.*, 2012) and *Pseudomonas* (Barragán-Huerta *et al.*, 2007).

For SENN DDT, most of the bacterial genera in the treated samples (Figure 6.17, left panel) have been associated with pesticide degradation: *Burkholderia-Caballeronia-Paraburkholderia* (Kikuchi *et al.*, 2012), *Asaia*, *Methylophilus* (Itoh *et al.*, 2018) and *Escherichia-Shigella.* For SENN DDT untreated samples, only some bacterial genera have been shown to be involved in pesticide degradation: *Yersinia*, *Raoultella*, *Citrobacter*, *Acinetobacter*, *Sphingobium* (Ahn *et al.*, 2018), *Streptococcus* (Lourthuraj, Hatshan and Hussein, 2022), *Rhodobacter* (Wu *et al.*, 2019), *Corynebacterium* (Hamada, Matar and Bashir, 2015), *Leuconostoc* (Haque *et al.*, 2020) and *Cedecea* (Ma *et al.*, 2023).

A similar pattern was observed when comparing the strains with each other. For both strains, untreated samples only had a few bacterial genera associated with pesticide degradation: *Acinetobacter*, *Flavobacterium* and *Pseudomonas* for SENN (Figure 6.18, left panel) and *Yersinia*, *Cutibacterium*, *Streptococcus* and *Cedecea* for SENN DDT (Figure 6.18, right panel). Both differentially-abundant bacterial genera in treated SENN samples (*Yersinia* spp. and *Micrococcus*) (Figure 6.19, left panel) have been shown to be involved in pesticide degradation (Lee *et al.*, 2004; Tallur, Megadi and Ninnekar, 2008), compared to only a few in treated SENN DDT samples (*Methylophilus* and *Burkholderia-Caballeronia-Paraburkholderia*) (Figure 6.19, right panel). The ratios of pesticide-degrading bacterial genera in treated vs. untreated SENN samples, compared to SENN DDT, suggest that SENN DDT likely rely on metabolic detoxification enzymes for pesticide degradation, while SENN may rely on gut bacteria.

Sub-lethal exposure to pesticides have been shown to alter gut bacterial composition and diversity (Juma *et al.*, 2020). Results from the current study corroborate this finding. Furthermore, the results from the current study show that there were less *Plasmodium*-inhibiting bacterial species present after exposure to temephos for both SENN (Figure 6.16) and SENN DDT (Figure 6.17). Notably, treatment with temephos reduced the number of *Plasmodium*-inhibiting species in untreated SENN samples from five to two in the treated samples (Untreated: *Elizabethkingia* spp. (Ngwa *et al.*, 2013), *Actinomyces* spp. (Prudhomme *et al.*, 2008), *Acinetobacter* spp. (Cirimotich *et al.*, 2011), *Chryseobacterium* spp. (Dong, Manfredini and Dimopoulos, 2009) and *Pseudomonas* spp. (Tchioffo *et al.*, 2013); Treated: (*Escherichia-Shigella* spp. (Tchioffo *et al.*, 2013) and *Asaia* spp. (Bassene *et al.*, 2018)). Similarly for SENN DDT, untreated samples had four *Plasmodium*-inhibiting species, while treated samples for SENN DDT

only had three *Plasmodium*-inhibiting species (Untreated: *Acinetobacter* spp., *Corynebacterium* spp. (Murphy, 1981), *Methylobacterium* spp. (Djihinto *et al.*, 2022) and *Cedecea* spp. (Pumpuni *et al.*, 1996); Treated: *Asaia* spp., *Escherichia-Shigella* spp. and *Burkholderia-Caballeronia-Paraburkholderia* (Bassene *et al.*, 2018)). There is therefore a risk that sub-lethal exposure to pesticides could potentially render the midgut more susceptible to *Plasmodium* infection, which may impact vector competence (Richards, White and Balanay, 2017). This may have epidemiological consequences for malaria transmission.

Comparing the strains with each other showed that untreated SENN samples had the same number of *Plasmodium*-inhibiting species compared to untreated SENN DDT samples (Figure 6.18), however the species were different between strains. *Plasmodium*-inhibiting species in untreated SENN samples were *Acinetobacter* spp. and *Pseudomonas* spp., while for untreated SENN DDT the species were *Methylobacterium-Methylorubrum* spp. and *Cedecea* spp. Of note, treated SENN samples had no differentially-abundant bacteria involved in *Plasmodium* inhibition while treated SENN DDT samples had two (*Burkholderia-Caballeronia-Paraburkolderia* spp. and *Elizabethkingia* spp.) (Figure 6.19). From these results it is evident that the two strains, SENN and SENN DDT, respond differently to sub-lethal exposure of temephos. Additionally, temephos treatment alters the species composition of the midgut bacteria and may create an environment within the gut that is more susceptible to infection by *Plasmodium.*

There were a few limitations to this body of work. Environmentally-relevant concentrations of temephos could not be used for the sub-lethal exposures, as the strains that were used in the study were too sensitive to these doses. The maximum sub-lethal dose was different for SENN and SENN DDT, which meant the two strains were exposed to different doses. This may have affected the outcomes. There was a possibility of using the same dose for both strains, however, there was a risk that the very low dose used for SENN may not have had any effect on SENN DDT. Ideally, sub-lethal exposures should be conducted on first instars, however this was not possible in this study, as the first and second instars were too sensitive to the dosages that were used. Therefore, third to fourth instar larvae were exposed. This means that larvae were only exposed for three to five days, which could explain the lack of differential abundance observed between the two strains. The current study aimed to examine

the effect of sub-lethal larviciding effects on the emergent adults, due to the epidemiological relevance. However, to better implicate gut bacteria in the capacity to survive the sub-lethal dose, the effect needs to be examined in larvae, rather than adults. Finally, because laboratory-reared strains were used, the gut microbiota may not have been fully characterised, since wild mosquitoes generally have much higher diversity of gut microbiota. However, since changes in the gut bacteria were observed in the laboratory strains after short sub-lethal exposures, it is likely that similar changes would be observed in wild *An. arabiensis*. The effects of sub-lethal exposure of pesticides on gut microbiota of malaria vectors in South Africa are not fully understood and needs to be further investigated in wild mosquitoes.

6.5 Conclusion

This study is one of the first that specifically investigates active selection due to sub-lethal exposure on strains that differ in insecticide susceptibility. Our results show that relatively short sub-lethal exposure to temephos alters gut bacteria of both the insecticide-selected and unselected strains of *An. arabiensis*. These changes were not observable in the alpha diversity analyses, but did reflect in the relative and differential abundance analyses. Treatment with temephos significantly affected beta diversity, while insecticide resistance phenotype did not. Treatment with temephos also reduced the number of unique bacterial species present in SENN DDT, while the opposite pattern was observed for SENN. The ratio of differentially-abundant bacteria in both strains that are associated with pesticide degradation was greater in the treated samples, compared to the untreated samples. This suggests that gut bacteria potentially play a role in pesticide degradation, in the absence of a specific resistance mechanism. Treatment with temephos also reduced the number of bacterial species associated with *Plasmodium*-inhibition, suggesting that sub-lethal effects of larviciding may impact vector competence, and subsequently transmission dynamics. Finally, treatment with temephos had strain-specific effects on longevity for SENN and SENN DDT, where SENN DDT suffered reduced longevity due to sub-lethal exposure. Results from this study highlight the importance of efficient larviciding in order to prevent or minimise the occurrence of sub-lethal exposures, as this may affect the efficiency of vector control programs.

CHAPTER 7
DISCUSSION AND CONCLUSION

The overall aim of this thesis was to investigate some of the factors that typically contribute to residual transmission of malaria in Ehlanzeni, Mpumalanga. The first objective was to gain a historical perspective of vector abundance dynamics in the province. This was investigated in **Chapter 3**. The second objective was to investigate the population dynamics of *An. gambiae s.l.* in Ehlanzeni, Mpumalanga. This was investigated in **Chapter 4** by characterising *Anopheles* larval breeding sites, environmental microbiota, as well as the midgut microbiota and insecticide resistance profiles of *An. gambiae s.l.* populations from Ehlanzeni. The third broad objective covered in **Chapter 5** investigated the impact of pollution on life-history traits of two *An. arabiensis* laboratory strains that differed in their insecticide resistance profile. Finally, **Chapter 6** investigated the sub-lethal effects of temephos, a common larviciding agent, on the longevity and gut microbiome of two strains of *An. arabiensis* that differ in insecticide susceptibility. Although *An. merus* is the dominant *An. gambiae* species in Ehlanzeni, Chapters 5 and 6 focused on *An. arabiensis*, as it is the only member of the *An. gambiae* complex implicated in malaria transmission (Dandalo *et al.*, 2017). South African mosquito populations were not included in our analysis, as we aimed to assess the effects of insecticide resistance, and the only available strains that were genetically related and differed in insecticide resistance phenotype were the Sudanese strains, SENN and SENN DDT. These strains therefore provided a suitable model to investigate whether pre-existing insecticide resistance phenotype influences the impact of pollution on the life-history traits of malaria vectors.

Malaria remains a consistent public health threat in South Africa. In 2023 there were 9 890 reported cases of malaria and 113 deaths due to the disease (World Health Organization, 2024b). South Africa has made significant strides in controlling the disease and is currently on track to eliminate malaria by 2028 (National Department of Health, 2024). However, shifting from a control to elimination framework faces several challenges. These include vectors that adapt their behaviours to avoid control interventions (Russell *et al.*, 2013) and knowledge gaps in vector distribution, insecticide resistance status, and malaria drug usage (Maharaj *et al.*, 2012; Raman *et al.*, 2016). This is compounded by sub-optimal coverage of vector control and surveillance activities (Moonasar *et al.*, 2021), as well as the lack of effective routine

surveillance of insecticide resistance and life-history traits (Killeen *et al.*, 2018). Another significant challenge, particularly in Mpumalanga, is the importation of malaria from neighbouring countries with high transmission rates (Li *et al.*, 2016; Raman *et al.*, 2016).There is also a critical need for case identification and notification within 48 hours (Moonasar *et al.*, 2013). Further challenges include health system bottlenecks (Raman *et al.*, 2021), the need for a skilled workforce and capacity development (Moonasar *et al.*, 2013, 2021). Most critically, however, is the challenge of sufficient funding to support elimination efforts (Maharaj *et al.*, 2012; Raman *et al.*, 2016; Björkman *et al.*, 2019; Moonasar *et al.*, 2021). Residual transmission of malaria will persist if these factors remain unsolved.

Analysis of the historical database provided several insights on the abundance and population dynamics of *An. gambiae s.l.* in Ehlanzeni over the last decade (Chapter 3). *Anopheles merus* was the dominant member of the *An. gambiae s.l.* complex in Ehlanzeni since at least 2008, while members of the *An. funestus* group and *An. arabiensis* were present at much lower abundance levels. This finding is significant for two reasons. Firstly, all three malaria vectors suspected to play a role in the ongoing residual transmission of malaria in Ehlanzeni have been present. These vectors, which include *An. vaneedeni* and *An. parensis* of the *An. funestus* group (Burke *et al.*, 2017, 2019), as well as *An. arabiensis* (Dandalo *et al.*, 2017), are predominantly outdoor-feeding and resting (Gillies and de Meillon, 1968). Secondly, while *An. merus* has not been implicated as a vector of malaria in South Africa, it has been implicated in the neighbouring countries of Mozambique (Cuamba and Mendis, 2009) and Madagascar (Tsy *et al.*, 2003). It is therefore possible that *An. merus* contributes to the transmission of malaria in Ehlanzeni. Indeed, Govere *et al*. (2000) and Mbokazi *et al*. (2018) both suggested that *An. merus* contributes to transmission in the province. The preference of these four species for outdoor- feeding and resting largely renders indoor control interventions poorly effective. This data suggests that supplementary interventions capable of targeting outdoor-biting and resting vector populations need to be considered in the province if elimination goals are to be achieved.

Anopheles gambiae s.l. abundance trends also shifted between 2008 and 2020. Notably, the increased rainfall in 2017 likely created several aquatic habitats that *An. arabiensis* could exploit, which would explain why *An. arabiensis* was more dominant than *An. merus* for the only year over the period of review. During 2017 there was a

significant outbreak of malaria in South Africa (Moonasar *et al.*, 2021). The abundance trends over the surveillance period suggest that, while *An. arabiensis* is likely the major vector transmitting malaria in the province, *An. merus* could nevertheless play an important role as a secondary vector. Secondary vectors have been shown to significantly hamper malaria elimination efforts (Afrane, Bonizzoni and Yan, 2016).

Because the *An. gambiae* complex species composition in Ehlanzeni changed from site-to-site over the surveillance period, it means different methods need to be employed to optimise collection of vector species. Fine-tuning of surveillance activities may be necessary to facilitate the shift from control to elimination, even though this may be more cost intensive. Moonasar *et al*. (2013) and Maharaj *et al*. (2012) likewise recommended more intensive and effective surveillance in order to facilitate early detection of changes in vector bionomics, rapid responses to epidemics/ outbreaks and identification of hotspots for targeted interventions. They also argue that, while more intensive surveillance requires increased funding, it is critical in order to facilitate the shift from a control framework to an elimination framework.

Successful elimination of malaria vectors were achieved in the 1900's in Brazil and Egypt (Soper and Wilson, 1943; Shousha, 1948). These elimination programs relied almost entirely on LSM as an elimination strategy. Larval source management is an effective method to address residual transmission as a result of outdoor-feeding and resting vectors (Amajoh *et al.*, 2013). However, LSM is most effective when larval habitats are "few, fixed and findable" (Amajoh *et al.*, 2013). This makes LSM an effective tool for control of *An. gambiae s.l.* vectors, but less so for *An. funestus s.l.* vectors. This is because, in general, *An. funestus s.l.* habitats are difficult to find (Gillies and de Meillon, 1968). Implementation of LSM in Mpumalanga as part of an area-wide integrated control program may be particularly effective in facilitating the shift from control to elimination in the province. However, successful LSM requires a better understanding of the larval breeding environment, especially in terms of the impact of polluted breeding environments and their effects on the life-history traits of vectors.

Early research investigating the breeding habitats of *An. gambiae s.l.* in South Africa found that *An. gambiae s.l.* typically prefers clear, sunlit, temporary pools of still standing water (Gillies and de Meillon, 1968). However, more recent research is finding that *An. gambiae s.l.* is increasingly becoming adapted to polluted breeding

environments. This has been shown for *An. arabiensis* and *An. gambiae s.s* (Awolola *et al.*, 2007; Antonio-Nkondjio *et al.*, 2011; Jones, Toé, *et al.*, 2012). This thesis has shown that *An. merus* is also adapting to polluted breeding environments in South Africa. What impact then, if any, does this adaptation have on the vector? Studies have shown that breeding in polluted habitats have far reaching consequences on vector life-history traits and insecticide resistance (Antonio-Nkondjio *et al.*, 2014; Nkya *et al.*, 2014; King *et al.*, 2017; Oliver and Brooke, 2018a; Jeanrenaud, Brooke and Oliver, 2020; Chen and Oliver, 2024).

The analysis of the larval breeding environment has shown that breeding in polluted habitats is associated with the upregulation of metabolic detoxification enzymes, as well as a suite of pesticide-degrading bacteria in the midguts of *An. gambiae s.l.* larvae. These two factors may be contributing to the development of insecticide resistance in *An. gambiae s.l.* in Ehlanzeni. Selection for resistance is likely occurring at the larval stage, based on the results of the detoxification enzyme assays. While wild adult *An. gambiae s.l.* from Ehlanzeni are still showing full susceptibility to DDT and deltamethrin, larval selection and the manifestation of resistance in adults is a growing concern (Nkya *et al.*, 2014).

One might ask whether the presence of insecticide resistance significantly hinders the efficacy of vector control programs. One school of thought is that if insecticide resistance significantly hindered vector control efforts, then why have we not seen large-scale failure of LLINs? A few studies have shown that insecticide-treated nets can offer significant protection against malaria, despite the presence of pyrethroid-resistant vector populations (Strode *et al.*, 2014; Lindblade *et al.*, 2015; Kleinschmidt *et al.*, 2018), seemingly reinforcing this theory. However, other studies have found that pyrethroid-resistant vectors have significantly reduced the efficacy of vector control programs (Hargreaves *et al.*, 2000; Churcher *et al.*, 2016). It is therefore likely that a range of factors contribute to the failure of control efforts, and it is not as straightforward as “high levels of insecticide resistance results in the failure of vector control”. These factors include reduced resistance in older mosquitoes (Chouaibou *et al.*, 2012; Jones, Sanou, *et al.*, 2012), as well as delayed effects of sub-lethal exposure (e.g. reduced survival after exposure) (Viana *et al.*, 2016). The interactions between insecticide resistance and parasite infection also play a role (Alout, Djègbè, *et al.*, 2014; Alout, Yameogo, *et al.*, 2014). Reduced blood feeding after exposure, the intensity of resistance present and the extent of net coverage are also important factors to consider

(Glunt, Coetzee, *et al.*, 2018). There is at least one instance where high levels of insecticide resistance contributed to a failure of control, which consequently led to a significant outbreak of malaria in South Africa (Hargreaves *et al.*, 2000). During this outbreak, 63 663 cases were reported- a more than 600% increase from 1995 (Maharaj *et al.*, 2012; Coetzee, Kruger, *et al.*, 2013). The relationship between insecticide resistance and control failure is clearly complex and warrants further investigation.

While insecticide resistance threatens the efficacy of vector control programs, changes in vector life-history traits present an equally significant challenge. Studies have shown that chronic exposure to pollutants have resulted in changes in fertility, larval and pupal survivorship, adult emergence, fecundity, reproductive rates, longevity, developmental time, metabolic and molecular processes and immune responses (Calabrese and Blain, 2005; Mireji *et al.*, 2010; Nkahe *et al.*, 2020). Changes in these life-history traits may have critical epidemiological consequences. For example, if sub-lethal exposure confers longevity advantages to female mosquitoes, it may result in an increased risk of transmission (Garrett-Jones and Shidrawi, 1969; Coosemans *et al.*, 1992).

Of particular concern is the potential impact of chronic/ long-term chloride exposure in conferring developmental advantages to *An. merus* in Ehlanzeni. Significantly decreased development time was observed in two laboratory strains of *An. arabiensis* larvae which were exposed to different concentrations of chloride, compared to their untreated counterparts (Chapter 5, Fig. 5.13). These concentrations were much lower than the environmentally-relevant concentrations present in breeding habitats in Ehlanzeni, which ranges from 50.00 mg/L Cl^- to 200.00+ mg/L Cl^- (Chapter 4, Figure 4.3.2 G). *Anopheles merus* is highly osmotolerant (Muirhead Thomson, 1951), and clearly capable of breeding in these highly saline breeding sites in Ehlanzeni. Faster developmental times result in an increased generational turnover, which in turn increases the risk of malaria transmission, as more generations of malaria transmitting mosquitoes are present. Will chronic exposure to high levels of chloride have the same, or perhaps more pronounced, effects on the development on wild *An. merus* as laboratory reared *An. arabiensis*? Will other life-history traits be impacted as well? These critical questions warrant further research to understand the impact of chloride exposure on *An. merus* development and the effect on its malaria vectorial capacity.

This is particularly important in Ehlanzeni, where *An. merus* is the dominant member of the *An. gambiae s.l.* complex and potentially a minor vector of malaria.

Related to this is the fact that sub-lethal exposure to xenobiotics, particularly insecticides, alters the composition of the gut microbiota. This has been shown for *Aedes albopictus* and *Culex pipiens* (Juma *et al.*, 2020). This thesis has shown that sub-lethal exposure of laboratory strains of *An. arabiensis* to insecticides resulted in changes in gut microbiota (Chapter 6). The gut microbiota of insects are important determinants of several life-history traits, including normal development, fertility, fecundity, survival, metabolism, longevity and blood digestion (Gaio *et al.*, 2011; Coon *et al.*, 2014; Coon, Brown and Strand, 2016; Harrison *et al.*, 2023). Critically, immune response and vector competence are also influenced by the midgut bacteriome (van Tol and Dimopoulos, 2016; Jayakrishnan, Sudhikumar and Aneesh, 2018). Chronic or long-term sub-lethal exposures to pollution and insecticides can shift the midgut bacteriome and potentially cause losses in bacterial species that inhibit *Plasmodium* development. This results in a situation, where on one hand pollution is driving the up-regulation of metabolic detoxification enzymes and increasing the number of pesticide-degrading bacteria, leading to an increase in insecticide resistance. On the other hand, pollution is reducing the number of *Plasmodium*-inhibiting species in the midgut (Chapter 6, Figs. 6.16 – 6.19). This ultimately may result in more resilient mosquito vectors that are more susceptible to infection by *Plasmodium* and are increasingly challenging to control with conventional vector control methods. It is therefore critical to gain a deeper understanding of larval breeding environments before implementing larval source management strategies, such as larviciding.

While selection for resistance at the larval stage in *An. gambiae s.l.* adults from Ehlanzeni has not manifested yet, it does not preclude insecticide resistance in this population. Incipient resistance may be present, which is not detectable by the standard WHO paper tube bioassays, due to the method's single discriminating dose. To detect developing resistance, other methods such as the CDC bottle assay need to be considered, as this method allows one to control the dose of exposure. While the CDC bottle bioassay requires a larger number of mosquitoes to be exposed (which may be challenging to achieve in an elimination framework), it has a higher chance of detecting developing resistance in vector populations. Detecting resistance timeously allows for alternative control efforts and insecticide resistance management strategies

to be implemented to delay the manifestation of insecticide resistance in the population. This will be critical to avoid further outbreaks of malaria and assist in achieving elimination goals.

This study has provided historical perspectives on vector abundance trends in Ehlanzeni and identified opportunities where surveillance can be fine-tuned to achieve malaria elimination goals. The incrimination of *An. merus* as a vector of malaria is a critical next step to push the province from control to elimination. Baseline characterisation of wild *An. gambiae s.l.* breeding habitats in Ehlanzeni, as well as preliminary information on insecticide resistance profiles of larvae, have been provided in this body of work. Further work should focus on establishing baseline resistance in adult *An. gambiae s.l.* and monitoring for incipient resistance with methods that are more sensitive than the WHO paper tube bioassay. This study has also investigated the potential effects of chronic sub-lethal exposures of pollution and larvicides on the life-history traits and gut microbiota of laboratory reared *An. arabiensis*. Further work needs to investigate these effects on wild *An. merus* in Ehlanzeni, particularly the effects of chloride exposure. There is also a need for a deeper understanding of the link between midgut microbiota, insecticide resistance and vector competence.

In conclusion, this study has shown that fine-tuning of surveillance activities may be necessary to shift Mpumalanga from a control framework to an elimination framework. Chronic exposure to pollutants in breeding habitats may have implications for the development of insecticide resistance and alteration of key life-history traits of *An. gambiae s.l.* in Ehlanzeni. Insecticide resistance is a major barrier to malaria elimination programs. Surveillance activities should be tailored to a per-region basis and include monitoring of the effects of pollution on insecticide resistance, life-history traits, gut microbiota and vector competence. Understanding and accounting for these factors in control programs will assist in achieving South Africa’s goal of eliminating malaria by 2028.

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APPENDIX

Appendix A: Manuscript

A Review of Historical Trends in *Anopheles gambiae* Giles (Diptera: Culicidae) Complex Composition, Collection Trends and Environmental Effects from 2009 to 2021 in Mpumalanga Province, South Africa.

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ORIGINAL ARTICLE

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A review of historical trends in *Anopheles gambiae* Giles (Diptera: Culicidae) complex composition, collection trends and environmental effects from 2009 to 2021 in Mpumalanga province, South Africa

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Abstract

South Africa is a frontline country for malaria elimination in the southern African region. It has three malaria-endemic provinces, each with its own transmission pattern. The elimination of malaria depends, in part, on controlling and/or eliminating vectors responsible for transmission. Sustained entomological surveillance is an important factor to consider when shifting from a control to elimination framework. The Ehlanzeni district in Mpumalanga province is a key entomological sentinel surveillance area. It is one of the malaria-endemic districts in South Africa with higher rates of malaria incidences. As such, entomological data about the *Anopheles gambiae* Giles (Diptera: Culicidae) complex have been collected in this province over a substantial period. These data are stored in a pre-existing institutional database. An analysis of the trends that can be observed from this database has not been performed before. This retrospective (longitudinal) analysis provides a summary of the *An. gambiae* complex vector composition in this region from 2009 to 2021. Routine surveillance data were correlated with climatic data (obtained from the NASA LaRC POWER project database) for the same period to assess the role of climatic factors in vector dynamics. This review also identifies a number of limitations in the data collection process across the sampling period and provides recommendations on how to strengthen the database going forward. The most abundant member of the

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An. gambiae complex since 2009 in the province was *An. merus* Dönitz followed by *An. arabiensis* Patton. Collection methods used showed that human landing catches were successful for collecting *An. arabiensis*, while pit traps were the most effective in collecting *An. merus* and *An. quadriannulatus* Theobald. The latter two species were mainly collected in spring, whereas *An. arabiensis* abundance was larger during autumn collections. Vector abundance was not significantly correlated with annual climatic data. The information gained from this database provides insights into the vector dynamics of the Ehlanzeni district of the Mpumalanga province.

KEYWORDS
Anopheles arabiensis, *Anopheles merus*, bionomics, surveillance

INTRODUCTION

Malaria is the most significant vector-borne disease in the world (World Health Organization, 2017). In South Africa, malaria has been a consistent public health threat since the early 1900s, being endemic in three provinces viz. Limpopo, Mpumalanga and KwaZulu-Natal (Coetzee, Kruger, et al., 2013). While significant efforts have been made to control malaria, local transmission of the disease still occurs presenting low-to-moderate risk (Moonasar et al., 2021). This low level and often outdoor transmission is known as residual malaria (Carnevale & Manguin, 2021). This threatens South Africa's malaria elimination agenda, which aims to achieve interruption of transmission and prevent re-establishment (National Department of Health, 2019).

The transmission of malaria depends on several factors. The entomological factors include vector species composition, abundance, breeding site characteristics, vector behavioural traits (e.g., feeding, resting and host preference behaviours) and insecticide resistance (Durnez & Coosemans, 2013; Ferguson et al., 2010; Russell et al., 2013; Sherrard-Smith et al., 2019). A variety of environmental factors are also involved. These include climatic factors such as ambient temperature, rainfall patterns and relative humidity (Adeola et al., 2017; Carnevale & Manguin, 2021). A clear understanding of these factors will assist in devising effective control strategies for the elimination or sufficient control of vector populations, which will in turn assist with achieving and maintaining malaria elimination. This is especially critical on a region-by-region basis, since vector bionomics have been shown to differ from one region to the next (Durnez & Coosemans, 2013).

Members of the *An. gambiae* Giles (Diptera: Culicidae) complex are some of the most abundant vectors in South Africa, along with the *An. funestus* Giles group. The *An. gambiae* complex consists of 10 species (Barrón et al., 2019; Coetzee, Hunt, et al., 2013; Mattingly, 1977; Tennessen et al., 2021; White, 1974, 1985). In South Africa only *An. arabiensis* Patton, *An. quadriannulatus* Theobald and *An. merus* Dönitz occur (Gillies & Coetzee, 1987; Spielman, 1970). Of these, only *An. arabiensis* has been directly implicated locally as a vector although it is highly likely that *An. merus* also contributes to local transmission (Govere et al., 2000; Mbokazi et al., 2018). Their ecological and biological characteristics vary between, and in some cases, within species (Gillies & Coetzee, 1987, Spielman, 1970), which poses a considerable challenge to understanding the bionomics of malaria vectors in South Africa. This involves understanding the role of secondary vector species, factors affecting life history traits (e.g., pollution), the impact of insecticide resistance on control interventions, and the feeding-, resting-, and host selection preferences of vectors.

Regular surveillance is critical to address these questions. While annual surveillance can give a snapshot of the situation in that year, an overview of multiple years of data can offer insight into bionomical trends over time. This information can assist in making evidence-based decisions for vector control interventions.

The Ehlanzeni district in Mpumalanga province is one of the malaria-endemic districts in South Africa affected with higher rates of malaria incidence compared with other malaria-endemic districts in KwaZulu-Natal and Limpopo (Raman et al., 2016). This district is a key surveillance area and has been the focus of surveillance for over a decade. As such, a large body of entomological data has been collected in this area, especially on the *An. gambiae* complex. Despite the surveillance programme being a government initiative with limited resources, funding, and standardised data collection methods, the accumulated data may still provide valuable insights into vector behaviour. Additionally, the database could help identify gaps in the data collection process.

In this review, surveillance data collected between 2009 and 2021 in Ehlanzeni district was analysed to provide insight into the historical trends of vector density and the potential impact of climate. Recommendations for improving the database were also investigated and have been provided.

MATERIALS AND METHODS

Overview

The dataset summarised in this study is curated by the Vector Control Reference Laboratory of the National Institute for Communicable Diseases in Johannesburg, South Africa. The data was collected in collaboration with the National Malaria Control Programme of the National Department of Health of South Africa or as part of past research projects. Data for the period 2009–2021 were analysed and summarised.

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Mosquito collection and identification methods

Adult *Anopheles* mosquitoes were captured using standard collection methods. These included human landing catches, natural resting shelters, man-made pit traps, indoor- and outdoor placed clay pots, modified plastic buckets and carbon dioxide (CO_2) baited tent traps (Braack et al., 2020; Dandalo et al., 2017; Muirhead Thomson, 1951; Muirhead-Thomson, 1958). Other methods included specimen collections from disused tyres, window traps and pit toilets (Dandalo et al., 2017). The immature stages were collected as larvae and pupae from natural pools, dams, stagnant ponds, water-filled potholes and other sources of still/standing water (Mbokazi et al., 2018).

Adult specimens were preserved on silica in 1.5-mL reaction tubes and were morphologically identified using external morphological characteristics (Coetzee, 2020; Gillies & Coetzee, 1987). Members of the *An. gambiae* complex were identified to species using the polymerase chain reaction (PCR) assay described by Scott et al. (1993).

Larval specimens were reared to adulthood and the subsequent generation of adults were identified to species as described for adult collections.

Data collection and organisation

The data from collections were recorded on standardised species identification sheets. The information recorded for each specimen included the unique identification number, identification result from the PCR assay, the date of collection, location of the collection, sex and the collection method. This information was collated in a single datasheet with 10,576 entries.

The data were subsequently cleaned by standardising the date format and spelling of location entries. Duplicate entries and entries without identification, date or location data were removed. This database with 7869 entries remaining was then enhanced by adding additional columns for each entry for ease of analysis. The date column (date of specimen collection) was expanded to include columns for month and year. A season column was added with the following variables: summer (01 December–29 February), autumn (01 March–31 May), winter (01 June–31 August) and spring (01 September–30 November). A municipality column was added which divided collection localities into one of three districts, viz Nkomazi, Bushbuckridge or Mbombela. Finally, a count column was added for each entry to convert the qualitative identification value to a numerical one.

Specimen collection sites

Anopheles mosquitoes were collected from 71 sentinel sites in three different municipalities in the larger Ehlanzeni district (Figure 1), viz., Nkomazi Municipality ($n = 40$ sites), Bushbuckridge Municipality ($n = 19$ sites) and Mbombela Municipality ($n = 12$ sites).

Data analysis

Sampling effort was defined as the number of collection days. A Spearman's rank correlation test was used to assess the correlation between sampling effort per year and the number of mosquitoes captured per year.

The total number of *An. gambiae* sensu *latu* (*s.l.*) mosquitoes collected were summarised for the entire collection period (2009–2021) by species. Sampling effort was determined for (1) each year across the sampling period, (2) each season across the sampling period, (3) each collection method across the sampling period and (4) each locality sampled across the sampling period. The number of *An. gambiae s.l.* captured per species was averaged for each year and divided by the sampling effort of each year, season and collection method.

Additionally, *An. gambiae s.l.* species density was correlated with the average temperature, precipitation and relative humidity for the Nkomazi and Bushbuckridge municipalities, as the data for these were more comprehensive than Mbombela Municipality. The total number of mosquitoes captured in each district was summed for each year across the collection period (13 years). This was correlated to the average temperature, precipitation and relative humidity for each year across the collection period. Similarly, the total number of mosquitoes were summed for each month across the collection period (i.e. all mosquitoes for the month of January across the 13-year collection period) and correlated with climatic parameters that were likewise averaged. A more microclimatic approach was investigated by focusing on a single site, Block A, in the Nkomazi Municipality and looking at daily climatic parameters. The total number of mosquitoes captured at Block A in Nkomazi for each day of collection was correlated with climatic data from the day of collection. The significance of the results was assessed using Spearman's Rank Correlation test (Spearman, 1904). Climatic data were obtained from the National Aeronautics and Space Administration (NASA) Langley Research Centre (LaRC) POWER Project, which is funded through the NASA Earth Science/Applied Science Program (NASA Langley Research Center Atmospheric Science Data Center, 2022).

The number of *An. gambiae s.l.* mosquitoes was averaged for each sampling locality and divided by the sampling effort of each sampling locality. Species richness (number of unique species per site, abbreviated 'R') was determined for each sampling locality and averaged for each sampling district. The following alpha-diversity indices were also calculated: Shannon-Weiner Diversity Index, Simpson's Diversity Index and the Pielou's Evenness Index. The Simpson Diversity Index (abbreviated 'D'; Equation 1) estimates species diversity within a community that accounts for both the number of unique species present as well as the relative abundance of each species present. The Shannon Diversity Index (abbreviated 'H'; Equation 2) estimates the species diversity within a community, taking into account the number of unique species present. The Pielou Evenness Index (abbreviated 'J'; Equation 3) measures whether species within a community are equally abundant (i.e., even). Equations are available in Figure S1. A Shapiro–Wilk (Shapiro & Wilk, 1965) test showed that the data for each alpha diversity index were not normally distributed. A Kruskal–

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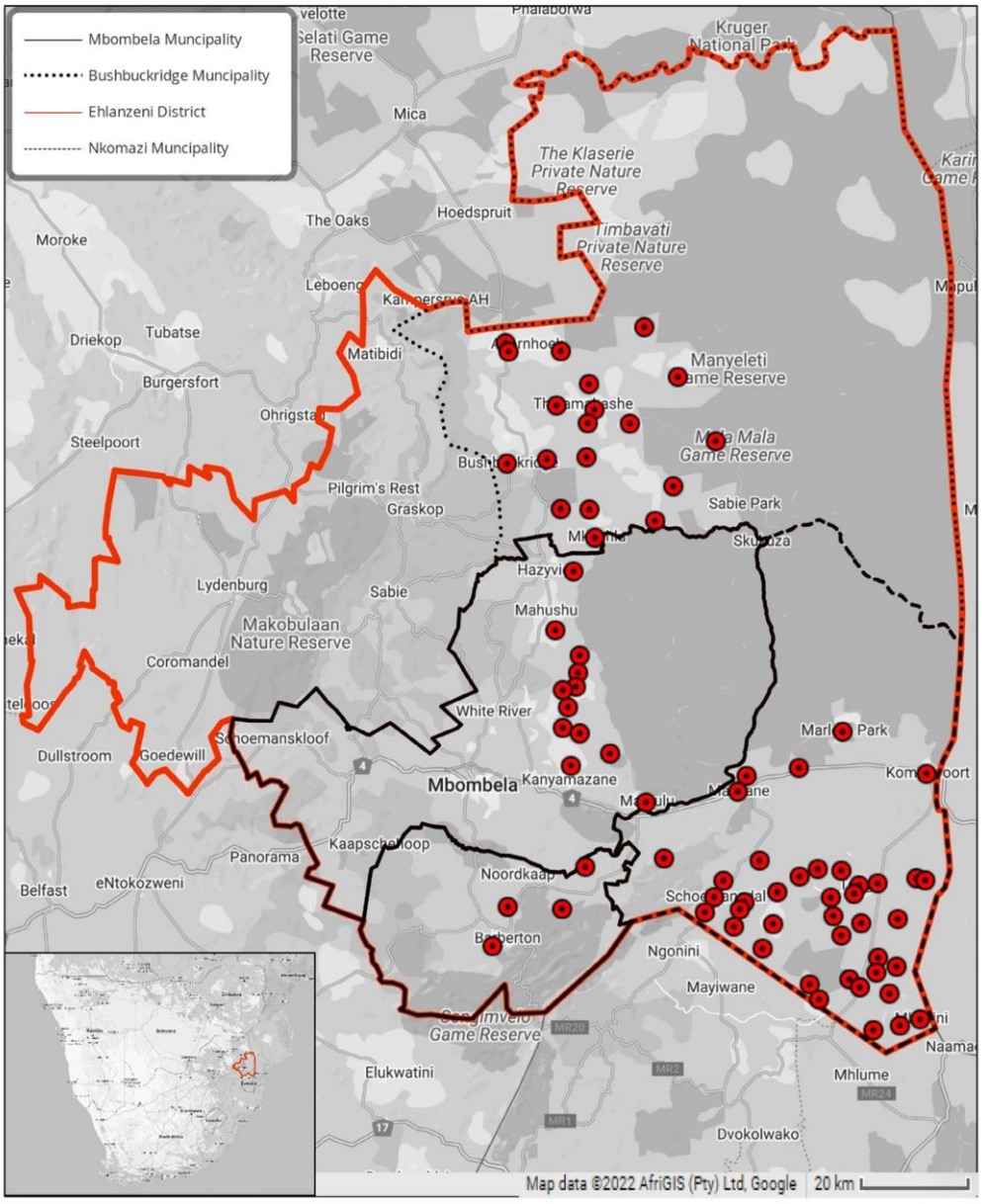


FIGURE 1 Collection sites in the surveillance areas of the Ehlanzeni district (bordered in red). The 75 sentinel sites within the Ehlanzeni district from which larval and/or adult *Anopheles* specimens were collected from 2009 to 2021 are indicated by red dots. The Nkomazi, Bushbuckridge and Mbombela municipalities are bordered in black.

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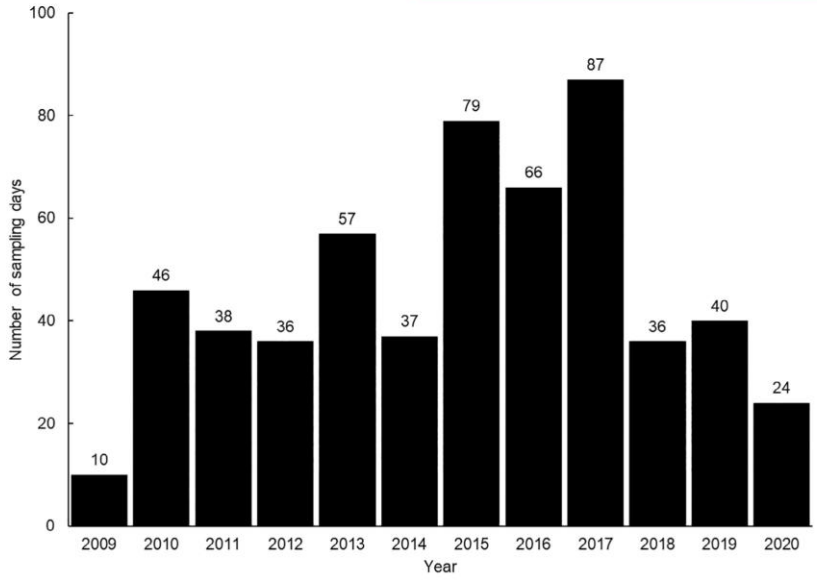


FIGURE 2 Sampling effort (defined here as the number of collection days) by year across the sampling period.

Wallis test (Kruskal & Wallis, 1952) was performed to assess the differences between districts for each index.

All statistical analyses were performed in R (R Core Team, 2023).

RESULTS

Sampling effort

Sampling effort was variable across the surveillance period, with the most sampling days occurring in 2017 ($n = 87$) and the least in 2009 ($n = 10$) (Figure 2). A Spearman's rank correlation test showed a significant positive correlation between sampling effort (number of collection days) and mosquito abundance ($r_s = 0.82$, $p = 0.0011$).

Total mosquitoes collected between 2009 and 2021

The total number of *An. gambiae* complex species collected and identified between 2009 to 2021 in Mpumalanga province was 7812. *Anopheles merus* accounted for 50.3% of collections ($n = 3933$), while *An. quadriannulatus* and *An. arabiensis* contributed 30.1% ($n = 2348$) and 19.6% ($n = 1531$), respectively.

Total mosquitoes collected per season

Anopheles merus and *An. quadriannulatus* were most abundant during spring ($n = 9.84$ captures per day and $n = 5.33$ captures per day, respectively), while *An. arabiensis* was most abundant during autumn ($n = 3.73$ captures per day) (Table 1). Mosquito abundance was lowest during spring for all *An. arabiensis*, autumn for *An. merus* and summer for *An. quadriannulatus* (Table 1).

TABLE 1 The number of *Anopheles arabiensis*, *An. merus* and *An. quadriannulatus* captured per day for each season from 2009 to 2021 in the Ehlanzeni district of Mpumalanga province, South Africa.

	An. arabiensis	*An. merus*	*An. quadriannulatus*	Total
Autumn	3.73	4.88	3.96	12.57
Spring	1.48	9.84	5.33	16.64
Summer	3.28	6.51	3.44	13.23
Winter	1.86	7.41	4.34	13.62
Total	10.35	28.64	17.07	56.06

Total mosquitoes collected per year

Anopheles relative abundance fluctuated over the 13-year period for all three species (Figure 3). *Anopheles merus* was the most abundant species for the majority of the 13-year surveillance period, with reduced abundance only during the period 2015 to 2018 (Figure 3). *Anopheles arabiensis* was present in lower numbers until 2015, then increased to become the dominant member of the *An. gambiae* complex in 2017 (Figure 3). The non-vector *An. quadriannulatus* was more abundant than *An. arabiensis* until 2015, after which it became less abundant than *An. arabiensis* (Figure 3). In 2018, all three species were

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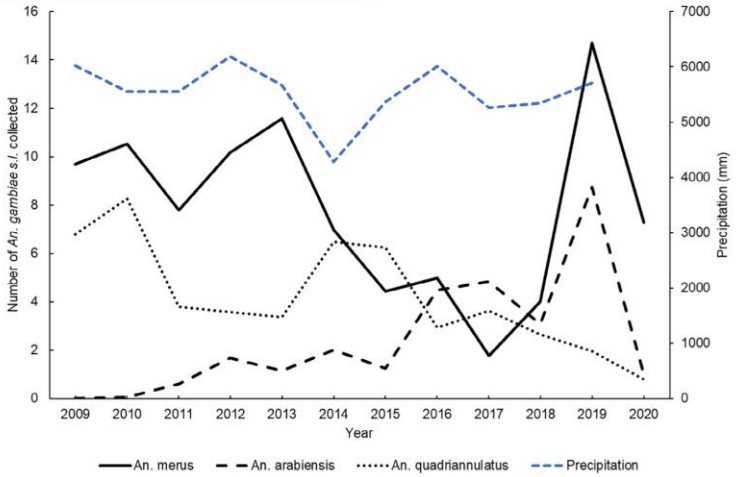


FIGURE 3 Average number of *Anopheles arabiensis*, *An. merus* and *An. quadriannulatus* captured annually per day from 2009 to 2021 in the Ehlanzeni district, Mpumalanga province, South Africa. The blue dotted line indicates the total annual precipitation in mm across the study period.

present in lower numbers (Figure 3). From 2019 to 2020, *An. merus* was the dominant species, and *An. quadriannulatus* numbers continued to decline (Figure 3).

Vector abundance correlated with climatic parameters

Anopheles arabiensis showed a significant negative correlation with the average monthly temperature in Bushbuckridge ($r_s = -0.7$, $p = 0.03$). *Anopheles arabiensis* at Block A in Nkomazi showed a significant positive correlation with temperature on the day of collection ($r_s = 0.17$, $p = 0.02$), whereas *An. quadriannulatus* showed a significant negative correlation with temperature on the day of collection ($r_s = -0.17$, $p = 0.02$). All other climatic factors, including precipitation, were not correlated with vector abundance, regardless of species ($p > 0.05$ in all instances).

Proportion of mosquitoes collected per collection method

The majority of samples were collected as larvae i.e. 90% of *An. arabiensis* collected were collected as larvae, while 85% of *An. merus* and 93% of *An. quadriannulatus* were collected as larvae.

Pit traps were the most effective method for capturing adult *An. quadriannulatus* while human landing catches were the most successful for capturing adult *An. arabiensis* (Figure 4). All three species were captured from CO_2 tents, and this was the most effective method for capturing *An. merus*. Clay pots were effective for capturing *An. arabiensis* and *An. quadriannulatus*, but not *An. merus* (Figure 4). Other collection methods, including disused tyres, traps, indoor pots and pit toilets, were generally ineffective at capturing *An. gambiae s.l.* adults (Figure 4).

Frequency of collection method usage across the sampling period

The frequency of the collection methods that were employed across the sampling period was not equal. Larval collections were the most consistent method used overall, while human landing catches were the most consistent method used to collect adult mosquitoes. Outdoor pots/buckets were used the least (Figure 5). With the exception of the clay pots, all other adult collection methods were used sporadically throughout the sampling period (Figure 5). The clay pots were introduced in 2018 and used consistently until the end of the sampling period (Figure 5).

Species diversity by surveillance locality

A total of 71 sites were sampled across three municipal areas, viz. Nkomazi ($n = 40$), Bushbuckridge ($n = 19$) and Mbombela ($n = 12$) from 2009 to 2021.

Nkomazi had a mean species richness (R) of 2.4 ± 0.75 (rounded down to represent a species richness of 2). The mean Shannon-Weiner Diversity index (H) for Nkomazi was 0.64 ± 0.36, while the mean Simpson's Diversity index (D) was 0.61 ± 0.23. The mean Pielou's Evenness index (J) for Nkomazi was 0.66 ± 0.35.

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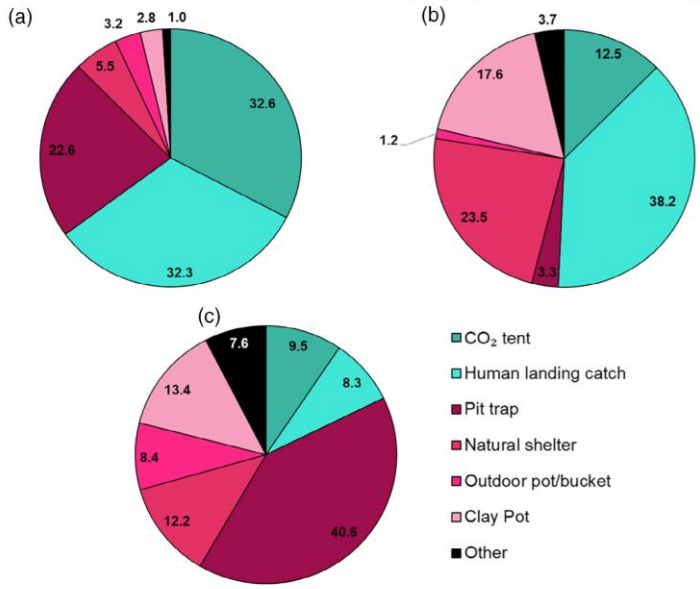


FIGURE 4 Number of *Anopheles gambiae s.l.* adults captured per day for each collection method used in the Ehlanzeni district, Mpumalanga province, South Africa, from 2009 to 2021. (a) *An. arabiensis* (b) *An. merus* and (c) *An. quadriannulatus*. Shades of cyan indicate catches of active mosquitoes and shades of magenta indicate passive catches. Numbers on the chart represent percentages.

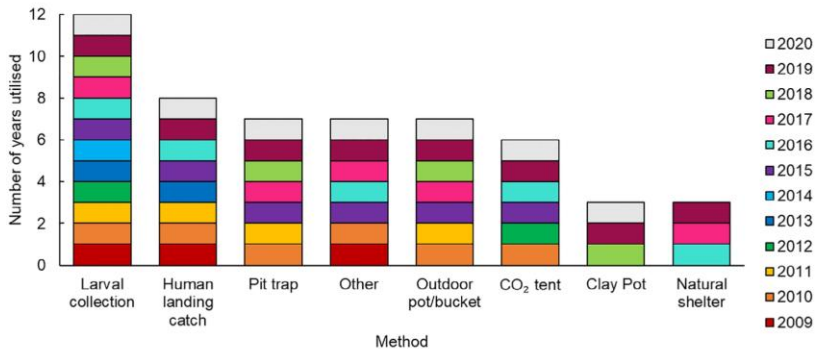


FIGURE 5 Frequency of different collection methods that were employed across the sampling period. Other methods include collections from dongas, indoor pots, pit toilets, window traps and disused tyres.

Mbombela had a mean species richness of 1.92 ± 0.79 (rounded up to represent a species richness of 2). The mean Shannon-Weiner Diversity index for Mbombela was 0.40 ± 0.38, while the mean Simpson's Diversity index was 0.75 ± 0.24. The mean Pielou's Evenness index for Mbombela was 0.47 ± 0.41.

Bushbuckridge had a mean species richness of 1.26 ± 0.45 (rounded down to represent a species richness of 1). The mean Shannon-Weiner Diversity index for Bushbuckridge was 0.12 ± 0.23, while the mean Simpson's Diversity index was 0.92 ± 0.16. The mean Pielou's Evenness index for Bushbuckridge was 0.18 ± 0.34.

The data for each of the indices were not normally distributed ($W_D = 0.83$, $p = 2.092e{-}07$; $W_H = 0.85$, $p = 1.112e{-}06$; $W_J = 0.81$, $p = 3.215e{-}08$ and $W_R = 0.78$, $p = 6.99e{-}09$). A Kruskal–Wallis test showed significant differences between districts for the Shannon-Weiner Diversity Index (H: $\chi^2[2, n = 71] = 22.94$, $p = 1.043e{-}05$), the Simpson's Diversity Index (D: $\chi^2[2, n = 71] = 21.55$,

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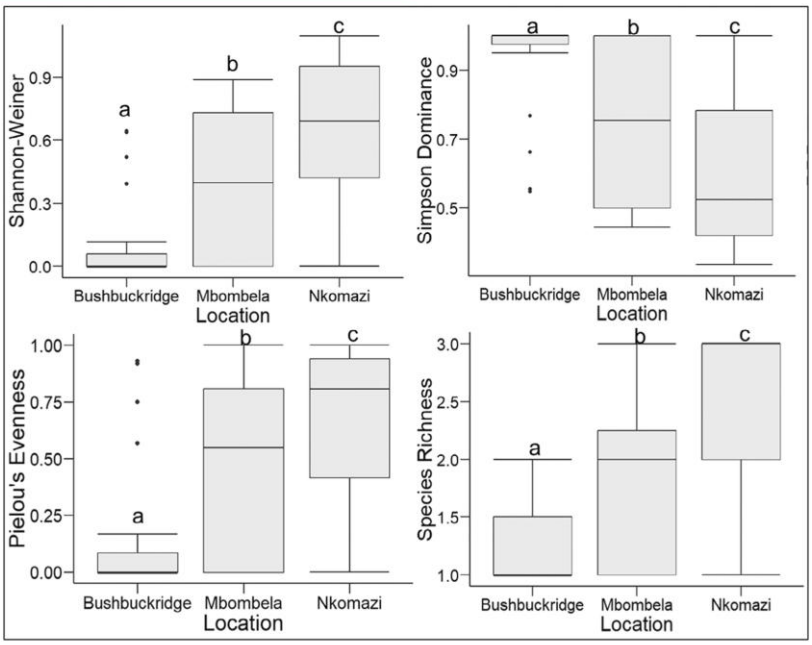


FIGURE 6 Alpha diversity of *Anopheles gambiae* complex species composition in the Ehlanzeni district in the three municipalities within the greater Ehlanzeni district, Mpumalanga province, South Africa, from 2009 to 2021. (a) species richness (b) Pielou's evenness index (c) Shannon-Weiner diversity index and (d) Simpson Diversity index. The mean is indicated by an 'x' for each box.

$p = 2.09e{-}05$), the Pielou's Evenness index (J: $\chi^2[2, n = 71] = 17.99$, $p = 0.00012$) and species richness (R: $\chi^2[2, n = 71] = 24.61$, $p = 4.534e{-}06$) (Figure 6).

DISCUSSION

This study was a retrospective examination of an existing entomological surveillance database, rather than a study of the historical bionomics of the Mpumalanga province. Several inferences on the historic trends in vector species composition, collection methodology and environmental effects could be made from the database that will aid South Africa's malaria elimination agenda. While no significant differences were present in sampling effort across the sampling period, there were a number of limitations in the data collection process, especially concerning the collection methodology. Addressing these limitations will strengthen the database going forward.

The database suggests that historically *An. merus* is the most abundant member of the *An. gambiae* complex in Mpumalanga province. This contrasts with KwaZulu-Natal province, where *An. arabiensis* is the predominant member of the *An. gambiae* complex (Dahan-Moss et al., 2020; Dandalo et al., 2017). A review of the anopheline diversity in Limpopo province showed that *An. merus* was the least prevalent member of the *An. gambiae* complex (Braack et al., 2020). This shows the heterogeneity in the distribution of anophelines in these study sites, highlighting the importance of understanding provincial malaria vector bionomics.

The dominance of *An. merus* in the Ehlanzeni district is congruent with previous surveillance data. Collections in the region between 1997 and 1998 showed that *An. merus* was the dominant species (56%), with *An. quadriannulatus* at a prevalence of 30.4% and *An. arabiensis* at 13.8% (Govere et al., 2000). Surveillance in the Ehlanzeni district between 2005 and 2014 indicated that *An. merus* made up 64% of the collections (Mbokazi et al., 2018).

Anopheles merus sampling abundance shows seasonality. Most specimens of this species were collected in spring, with the numbers declining to their lowest levels in winter. By contrast, most *An. arabiensis* specimens were collected in autumn, followed by summer, spring and winter seasons. For *An. quadriannulatus*, the greatest numbers were collected in spring, followed by autumn, summer and winter. Although this represents absolute numbers, it is worth noting that when examining the percentage distribution, *An. arabiensis* only constituted 8.8% of the spring total, but 13.8% of the winter total. Therefore, although as with both the other species, the total captures were lowest in winter, *An. arabiensis* had a greater proportional representation in winter than in spring. Autumn was the most evenly

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represented season, where the species proportions were approximately a third each and *An. merus* was at the lowest proportional representation. These differences in seasonality suggest either competition between the species (Ewing et al., 2019), or variation in ecological adaptation whereby *An. arabiensis* are better adapted to drier climatic conditions.

From the information available in the database, in general, there was no correlation with annual climatic parameters. However, this does not mean that there is no association between climate and vector abundance in the province. When examining rainfall in Nkomazi at Block A on a daily level, the correlation between climate and vector abundance increases and is significant at the 95% confidence interval. Several other studies found a significant correlation between vector abundance and climatic parameters at a site-specific level on a monthly basis (Govere et al., 2000; Konwar et al., 2021; Manyi et al., 2015; Mosha & Mutero, 1982; Simon-Oke & Olofintoye, 2015). This suggests that there may be significant correlations when examining abundance and rainfall/ temperature at a daily or weekly level. It is possible that there may be unexamined effects of microclimate on vector abundance. For instance, microclimatic variation has been demonstrated to affect the biting behaviour of *An. arabiensis* (Ngowo et al., 2017). As such, climatic variation may play a complex role in species abundance, but may not be detected on the scale of climate data available.

There is a notable climate anomaly though. In 2017, the numbers of *An. arabiensis* exceeded that of *An. merus* and *An. quadriannulatus* for the only time during the period under review. During 2017, the average daily rainfall in the province was higher than the average daily rainfall from 2007 to 2016 (NASA Langley Research Center Atmospheric Science Data Center, 2022). The higher rainfall may have hindered the population growth of *An. merus* and *An. quadriannulatus* by flushing out larval habitats (Paaijmans et al., 2007). Prolonged rains may also have diluted the salinity levels of water bodies primarily used by *An. merus*, thus enabling *An. arabiensis* to utilise these habitats. It is also not coincidental that 2017 marked South Africa's highest number of malaria cases since the epidemic of 2000 (Maharaj et al., 2019). Several studies have shown that rainfall is a significant driver of malaria transmission (Abiodun et al., 2018; Adeola et al., 2017; Adeola, Botai, et al., 2019; Adeola, Ncongwane, et al., 2019). This highlights the fact that climate's effect on the intensity of malaria transmission cannot be ignored.

The analysis of the collection methods was also informative. The relative efficiencies of the adult collection methods can be used to infer some mosquito behavioural characteristics. The dominant species, *An. merus*, were primarily collected by human landing catches, pit traps and CO_2 tent catches at approximately equal ratios. These were also the most successful methods for collecting *An. quadriannulatus*, although pit traps were more successful than human landing catches, which in turn was more successful than CO_2 tent catches. By contrast, there was more variability in the *An. arabiensis* catches. Human landing catches, however, were the most efficient mechanism for sampling this species. This suggests that although all three species engaged in human seeking, *An. arabiensis* was the most anthropophilic. Outdoor-placed clay pots are a successful method for sampling *An. arabiensis* in northern KwaZulu-Natal province (Burke et al., 2017; Dandalo et al., 2017; Munhenga et al., 2022), but their deployment was less successful in Mpumalanga. It is worth noting that clay pot collections made up a more substantial portion of *An. arabiensis* than the other two species. The results also suggest tailored methods to collect species of interest on a regional basis. For example, when trying to incriminate *An. arabiensis* as a potential vector, pit traps and natural shelters are not suitable trapping methods. Rather, human landing catches would be more efficient in collecting *An. arabiensis*. These are crucial considerations for the optimisation of the control programme in Mpumalanga.

There were several limitations identified during the analysis of the collection methodology. The number of traps, sampling hours and sampling personnel were not captured during data collection. Additionally, different collection methods were not employed with equal frequency across the sampling period. Finally, several different sampling sites were used across the sampling period, with no clear indication of whether the same sampling sites were used or whether sites were randomised. While there were no significant differences in sampling effort across the sampling period, standardising the collection methodology may strengthen the database and allow for more powerful analyses to be performed in future.

We suggest a standardised metadata template for collection, outlining the necessary data to capture when collecting field samples (Figure S2). Importantly, detailed locality data with GPS coordinates, trap number, number of sampling personnel and sampling hours should be added to the current body of data collected from the field. Further, employing the three best-performing collections methods (i.e., larval collections, pit traps and CO_2 tents) exclusively would be most suitable to standardise the data collection process. We also encourage sampling from the same localities as far as possible. Challenges arise when streams in certain areas dry up. To address this, permanent sampling sites can be established in localised areas such as Vlakbult or Masibekela, with collections carried out at micro-sampling sites within those areas.

The Nkomazi Municipality had higher diversity and evenness indices than the other two municipalities, indicating that the diversity of *An. merus*, *An. arabiensis* and *An. quadriannulatus* in this district is comparatively high and that all three species are more or less evenly distributed, as opposed to Mbombela and Bushbuckridge where the diversity was low, and the three species were not evenly distributed. This may be because Mbombela and Bushbuckridge had a notably lower number of sampling sites compared to Nkomazi. Further, regular sampling in Mbombela only started in 2015 and for Bushbuckridge in 2014. All three species occurred in all three municipal districts, but did not necessarily all occur together at the same time. Previous studies also found high diversity and species evenness at Nkomazi (Govere et al., 2000; Mbokazi et al., 2018; Ramalwa et al., 2014). Mbokazi et al. (2018) study similarly found that species diversity was low for the Mbombela and Bushbuckridge municipalities, as well as a low species evenness for Bushbuckridge. Species richness for Mbombela was however found to be fairly evenly distributed in their study.

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Significant differences existed between the three sites for both the Shannon-Weiner and Simpson diversity index, which takes into consideration both species richness and evenness. Crucially, these patterns are inverted (e.g. Nkomazi has the highest Shannon-Weiner index and the lowest Simpson index), as expected. This indicates that, even with the limited amount of data, the distribution that can be determined from the database is valid.

This review has provided valuable insights into historical trends of *Anopheles gambiae* complex species composition, collection trends and environmental effects. Additionally, this review has identified a number of limitations in the data collection process. Going forward, what this review also presents are considerations on how to improve and strengthen the database for future analyses. This includes using permanent sentinel sampling sites, employing the three best-performing collection methods and collecting additional information during the collection process. Additionally, recording climatic variables at the sampling site level, as opposed to the regional level, is essential for understanding the relationship between climate and vector abundance. These all, however, are limited by logistical factors. Despite this, if such factors could be employed to strengthen this database, it would make a greater impact on South Africa's elimination agenda.

In conclusion, a substantial amount of data could be extracted from the VCRL database. These data provide information on the population fluctuations of the *An. gambiae* complex in Mpumalanga province. It enables some inferences concerning adaptability to the climate variables, rainfall and temperature, and they indicate seasonal abundance and geographical distribution by species. Importantly, these data show the efficacy of various collection methods for the capture of *An. gambiae* complex mosquitoes in the province. This information also highlights the need for deeper investigations into the role of climate, and microclimate in particular, on vector biology. This information can be used to tailor vector control in this province in terms of timing of interventions and also indicates risk and receptivity for malaria based on vector occurrence and distribution—an important indicator for malaria elimination certification.

AUTHOR CONTRIBUTIONS

Kayla P. Noeth: Conceptualization; investigation; writing – original draft; formal analysis; project administration; writing – review and editing. **Maria L. Kaiser:** Writing – review and editing; investigation; resources; data curation. **Thabo Mashatola:** Writing – review and editing; investigation; resources. **Yael L. Dahan-Moss:** Data curation; writing – review and editing. **P. Avhatakali Matamba:** Formal analysis; writing – review and editing. **Belinda Spillings:** Formal analysis; writing – review and editing. **Riann Christian:** Writing – review and editing; formal analysis; data curation. **Erika Erlank:** Investigation; resources; formal analysis; writing – review and editing. **B. Power Tshikae:** Writing – review and editing; investigation; resources. **Eunice Jamesboy:** Writing – review and editing; formal analysis. **Silindile Sibambo:** Investigation; resources; writing – review and editing. **Busisiwe G. Nkosi:** Investigation; writing – review and editing; resources. **Brian T. Silawu:** Investigation; writing – review and editing; resources. **Lazarus J. Mkhabela:** Investigation; writing – review and editing; resources. **Fanuel S. Ndlovu:** Investigation; writing – review and editing; resources. **Thembekile P. Mgwenya:** Investigation; writing – review and editing; resources. **Maureen Coetzee:** Funding acquisition; writing – review and editing; supervision. **Basil D. Brooke:** Funding acquisition; writing – review and editing; supervision. **Lizette L. Koekemoer:** Writing – review and editing; supervision; funding acquisition. **Givemore Munhenga:** Funding acquisition; writing – review and editing; supervision. **Shüné V. Oliver:** Funding acquisition; supervision; project administration; conceptualization; writing – review and editing; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are not publicly available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Equations used to calculate diversity indices.

Figure S2. Proposed metadata template to be used with example information to be collected during future surveillance activities.

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Appendix B: Ethical Clearance

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

Date: 29/02/2024

Certificate reference: Waiver 29-02-2024-O
Category: O
Applicant: Dr Shüné Oliver
Department: Vector control Reference Laboratory, National Institutes for Communicable Diseases, Sandringham, Johannesburg, South Africa.
Tel: 011 386 6483 **Email**: Shuneo@nicd.ac.za

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

This letter is to confirm that following studies to be undertaken in the Vector Control Reference Laboratory at the National Institutes for communicable Diseases, do not require a full Animal Ethics Research Committee clearance.

A. Singh_29-02-2024-O
Student Details: Ashmika Singh, PhD Candidate (859507)
Project Title: Characterisation of the dynamic gut microbiota of members of the An. gambiae complex.

K. Noeth_29-02-2024-O
Student Details: Kayla Noeth, PhD Candidate (2525673)
Project Title: Bionomics of Anopheles mosquitoes in Ehlanzeni District Municipality, Mpumalanga

S. Misser_29-02-2024-O
Student details: Shristi Misseer, MSc Candidate (1388499)
Project Title: The effect of larval exposure to plastic pollution on the major malaria vector, Anopheles arabiensis Patton (Diptera: Culicidae)

W. Rasikhanya_29-02-2024-O
Student details: Wendy Rasikhanya, MSc Canidate (1884976)
Project Title: The effect of rural and urban pollutant exposure on the major malaria vector Anopheles arabiensis.

Reason for waiver
These projects entail metagenomic data collection from invertebrate species, of laboratory reared and wild *Anopheles arabensis* mosquitoes and thus do not require a full animal ethics clearance.

Details of the study
These projects aim to generate metagenomic data on the Anopheles gambiae complex (including An. arabiensis species) gut microflora using next generation sequencing (NGS) technology. Changes in microflora will determined in response to the exposure of heavy metals and salt (A. Singh), organosulphate larvicides (K. Noeth), plastic pollutants (S. Misser) and nitrate, nitrites, sulphides and sulphates (W. Rasikhanya) and the subsequent effect on larval development, adult longevity and insecticide resistance.

The individuals covered by this waiver include students; Ashmika Singh, Kayla Noeth, Shristi misser Wendy Rasikhanya and supervisors Dr Shüné Oliver (A002517), Dr Givemore Munhenga (A002589) and Dr Chia-yu Chen (A0048530) in the Vector control Reference Laboratory, NICD and WITS joint staff.

Please contact me should you require further information.

Yours sincerely,

Dr Mark Killick
Deputy Chair: Animal Research Ethics Committee
University of the Witwatersrand.
Mail: Mark.Killick@wits.ac.za Office number: 011 717 2362

Appendix C: Section 20 Permit



agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA



Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: MarnaL@dalrrd.gov.za Website: www.dalrrd.gov.za
Reference: 12/11/1/1/13 (3039PM)

Prof Basil Brooke
NICD
1 Modderfontein Road,
Sandringham, 2131
Johannesburg
E-mail: basilb@nicd.ac.za

Dear Prof Brooke

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 12 May 2023 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa.
2. The research project is approved as per the application form dated 12 May 2023 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za.
3. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za.
4. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National *Road Traffic Act*, 1996 (*Act* No. 93 of 1996).



5. *Anopheles* mosquitoes used for this project must only be collected from Ilembe, Umkhanyakude, King Cetshwayo and Zululand districts of KwaZulu-Natal province as well as Ehlanzeni district of the Mpumalanga province. *Anopheles* mosquitoes to be used in this research project must not be sourced from any area under veterinary restriction of mosquito-borne pathogens.
6. Prior to collection of mosquitoes, please consult with the local State Veterinarian to ensure that the area is still not under any veterinary restrictions, as disease status of area may change.
7. All potentially infectious material utilised or generated during or by the research project is to be destroyed at completion of the research project except for:
   7.1. DNA extracted must be stored in -20°C freezers at Vector Control Reference Laboratory (VCRL).
   7.2. The original silica tubes containing *Anopheles* specimen must be sealed and stored in the VCRL museum in a sealed packet.
   7.3. DNA extracted and Anopheles specimens must not be used or outsourced for further research purposes without written permission from the Director: Animal Health. Please apply in writing to MamaL@dalrrd.gov.za.
8. Any incidence or suspected incidence of a controlled or notifiable disease in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984), must be reported immediately to the responsible state veterinarian.
9. All potentially infectious waste from the research project must be removed and disposed by incineration with a waste disposal company registered to remove biohazardous waste.
10. Records must be kept for five years for auditing purposes.

Title of research/study: Malaria vector surveillance in South Africa.
Researcher: Prof. Basil Brooke
Institution: National Institute for Communicable Diseases, Centre for Emerging Zoonotic and Parasitic Diseases, Vector Control Reference Laboratory.
Permit Expiry Date: 31 May 2026
Our ref Number: 12/11/1/1/13 (3039PM)
Your ref: No reference

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2023-10-05

Appendix D: Additional Information

Additional information is available using the following link:

Appendices

The information includes:

• Chapter 3: raw climatic and mosquito abundance data

• Chapter 6:

- OTU taxa tables
- OTU abundance tables
- Overlapping bacteria
- Processed reads