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The effect of larval exposure to plastic pollution on the major malaria vector, *Anopheles arabiensis* Patton (Diptera: Culicidae)

by

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

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Johannesburg, 2025

Declaration

I, Shristi Misser (1388499), declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



_____

(Signature of candidate)

26th of May 2025 in Johannesburg

Abstract

Anopheles arabiensis is a dominant vector species of malaria within Southern Africa. Previous studies have demonstrated that *Anopheles* mosquitoes are capable of breeding in polluted water bodies, raising concerns about the role of environmental contaminants in vector biology. Among the most prevalent pollutants in aquatic environments is plastic. This study aimed to investigate the effects of plastic exposure on various biological factors of *Anopheles arabiensis*, including life history traits, insecticide tolerance, midgut microbiota composition, and epigenetic modifications. Larvae were exposed to artificially degraded disposable nappies, two plastic additives (Bisphenol-A and phthalic acid), and a representative of microplastics (commercially available latex beads). Two laboratory strains were used: an insecticide-selected strain (SENN DDT) and an unselected strain (SENN), allowing for the assessment of the effects of plastic exposure on insecticide resistance phenotypes. The results indicated that larval exposure to nappies and latex beads significantly delayed larval development in both strains. Conversely, exposure to plastic additives accelerated larval development in SENN but slowed it in SENN DDT. Adult longevity was significantly increased in SENN females exposed to nappies and BPA. An overall increase in tolerance to the insecticide deltamethrin was observed across both strains, with the most significant increase following phthalic acid exposure. Midgut microbiota analysis revealed greater shifts in SENN compared to SENN DDT following plastic exposure. Particularly, an increase in the abundance of pesticide and plastic-degrading bacteria was observed upon larval exposure to plastics. Additionally, strain-specific epigenetic changes were observed, with SENN males exhibiting higher histone acetyltransferase (HAT) activity post plastic exposure. Importantly, the effect of plastic exposure is contingent upon the specific insecticide resistance phenotypes present in *An. arabiensis*. However, further studies on the effect of plastics on wild populations of *An. arabiensis* are warranted as this will deepen our understanding of the real-world impact of plastic pollution on mosquito biology, insecticide tolerance, and vector-borne disease dynamics. This study enhances the understanding of the impact of pollution on the biology of *An. arabiensis* and underscores the role of plastic pollution as a potential contributor to the challenges encountered in vector control and malaria transmission. In conclusion, this research highlights the critical intersection of environmental pollution, vector biology, and public health, underlining the urgency of addressing and mitigating the challenges of pollution, especially as it pertains to malaria transmission and effective vector control strategies.

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Misser, S., Chen, C-Y., Oliver, S.V. The effect of plastic pollution on the gut microbiota of the major malaria vector, *Anopheles arabiensis* Patton (Diptera: Culicidae). The South African Society of Biochemistry and Molecular Biology (SASBMB) Congress 2024, Limpopo, South Africa. 7-10 July 2024. (Poster Presentation)

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List of Abbreviations and Acronyms

Abbreviations	Definitions
5mC	5-Methylcytosine
5hmC	5-Hydroxymethylcytosine
ANOVA	Analysis of variance
ASVs	Amplicon sequence variant
BPA	Bisphenol A
CDC	Centres for Disease Control
CpG	Cytosine-Guanine dinucleotide
DDT	Dichlorodiphenyltrichloroethane
DEHP	di-2-ethylhexyl phthalate
DNMT	DNA methyltransferase
DVS	Dominant vector species
GABBA	Gamma-aminobutyric
GMEP	Global Malaria Eradication Programme
GST	Glutathione S-transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HMT	Histone methyltransferase
Imd	Immune deficiency
IRS	Indoor Residual Spraying
ITN	Insecticide treated nets
JAK/STAT	Janus kinase/signal transducers and activators of transcription
Kdr	Knockdown resistance
LC_{50}	Median lethal concentration to 50% mortality
LT_{50}	Median lethal time to 50% mortality
LDPE	Low density polyethylene
LLINs	Long lasting insecticide treated nets
miRNA	microRNA
MP	Microplastic
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide

NICD	National Institute for Communicable Diseases
NMDS	Non-metric multidimensional scaling
PAE	Phthalic acid ester
PCR	Polymerase chain reaction
PE	Polyethylene
PERMANOVA	Permutational multivariate analysis of variance
PET	Polyethylene terephthalate
PHB	Polyhydroxybutyrate
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinyl chloride
Rdl	Resistance to dieldrin
RO	Reverse osmosis
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SIT	Sterile insect technique
SD	Standard deviation
SEM	Standard error of the mean
VBD	Vector borne disease
WHO	World Health Organization
YFP	Yellow Fluorescent Protein

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Chapter 1: General Introduction

1.1. Vector-borne diseases

Vectors- organisms that facilitate the transmission of infectious pathogens between humans or from animals to humans- play a central role in the spread of vector-borne diseases (VBDs) (World Health Organization, 2020). These infectious diseases are caused by parasites, bacteria, or viruses and are transmitted by hematophagous (blood-feeding) arthropods such as mosquitoes, ticks, fleas, sandflies, tsetse flies, blackflies, lice, and triatome bugs (World Health Organization, 2020). VBDs, including Dengue, Chagas disease, Japanese encephalitis, yellow fever, and malaria, disproportionately impact impoverished populations, with VBDs accounting for 17% of global infectious diseases (Golding *et al.*, 2015; World Health Organization, 2020). Low-income countries are particularly vulnerable to VBDs due to limited resources for effective disease control and prevention. This vulnerability restricts their ability to mitigate public health risks, which in turn heightens the impact of infectious diseases (Golding *et al.*, 2015). The distribution and spread of these diseases are significantly shaped by numerous demographic, environmental and socioeconomic factors (Institute of Medicine (US) Forum on Microbial Threats, 2008; Pala *et al.*, 2022).

A factor that is particularly important to the spread of VBDs is the convergence of rapid population growth and urbanisation that has significantly altered landscapes, often transforming rural areas for agricultural use and inadvertently increasing VBD prevalence (Institute of Medicine (US) Forum on Microbial Threats, 2008; Pala *et al.*, 2022). Moreover, globalisation and increased travel have facilitated VBD outbreaks in previously unaffected regions. This phenomenon is particularly prominent in the context of migration patterns from rural to urban areas, which facilitates the spread of these diseases (Pala *et al.*, 2022). The interplay between urbanisation and VBDs, particularly malaria, underscores the need for integrated approaches to disease prevention and control. Understanding how urbanisation influences vector behaviour and disease transmission is critical for developing targeted strategies to combat malaria and other VBDs.

1.2. Malaria

Malaria is the most extensively documented VBD, a phenomenon largely attributable to its endemic presence across the African continent. During the period of the COVID-19 pandemic, specifically from 2020 to 2021, there was a notable surge in malaria cases, with an increase of 13 million reported instances and an additional 63 000 fatalities directly associated with the disease. This data underscores the ongoing public health challenges posed by malaria, particularly in the context of global health crises (World Health Organization, 2023). The WHO African Region accounted for 95% of all malaria cases and 96% of malaria deaths in 2021 (World Health Organization, 2023).

The burden of malaria is caused by the five protozoan parasites within the *Plasmodium* genus, namely: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi* (Antinori *et al.*, 2012, 2013). Among these, *P. falciparum* is particularly noteworthy for its ability to induce severe forms of malaria, primarily due to its rapid replication in the blood, resulting in severe blood loss (as reviewed in Oaks *et al.,* 1991). The pathophysiological mechanisms of *P. falciparum* include sequestering within the small blood vessels, causing blockages (Center for Disease Control and Prevention, 2024). Upon the sequestering of *P. falciparum* in the brain, cerebral malaria, a potentially fatal consequence, is the likely outcome (Center for Disease Control and Prevention, 2024).

Plasmodium falciparum is widely distributed across tropical and subtropical regions globally, with the highest prevalence observed in Africa (Center for Disease Control and Prevention, 2024). In contrast, *P. vivax*, represents the most commonly encountered protozoan associated with infectious diseases, predominantly found in Asia, Latin America, and select regions of Africa (as reviewed in Oaks *et al*., 1991). Due to *P. vivax's* wide distribution within these regions, particularly within Asia, it is recognised as the most common malaria parasite (Centers for Disease Control and Prevention, 2024).

Two host organisms are essential for the life cycle of the *Plasmodium* parasite. The first is an intermediate host (i.e. human), and the second is a definitive host (i.e. mosquito) (Centers for Disease Control and Prevention, 2024). The transmission of the *Plasmodium* parasite to the intermediate vertebrate host involves a two-week infection cycle in the mosquito vector. Upon feeding on an infected individual, female mosquitoes ingest *Plasmodium* gametocytes. In the

mosquito midgut lumen, male and female gametocytes mature into male microgametes and female macrogametes, respectively, which, upon fertilisation, produce zygotes (Clayton *et al.*, 2014). These zygotes further develop through meiosis into motile ookinetes. The ookinetes navigate and invade the mosquito midgut epithelium. When the ookinetes reach the basal lamina, they develop into oocysts. The process of sporogony within the oocysts results in multiple rounds of mitotic replication. This results in the production of haploid sporozoites that are released into the haemocoel and thus result in the invasion of the salivary glands. Once the sporozoites are within the salivary glands, they can then be transmitted to the intermediate host during another blood meal (Lee *et al.*, 2014; Simões *et al.*, 2017).

1.3. Malaria and the *Anopheles* vector

Mosquitoes that can transmit the *Plasmodium* parasite have adapted and evolved in a way in which the fitness cost of infection is minimised and concurrently both organisms benefit through the sharing of nutrient resources. Additionally, parasite immune suppression and mosquito tolerance to infection play a role in the interactions between the two organisms (Shaw *et al.*, 2022). Currently, the only known genus of mosquitoes to transmit malaria to humans is *Anopheles*. The competency of a vector measures how susceptible the vector is to the *Plasmodium* parasite (Habtewold *et al.*, 2017; Simões *et al.*, 2017). Thus, only certain species within the *Anopheles* genus are competent vectors due to their inability to develop an immune response to the *Plasmodium* parasite (Habtewold *et al.*, 2017). As such, vector competency differs between closely related species such as members of the *Anopheles gambiae* complex (Lambrechts *et al.*, 2005; Molina-Cruz *et al.*, 2012; Eldering *et al.*, 2016).

Sub-Saharan Africa has the highest burden of malaria (Sinka *et al.*, 2010). This is due to the most effective and efficient dominant vector species (DVS) of human malaria found in Africa (Coluzzi, 1999). The *An. gambiae* complex is of particular interest due to its efficiency as a DVS in Africa (Sinka *et al.*, 2010). The *An. gambiae* complex consists of at least nine morphologically identical species that differ in genetic makeup, behaviour, and vectorial capacity (Coetzee *et al.*, 2000; Barrón *et al.*, 2019). The *An. gambiae* complex includes the major malaria vectors *Anopheles gambiae*, *Anopheles coluzzii* and *Anopheles arabiensis* (Gillies and Coetzee, 1987) as well as the saltwater tolerant coastal secondary vectors *Anopheles melas* and *Anopheles merus* (Harbach, 2004). The remaining members of this complex are restricted by their distribution in areas with low population densities and their

zoophilic (animal-biting) behaviour thus not making them efficient vectors of human malaria (Sinka *et al.*, 2010).

The variability of susceptibility to the parasite is due to genetic variations (Lambrechts *et al.*, 2005; Molina-Cruz *et al.*, 2012; Eldering *et al.*, 2016; Simões *et al.*, 2017). These genetic variations have been linked to the innate immunity of mosquitoes and rely on specific immune gene alleles (Blandin *et al.*, 2009; White *et al.*, 2011; Mitri *et al.*, 2015). As such, the mosquito's immune system is vital in determining whether *Plasmodium* infection occurs within the vector and if the parasite is transmitted to the intermediate host (Habtewold *et al.*, 2017). The parasite is subject to vital immune responses during the invasion and development within the midgut, which may lead to the elimination of the parasite (Habtewold *et al.*, 2017). The primary locations in which the parasite may be eliminated are within the midgut lumen and haemocoel of the mosquito (Clayton *et al.*, 2014; Habtewold *et al.*, 2017; Mizushima *et al.*, 2023). Therefore, the early stages of infection, particularly from ingestion to invasion into the midgut epithelium, act as obstacles for the parasite infection cycle within the mosquito (Mizushima *et al.*, 2023). Therefore, these sites are ideal targets for blocking transmission (Mizushima *et al.*, 2023). However, the current methods of vector control employ the use of insecticides.

1.4. Vector control strategies

The strategies implemented to combat malaria have primarily focused on vector control and the systematic management of the disease through effective diagnostic methods and treatment protocols (World Health Organisation, 2009). Either mechanical, biological, or chemical means can be used for vector control. Of these, chemical control is the most significant for malaria control (Rose, 2001; Bhatt *et al.*, 2015). Chemical means of vector control involve the use of insecticides, with synthetic insecticides playing a vital role in the control of disease vectors (Rose, 2001; Silva *et al.*, 2014). The WHO commenced the Global Malaria Eradication Programme (GMEP) in 1955 to intersect transmission through indoor residual spraying (IRS) with dichlorodiphenyltrichloroethane (DDT) and other residual insecticides (Nájera *et al.*, 2011). The use of insecticide-treated bed nets (ITNs) arose as another vector control method. The use of DDT was the first documented insecticide used against anophelines (Mellanby and Council, 1992). Since then, four classes of insecticides have been used for public health. Synthetic pyrethroids have also been employed as IRS and ITN and are the common agents for malaria control worldwide (Silva *et al.*, 2014). However, resistance to all classes of insecticides

used for public health has been reported. This has resulted in operational challenges in the use of insecticides (Hancock *et al.*, 2024).

Insecticide resistance refers to the acquired capability of individuals within a species to withstand lethal concentrations of a toxic agent (Subramanyam and Hagstrum, 1995). Consequently, the widespread and prolonged application of insecticides has led to the emergence of select populations that exhibit increased resistance to these chemical compounds (Dong, 2007). The *An. gambiae* complex can mediate resistance to pyrethroids due to a single base-pair mutation present in the voltage-gated sodium channel. This mutation is referred to as knockdown resistance (*Kdr*) (Martinez-Torres *et al.*, 1998). Two distinct forms of this mutation are identified within Africa. The West African variant arises from the substitution of phenylalanine for leucine (L1014F) (Martinez-Torres *et al.*, 1998). Conversely, the East African variant is characterised by the substitution of serine for leucine (L1014S) (Ranson *et al.*, 2000). Beyond conferring resistance to pyrethroid insecticides, mutations have also been associated with resistance to dieldrin (*Rdl*), observed in both laboratory populations of *An. gambiae* and in wild populations (Coetzee *et al.*, 2006). *Rdl*-based resistance is due to one of two target site mutations caused by a single point mutation in the M2 transmembrane domain of the gamma-aminobutyric acid (GABBA) receptor (ffrench-Constant *et al.*, 2000; Du *et al.*, 2005). The *Rdl* mutation prevents dieldrin from interacting within the active site of the chloride channel therefore disrupting the normal nerve potential of the neurons (Casida and Durkin, 2013). Research has additionally indicated that the *Rdl* mutation is due to a cross-resistance to modern insecticides utilised such as deltamethrin and neonicotinoids (Kolaczinski and Curtis, 2001).

The emergence of insecticide resistance necessitates the development of innovative vector control strategies. Recent advancements in vector control emphasise strategies that are independent of insecticides. Among these innovative approaches, transgenic methods including gene drives (James, 2005; Eckhoff *et al.*, 2017), *Wolbachi*a-based interventions (Zhang *et al.*, 2015a), and the sterile insect technique (SIT) (Munhenga *et al.*, 2011; Lees *et al.*, 2015), are currently under rigorous investigation. Additionally, paratransgenesis represents an innovative approach currently under investigation. This transmission-blocking strategy involves the introduction of either native or genetically engineered bacterial symbionts into mosquito populations (Wilke and Marrelli, 2015). The primary objective of this intervention is to elicit an anti-*Plasmodium* effect, thereby reducing the transmission of malaria. Through this

innovative method, researchers aim to manipulate the mosquito microbiota to enhance resistance to malaria pathogens. These methodologies aim to provide sustainable alternatives for controlling insect populations while mitigating the ecological impact associated with traditional chemical controls.

1.5. The biology of *Anopheles arabiensis*

Anopheles arabiensis holds significant importance within the South African context, as it is one of the two DVS identified in Southern Africa (Dandalo *et al.*, 2017). *Anopheles arabiensis* has extremely variable behaviour. This species has been described as a zoophilic (animal biting) and anthropophilic (human biting), exophagic/endophagic (outdoor/indoor feeding) and exophilic/endophilic (outdoor/indoor resting) species (White, 1972). This highly variable behaviour allows for adaptation to environmental conditions as well as geographical location (Gillies and Coetzee, 1987; Sharp and le Sueur, 1991; White, 1974). It has been observed that *An. arabiensis* adapted through behavioural avoidance (Ameneshewa and Service, 1996), which references the behaviour of the species to avoid resting on surfaces treated with insecticides (Enayati and Hemingway, 2010). It is important to note that this variation in the behaviour of *An. arabiensis* has been observed in Kwa-Zulu Natal, South Africa (Sharp and le Sueur, 1991). Therefore, *An. arabiensis* can adapt to control mechanisms that employ insecticides and use physical barriers such as nets (Coluzzi *et al.*, 1979). As an example, long-lasting insecticide nets (LLINs) are particularly ineffective against *An. arabiensis* due to their shift to outdoor feeding in the presence of such barriers (Kitau *et al.*, 2012). As such, the avoidance behaviours make it a challenging species to control.

Anopheles arabiensis is found predominantly in arid, savannah-like environments (Coetzee *et al.*, 2000; Sinka *et al.*, 2010; Hamza and Rayah, 2016). Their larval habitats are generally small, temporary, sunlit, clear, and shallow freshwater pools (Machault *et al.*, 2009; Sinka *et al.*, 2010; Kenea *et al.*, 2011). However, *An. arabiensis* is known to develop within a diverse range of locations, such as irrigated paddies (Sinka *et al.*, 2010). In rural as well as urban areas, members of the *An. gambiae* complex, specifically *An. gambiae* and *An. arabiensis* has adapted to breeding in heavy metal-polluted waters (Kabula *et al.*, 2011; Gunathilaka and Karunaraj, 2015). Breeding in polluted water increases the breeding range of the species as these species can now breed in sites that were previously thought to be inaccessible. Additionally, breeding in polluted waters serves as a selection pressure for insecticide resistance (Jeanrenaud *et al.*,

2020). Therefore, this adaptation is becoming an increasingly important consideration for vector control.

1.6. *Anopheles arabiensis* and pollution

Anthropogenic activities such as urbanisation, industrialisation, agriculture, and mining have resulted in global issues relating to environmental pollution (Ukaogo *et al.*, 2020; Nishat *et al.*, 2022). Environmental pollution affects both the developed and developing world, with the impact of pollution having long-term consequences on living organisms (Ukaogo *et al.*, 2020). Fertilisers, pesticides, chemicals, heavy metals, non-degradable products (i.e. plastics), nutrients, biological agents and toxic substances all contribute to water pollution (Nishat *et al.*, 2022). The contamination of water bodies exposes aquatic organisms to pollutants, impacting their health (Arambourou *et al.*, 2019). The organisms impacted include invertebrate species such as mosquitoes that breed and spend their immature life stages within these water bodies (Arambourou *et al.*, 2019).

Unplanned urbanisation has resulted in a change in landscape in many parts of Africa thus resulting in a change in the natural ecosystems. These changes contribute to modifications and adaptation of *Anopheles* mosquito breeding sites (Awolola *et al.*, 2007). Consequently, in urban Lagos, Nigeria, *An. gambiae s.s* adapted to breed within a wide range of polluted water (Awolola *et al.*, 2007). Additionally, in Kenya, it was observed that *An. arabiensis* breed in bodies of water contaminated with agricultural run-off such as nitrogenous pesticides and fertilisers (Mwangangi *et al.*, 2006). The increased use of fertilisers and agrochemicals may potentially influence the breeding patterns of *An. arabiensis,* therefore resulting in an adaptation for greater tolerance for breeding within polluted waters (Antonio-Nkondjio *et al.*, 2011).

Consequently, various studies have shown that when clean water breeding anopheline larvae are exposed to polluted waters, they result in increased tolerance to insecticides (Antonio-Nkondjio *et al.*, 2011; Kabula *et al.*, 2011; Jeanrenaud *et al.*, 2019). A study in Ghana illustrated that the resistance of *An. gambiae* to deltamethrin was influenced by breeding within water polluted with fluoride, ammonium, nitrites and nitrates (Kabula *et al.*, 2011). Additionally, in Burkina Faso, *An. arabiensis* adults emerging from sites polluted with organic and domestic waste displayed high levels of DDT resistance (Jones *et al.*, 2012). It was postulated that one

of the factors driving the selection for DDT resistance in *An. arabiensis* was the exposure to organic and anthropogenic pollutants (Jones *et al.*, 2012). Therefore, it was not surprising that in Cameroon, the use of insecticides in agricultural practices was linked to the prevalence of insecticide resistance in *Anopheles* mosquitoes in industrial areas, cotton cultivation and market gardening (Antonio-Nkondjio *et al.*, 2015). Therefore, understanding and mitigating the selection pressures such as pollution that drive insecticide resistance is vital, especially regarding vector control implementation. Furthermore, understanding the biological significance of these adaptations is also important as it would involve various molecular and genetic adaptations.

1.7. The challenge of plastic pollution

Since the advent of plastic manufacturing, there has been a rapid increase in the production and use of plastics worldwide (Geyer *et al.*, 2017). However, the extensive use and application of plastic are increasing concerns regarding the disposal, persistence, and accumulation of this material within the environment (Barnes *et al.*, 2009). Plastics are known to biodegrade poorly and accumulate in the environment, particularly within bodies of water. Additionally, plastic pollutants can fragment into smaller pieces that can accumulate in the environment, thereby increasing environmental concerns (Thompson *et al.*, 2004; Ryan *et al.*, 2009). Plastic particles less than 5 mm in diameter are defined as microplastics (MPs) (Wagner *et al.*, 2014; Bouwman *et al.*, 2018; Jiang, 2018). Microplastics can enter the environment either as manufactured MPs or as a result of the degradation of larger plastics (Ivar do Sul and Costa, 2014).

Previous studies on plastic pollution have identified polymers such as polyethylene (PE), polypropylene, acrylic, polyamide, polyester, polyoxymethylene, polyurethane (PU), polybutylene terephthalate, polyethylene terephthalate (PET), polyvinyl chloride (PVC), polystyrene (PS) as sources of MPs (Browne *et al.*, 2007). Plastic is generally considered durable, however, when exposed to oxidative conditions, UV radiation and physical stress, it begins to fragment (Andrady, 2011). Additionally, a major contributor to MPs found in aquatic systems is fibres released during fabric washing (Bouwman *et al.*, 2018). Within urban settings, it has been reported that fibres are the most common MPs (Zhou *et al.*, 2020, 2023). Microplastics are particularly concerning because the smaller the fragment size, the greater the potential for bioaccumulation (Wagner *et al.*, 2014).

Plastic fragments have been identified to be present within both freshwater and marine environments (Dris *et al.*, 2015; Eerkes-Medrano *et al.*, 2015). Research has indicated that the vast majority of plastic waste in the oceans comes from terrestrial environments (Wagner *et al.*, 2014; Jambeck *et al.*, 2015; Lebreton *et al.*, 2017). However, previous research on plastic pollution focused on marine environments. The first reports of plastic pollution focused on the open seas and coastal regions (Carpenter *et al.*, 1972; Thompson *et al.*, 2004). In South Africa, the first known reports on MP pollution have been on marine environments (Lamprecht, 2013; Naidoo *et al.*, 2015; Nel and Froneman, 2015). However, in recent years, greater interest in the impact of plastic pollution on terrestrial environments such as inland aquatic and soil environments has gained greater focus.

Freshwater environments that have been reported to contain MPs are groundwater, freshwater lakes, rivers, dams (Wong *et al.*, 2020), and both constructed and temporary wetlands (Reynolds and Ryan, 2018; Atugoda *et al.*, 2022). Microplastics are a common urban pollutant and thus are more frequently found within urban freshwater environments. Additionally, MPs have been identified in remote locations such as high-altitude streams (Free *et al.*, 2014). Microplastics were found within fish species inhabiting African Great Lakes such as Lake Victoria in Tanzania, therefore indicating the presence of MPs within African water systems (Biginagwa *et al.*, 2016). The polymers that were identified within the fish were polyethylene, polyurethane, polyester, and silicone rubber (Biginagwa *et al.*, 2016). In a scoping study on microplastics in freshwater environments in the North West and Gauteng provinces of South Africa, it was determined that MPs occurred at a range of 1.9 to 5.12 MPs per litre of water (Bouwman *et al.*, 2018).

The extent of plastic pollution in South Africa is evident in both urban and rural areas and along coastlines. The improper management of plastic waste, coupled with inadequate waste infrastructure, has resulted in significant quantities of plastic waste discarded into the environment. According to Plastic SA, a significant amount of waste, including recyclable plastic, still ends up in landfills and the environment (Plastic SA, 2022). One particular plastic pollutant of concern in South Africa is disposable nappies. The growing use of single-use disposable nappies has resulted in improper disposal and subsequent accumulation in South African environments (Schenck *et al.*, 2023, 2024). The issue of disposable nappy pollution is also present in other Southern African countries like Zimbabwe (Muringaniza *et al.*, 2023). The accumulation of nappies in the environment can degrade and contribute to the increasing

concerns of MP pollution. Evidence suggests that nappy waste tends to accumulate in proximity to rivers and in regions where livestock graze (Kordecki *et al.*, 2022). This occurrence raises significant concerns, as these locations are frequently associated with *An. gambiae* complex larval breeding sites. This association may worsen ecological and public health challenges, particularly the increased insecticide resistance and increased malaria transmission.

However, there is a complexity in understanding the impact of MPs due to the varying physical and chemical properties associated with plastic (Campanale *et al.*, 2020). The physical properties relate to the size, shape, and concentration of MPs (Campanale *et al.*, 2020). Two types of chemical properties are associated with MPs. The first is the chemicals that are absorbed from the environment. Plastics may act as sinks for contaminants that are already present in the environment, such as persistent organic pollutants (Wagner and Lambert, 2018) and heavy metal pollutants (Graca *et al.*, 2014; Holmes *et al.*, 2014). The second type of chemical property is the additives intentionally added during production to give plastic the desired qualities and prolong the plastic life span (Campanale *et al.*, 2020; Pereao *et al.*, 2020). These additives include reinforcing fillers, plasticisers, antioxidants, UV stabilisers, lubricants, dyes, and flame retardants (Hahladakis *et al.*, 2018). However, there is a lack of knowledge regarding the major additives used in plastic production. In particular, there is a lack of knowledge regarding the fate upon disposal into the environment and subsequent effects on living organisms (Campanale *et al.*, 2020). Additives such as phthalic acid esters (PAE) and bisphenol A (BPA) are added to plastics during production to obtain and improve desired properties (Pereao *et al.*, 2020). One of the environmental concerns regarding additives is that they may leach into the environment (Hahladakis *et al.*, 2018).

1.7.1. Phthalic acid esters

The most commonly used additive is phthalic acid esters (PAE) which is commonly known as phthalates. Phthalic acid esters are plasticizers which function to make the polymers softer, more flexible, and more durable (Braun *et al.*, 2013) and are widely used in polyvinyl chloride (PVC) production (Lü *et al.*, 2018). Additionally, they are greatly used in the manufacturing of various personal care products, cosmetics, paints, and adhesives (Saeidnia, 2014). Notably, PAEs are used in products other than plastic, such as fertilisers and insecticides (Schettler, 2006). The nature of PAE allows it to easily leach into the environment (Fromme *et al.*, 2002).

PAE are not covalently bonded to the polymer matrix of plastics, therefore making them extremely prone to leaching (Halden, 2010). Therefore, they can enter the environment either directly or indirectly during the production, use, and release of plastic products (Wang *et al.*, 2013).

PAE were found to have adverse effects on humans and ecosystems (Jobling *et al.*, 1995; Hauser and Calafar, 2005; Campanale *et al.*, 2020). The routes of exposure in humans occur through either inhalation, ingestion, or dermal contact from the use of products containing them as well as direct contact with a contaminated environment (Anderson et al., 2001; Schettler, 2006). PAE is noted to leach into the environment from plastic that has been dumped at landfills (Bauer and Herrmann, 1998) and has been found in the atmosphere, water, sediment, soil, plants, animals, and food (Net *et al.*, 2015; Abtahi *et al.*, 2019; Arfaeinia *et al.*, 2019; He *et al.*, 2020; Alkan *et al.*, 2021; Hajiouni *et al.*, 2022; Mohammadi *et al.*, 2022, 2023).

With the ease with which PAEs can enter the environment, it is unsurprising that they have health consequences for living organisms. The Environmental Protection Agencies of various countries have categorised PAEs as priority pollutants due to their teratogenic, mutagenic and carcinogenic impacts (Koch and Calafat, 2009; Meeker *et al.*, 2009; Oehlmann *et al.*, 2009; Zhang *et al.*, 2015b; Chang *et al.*, 2021; Urmi *et al.*, 2023). Studies have shown that exposure to PAEs results in adverse effects on living organisms such as humans (Arrigo *et al.*, 2023), rats (Sellinger *et al.*, 2020; Repouskou *et al.*, 2021; Tanoren *et al.*, 2021; Willson, 2021; Adam and Mhaouty-Kodja, 2022), loggerhead marine turtles (Sanjuan *et al.*, 2023), fish (Verma *et al.*, 2023; Khoshmanesh *et al.*, 2024) and aquatic invertebrates (Burgos-Aceves *et al.*, 2021; Prieto-Amador *et al.*, 2021; Gambardella *et al.*, 2024). Particularly, adverse effects on the reproductive system and immune responses have been observed (Agency for Toxic Substances and Disease Registry (ATSDR), 2022; Ullah *et al.*, 2023). Therefore, PAEs are classified as endocrine-disrupting organic compounds with negative effects on living organisms (Chen *et al.*, 2024b).

In South Africa, a study was conducted to assess the levels of PAEs in freshwater systems in Venda and determine the potential health effects (Fatoki *et al.*, 2010). The study determined that levels of PAEs in the rivers varied between 0.16 and 10.17 mg/L. In dams, it was determined that PAE levels ranged between 0.30 and 5.70 mg/L (Fatoki *et al.*, 2010). Therefore confirming the presence of PAEs in South African water systems.

1.7.2. Bisphenol-A (BPA)

Like PAE, BPA is a significant pollutant due to its multitude of applications, especially within the plastic industry (Li and Suh, 2019). BPA is a common plasticiser reported to be used in the manufacturing of food and beverage containers (Gálvez-Ontiveros *et al.*, 2021; Ugboka *et al.*, 2022) and primarily in the production of polycarbonate plastics and epoxy resins (Huang *et al.*, 2012). BPA is most commonly used as an additive in PVC (Halden, 2010). BPA-based plastics are strong and stable, allowing them to withstand high temperatures and high-impact collisions (Campanale *et al.*, 2020). However, the instability of the BPA compound within plastic products makes it vulnerable to leaching (Halden, 2010). Therefore, BPA can leach from products, resulting in contamination of food, drinking water, wastewater, air, and dust (Biles et al., 1997; Brotons et al., 1995; Crain et al., 2007; Markey et al., 2001).

Additionally, BPA is considered an endocrine-disrupting compound and has been shown to act as an oestrogen receptor agonist (Li *et al.*, 2013). Various studies have linked exposure to BPA to human chronic diseases such as cardiovascular disease (Lang, 2008), cancer (Ho *et al.*, 2006) and fertility problems (Sugiura-Ogasawara *et al.*, 2005). Exposure to BPA from early development results in neurobehavioral alterations in children (Braun *et al.*, 2011) as well as secondary sexual developmental changes and immune disorders (Erler and Novak, 2010).

There is a high prevalence of BPA within aquatic environments. BPA has been detected within wastewater runoff from industrial and urban sources at varying concentrations of 0.23 µg/L up to 149 µg/L (Höhne and Püttmann, 2008). However, it should be noted that the reported concentrations of these pollutants found within runoff and surface water are inconsistent and vary between different geographical regions (Corrales *et al.*, 2015). A study conducted in the Eastern Cape, South Africa, determined that the mean concentration of BPA in the water sampled from the rivers was found to be 0.415 µg/L (Farounbi and Ngqwala, 2020). The highest mean concentration was found in the Uitenhage-treated effluents with a concentration of 1.684 µg/L (Farounbi and Ngqwala, 2020). Additionally, a study conducted by Wanda *et al.* (2017) determined the levels of BPA present within water samples collected from Gauteng, Mpumalanga and North-West. In Mpumalanga, the mean BPA concentrations in water treatment plants ranged between 4 and 20 ng/L (Wanda *et al.*, 2017). However, the mean concentration of BPA from two rivers in Mpumalanga was determined to be between 9.3 and 13 ng/L (Wanda *et al.*, 2017). The highest mean BPA concentration was determined to

originate from a spring from the Sisukumile Secondary School and Lower Lochiel Community in Mpumalanga (Wanda *et al.*, 2017). It should be noted that the study concluded that BPA was found within 62% of the sampling sites and was considered the most widely distributed micropollutant within bodies of water (Wanda *et al.*, 2017). Therefore, this highlights the presence of BPA as a common pollutant within South African water systems in both urban and rural settings.

The presence of plastic additives in South African aquatic systems raises significant environmental concerns. However, there is a notable lack of data regarding the impact of these additives on aquatic organisms, particularly invertebrates. It is essential to examine the implications of these chemical additives on malaria vector populations and the subsequent effect on disease transmission. This gap in knowledge underscores the need for comprehensive research to elucidate the ecological implications of plastic contamination in these ecosystems.

1.8. Microplastics and invertebrates

Due to their small size, MPs can be easily ingested by a wide range of organisms including invertebrates. Various studies have demonstrated that aquatic organisms can ingest and transfer MPs. Ingestion of MPs has been shown to have adverse effects on feeding, growth, reproduction and overall survival (Cole *et al.*, 2013). Additionally, baseline toxicity of MPs has been investigated in earthworms (Rodríguez-Seijo *et al.*, 2018), *Daphnia magna* (Schrank *et al.*, 2019) and nematodes (Fueser *et al.*, 2020). In earthworms, the lowest concentration of MPs caused the most significant effect (Rodríguez-Seijo *et al.*, 2018). In *Daphnia magna*, it was determined that flexible PVC rather than rigid PVC caused changes to body length and reduced the number of offspring. Additionally, none of the MP treatments administered resulted in a significant change in mortality (Schrank *et al.*, 2019). In nematodes, it was observed that ingestion rates were influenced by exposure time, concentration and the size of the polystyrene beads (Fueser *et al.*, 2020). Additionally, polystyrene MP ingestion has been demonstrated in the water flea *Daphnia magna* (Al-Jaibachi *et al.*, 2018) and in zooplankton (Cole *et al.*, 2013). The uptake of these plastics depends on the size and concentration of these particles (Scherer *et al.*, 2017). Among aquatic invertebrates, mosquitoes present a unique case when studying the impacts of MPs. During the larval stages of mosquitoes, they inhabit a wide range of aquatic environments from clean freshwater bodies to polluted puddles, rice paddies

and urban drainage systems. This adaptability allows mosquitoes to encounter MPs in various contaminated water bodies.

Larval MP exposure has been examined in mosquitoes. The ingestion of MPs by larvae resulted in the accumulation within the adult gut of *Culex pipiens* (Al-Jaibachi *et al.*, 2019). A similar study was performed on *Aedes aegypti.* The larvae of this species readily feed on MPs, and this was transferred to the adult, notably to the salivary glands (Gopinath *et al.*, 2022). The emergence of MP pollution has prompted significant concerns regarding the potential for transmission of MPs from female mosquitoes to humans and animals during blood-feeding activities. Of particular significance is the ingestion of MPs by mosquito larvae, given their holometabolous life cycle, which encompasses a complete metamorphosis. This transformation from immature aquatic larvae to terrestrial adults may facilitate the translocation of MPs across various ecosystems (Jones *et al.*, 2024; Li *et al.*, 2024a). Mosquitoes are a crucial prey species for various organisms and may facilitate the transfer of microplastics from their larval stages to adults. This transference has the potential to result in the bioaccumulation of microplastics throughout food webs, leading to unpredictable consequences for other species within the ecosystem (Cui *et al.*, 2022b). More critically, MPs can influence the ecological development and survivability of mosquito populations, which may subsequently impact population dynamics and the transmission of diseases, considering that mosquitoes serve as vector species (Jones *et al.*, 2024).

Previous research investigating the effects of MPs on mosquito populations has largely concentrated on *Aedes* and *Culex*. However, there is a notable absence of data regarding the impact of MPs on the biology and development of malaria vectors. This research gap encompasses critical aspects of mosquito biology. Investigating the impact of plastic pollutants on malaria vectors, specifically *An. arabiensis* is vital for understanding the dynamics of the insecticide-resistant phenotype, life history traits, associated microbiota, and potential epigenetic modifications affecting inheritance. This research is essential for addressing existing research gaps and advancing our comprehension of these complex interactions.

1.9. Aim and Objectives

This study aimed to characterise the effects of larval exposure to plastic and its additives on both insecticide-selected and unselected laboratory strains of *Anopheles arabiensis*. The objectives were:

i. To characterise the effect of plastics on larval development, longevity, insecticide tolerance, and median lethal concentration (LC_{50}) of insecticide-selected and unselected strains of *An. arabiensis*

ii. To determine the effect of microplastic exposure on the midgut microbiota of insecticide-selected and unselected strains of *An. arabiensis*

iii. To trace the fate of microplastic particulates of three different sizes on fourth instar larvae and emergent adults of the insecticide-selected and unselected strains of *An. arabiensis*.

iv. To determine the transgenerational effect of plastic on the histone acetyltransferase (HAT) activity of the insecticide-selected and unselected strains of *An. arabiensis*

Chapter 2: Common methods and materials

2.1. Study site and mosquito strains

This study used two laboratory strains of *An. arabiensis*. The SENN strain was colonised from the Gezira region of Sudan (Hemingway, 1983) and is considered an insecticide-unselected strain. The SENN DDT strain was selected from SENN by continuous exposure to 4% DDT since 1995. SENN DDT has exhibited resistance to DDT, permethrin, deltamethrin, λ-cyhalothrin, and malathion (Oliver and Brooke, 2017). Resistance in SENN DDT is mediated by elevated metabolic detoxification enzyme activity (Oliver and Brooke, 2013). Additionally, resistance in SENN DDT is mediated by fixation of the L1014F mutation. This is a point mutation in the sodium channel gene that makes this neurological target insensitive to DDT and pyrethroids (Matambo *et al.*, 2007; Oliver and Brooke, 2013). Both the SENN and SENN DDT strains are housed in the Botha de Meillon insectary of the National Institute for Communicable Diseases (NICD) located in Sandringham, Johannesburg and reared according to standard procedures (Hunt *et al.*, 2005). The strains were reared at 25 ºC (± 2 ºC), and 75% humidity (± 5%). The insectary functioned on a 12:12 hour light: dark photoperiod with a 30-minute dawn/dusk cycle. Larvae were fed a combination of Beano™ dog biscuits and brewer's yeast at a ratio of 3:1. The adults were allowed *ad libitum* access to 10% sucrose.

2.2. Resistance profile of *Anopheles arabiensis* strains

The colonies were characterised for baseline insecticide resistance in June 2023 as per the WHO insecticide susceptibility test procedure (World Health Organization, 2016). In brief, three-day-old adult SENN and SENN DDT mosquitoes were exposed to different filter papers infused with known standard concentrations of various insecticides for one hour. The insecticides used were DDT, deltamethrin, permethrin, pirimiphos-methyl and malathion. Twenty-five mosquitoes per strain per insecticide were used. The mortality was recorded 24 hours after exposure. An environmental control (unexposed) and a solvent control, which functioned as a handling control, were included. This process was repeated five times.

2.3. Plastic treatments

Various plastic treatments were used in this study. To represent the complex plastics found in larval breeding sites, artificially degraded disposable nappies (Huggies® extra care disposable

nappies: Kimberly-Clark South Africa; Serial no.: 5029053548647) were utilised. These disposable nappies consisted of wood pulp, sodium polyacrylate, polypropylene, polyethene, adhesives, polyester, polyurethane elastics and polyolefin elastics. The nappies were degraded under insectary conditions by placing a single nappy in 2000 mL of reverse osmosis (RO) water and allowed to degrade for three months. The nappies were turned daily to stimulate mechanical disturbance and aid in the degradation. Bisphenol-A (BPA) (Sigma-Aldrich, Catalogue no.: 80-05-7) was used as a representative of a plastic breakdown product. A final concentration of 12.5 µM per 1000 mL of RO water was used. Phthalic acid dimethyl ester (Carl Roth GmbH+Co. KG, Catalogue no.: 131-11-3) was used as a representative of a common plasticiser that can leach into the environment. A final concentration of 59.5 µM per 1000 mL of RO water was used.

Three different sizes of commercially available fluorescently labelled latex beads were used. The three sizes consisted of a 0.5 µm fluorescently labelled sulphate-modified polystyrene bead (Sigma-Aldrich: Product no.: L1403), 1 µm fluorescently labelled amine-modified polystyrene beads (Sigma-Aldrich: Product no.: L2778) and a 2 µm fluorescently labelled latex amine-modified polystyrene beads (Sigma-Aldrich: Product no.: L0280). A final volume of 7 µL of the latex bead solutions per 1000 mL of RO water was used for each size of bead. Any variation in concentrations is detailed in the experiments.

Chapter 3: Characterisation of the effect of larval plastic exposure on the life history and insecticide tolerance of *Anopheles arabiensis*

3.1. Introduction

Malaria vectors, particularly *Anopheles arabiensis,* have adapted to breed within various environmental conditions and locations. Therefore, there is significant interest in understanding the effects of environmental factors on mosquito populations (Rochlin *et al.*, 2016; Barreaux *et al.*, 2018). Mosquitoes are holometabolous insects that spend their immature life stage within aquatic environments, whereas emergent adults are confined to aerial and terrestrial environments. However, despite the differing environments, the immature larval aquatic habitat influences the adult life history traits such as longevity, fertility and fecundity. Nutrient storage and acquisition at the larval stage have been shown to affect the resultant adults (Noden *et al.,* 2016; Vantaux *et al*., 2016). Therefore, the larval habitat is a crucial determinant of adult life history parameters. Additionally, the life history traits, including larval development time, adult longevity, fertility and fecundity, are key determinants of mosquito population dynamics and disease transmission. Therefore, the residual effects from the immature larval stage can influence vectorial capacity in addition to life history traits (Reiskind and Lounibos, 2009; Roux *et al.*, 2015). Notably, the greatest influence on vector capacity is the biting rate followed by adult longevity (Cohuet *et al.*, 2010; Barreaux *et al.*, 2016). Studies on adult longevity of vector species are vital as longevity influences disease transmission. The increased survival of female mosquitoes impacts the possibility of females acquiring the *Plasmodium* parasite and subsequently infecting humans (Garrett-Jones and Shidrawi, 1969). Additionally, older female mosquitoes enable the full incubation of the parasite. Therefore, the longevity of females is of epidemiological significance (Oliva *et al.*, 2011). Understanding the effects of environmental stressors such as pollutants on life history traits is an important area of research concerning vectorial capacity and vector control.

3.1.1. The effect of pollutants on life history traits of *Anopheles arabiensis*

Chapter 1 highlighted the potential of vector populations, particularly the adaptable *An. arabiensis* to breed within polluted waters. The changes to landscapes through urbanisation and agriculture have resulted in changes in larval breeding habitats (Awolola *et al.*, 2007).

Cosmopolitan species such as *Aedes* and *Culex* typically breed within environments characterised by polluted water bodies. However, there are increasing incidences of clean water breeding anopheline mosquito populations, particularly *An. gambiae* and *An. arabiensis*, breeding in polluted waters. This is crucial as wild populations that breed in polluted waters have been observed to garner a greater tolerance to insecticides (Antonio-Nkondjio *et al.*, 2011, 2015; Kabula *et al.*, 2011; Jones *et al.*, 2012). This is concerning, especially with regard to the conventional vector control strategies currently being implemented. Additionally, the larvae of the laboratory strains of *An. arabiensis,* SENN and SENN DDT, have previously been exposed to pollutants such as heavy metals (Oliver and Brooke, 2018b; Jeanrenaud *et al.*, 2020), pesticides (Oliver and Brooke, 2018a), fertilisers (Jeanrenaud *et al.*, 2019; Samuel *et al.*, 2020) and acid and detergents (Chen and Oliver, 2024). These studies indicated that pollutant exposure can influence the development and survival of larvae and the subsequent life history traits of the emergent adults of SENN and SENN DDT.

Metal larval exposure on laboratory strains of *An. arabiensis* did not have a noticeable effect on adult longevity and larval development. However, a significant increase in insecticide tolerance was observed after larval exposure to metal pollutants (Oliver and Brooke, 2018b). In a study examining the transgenerational effects of larval metal exposure on *An. arabiensis*, it was determined that the increase in insecticide tolerance seen in the first generation was carried over to the second generation (Jeanrenaud *et al.*, 2020). This finding was significant as the increased insecticide tolerance was carried over even when the second generation was reared in metal-free water (Jeanrenaud *et al.*, 2020). Larval exposure to inorganic fertilisers was determined to cause a faster developmental rate and increased insecticide tolerance in *An. arabiensis* (Samuel *et al.*, 2020). However, the adult longevity of the males was significantly reduced. Conversely, the study examining the effects of organic fertilisers determined that exposure resulted in shorter larval development time, increased adult longevity and increased insecticide tolerance in insecticide-selected (SENN DDT) and unselected (SENN) strains of *An. arabiensis* (Jeanrenaud *et al.*, 2019). Additionally, exposure to detergents and acids had differing effects on *An. arabiensis*. Detergents were seen to induce a longer larval development time, increased adult longevity and increased insecticide tolerance (Chen and Oliver, 2024). Conversely, larval acid exposure resulted in varying effects on larval development, with nitric acid resulting in a shorter larval development time in both strains. In contrast, hydrochloric acid resulted in a shorter developmental time only in the unselected SENN strain of *An.*

arabiensis (Chen and Oliver, 2024). Additionally, no significant difference in longevity was observed upon acid exposure in SENN and SENN DDT.

3.1.2. The effect of plastic pollution on mosquito life history traits

Despite the growing body of knowledge on the effects of pollutants on the life history traits of *An. arabiensis*, there is a clear lack of information on plastic pollution. This is concerning as plastic pollution is a major environmental concern impacting larval breeding sites. However, the majority of research on MP has focused on marine and aquatic model organisms such as the water flea *Daphnia magna* and zebrafish *Danio rerio,* with little research focusing on insect species, particularly mosquito species (Lu *et al.*, 2018; Elizalde-Velázquez *et al.*, 2020).

An invertebrate study examining larval MP ingestion in the midge *Chironomus riparius* determined that MP ingestion reduced the developmental and emergence rate (Silva *et al.*, 2019). However, in recent years, there have been a few studies that have examined the effects of MPs on mosquito species. However, these studies have only focused on the *Culex* and *Aede*s mosquito species. In a recent study examining the effect of MP ingestion on *Aedes,* it was determined that the size and concentration of the MP had very little effect on larval and pupal survival and emergence (McConnel *et al.*, 2024). Notably, the authors determined that a significant difference in adult longevity was observed in *Ae. aegypti* males after ingestion of 1.0 µm polystyrene beads (McConnel *et al.*, 2024). These results were similar to a study conducted by Thormeyer and Tseng (2023) that observed minimal effects on the growth and development of polystyrene MP ingestion in *Cx. tarsalis* and *Cx. pipiens.*

As was mentioned in Chapter 1, plastic is a complex pollutant as there is the cumulative effect of plastic fragments and the chemical additives that can leach into the environment. Currently, few studies on the effects of chemical additives on mosquito species have focused solely on BPA. The study that examined the effect of BPA on *Cx. quinquefasciatus* determined that exposure to BPA to above environmentally relevant concentrations resulted in reduced developmental time (Valsala and Asirvadam, 2022). However, in the study on *Ae. aegypti*, it was determined that BPA had no significant impact on the development time (Prud'homme *et al.*, 2017). Therefore, it is plausible that different mosquito populations will respond differently to plastic leachates.

However, despite the growing body of knowledge, there is a lack of studies on the effect of plastic pollution on the life history traits of malaria vector species. Currently, only one study on the effect of MPs on *An. gambiae s.s* has been published. The study determined that exposure to polyethylene terephthalate (PET) MPs affected survival rates, larval development time and insecticide tolerance (Shilla *et al.*, 2024). The authors concluded that prolonged exposure of *An .gambiae s.s* larvae to MPs could potentially increase their tolerance to MP pollution and potentially exacerbate insecticide resistance (Shilla *et al.*, 2024). However, to the best of our knowledge, no other studies have examined the effect of plastic pollution on *An. arabiensis*. The lack of studies on malaria vectors potentially relates to the lack of information on the concentration of plastic found within larval breeding sites. Additionally, studies have examined primarily the effects of polystyrene, with the exception of two studies on BPA. Given the multiplicity and nature of plastic polymers, there is a lack of knowledge on the effects of common plasticisers and other plastic compounds. Therefore, this study seeks to bridge these knowledge gaps. The aim of this chapter, therefore, was to determine the effect of plastic and plastic additives on the median lethal concentration, life history and insecticide tolerance of insecticide-selected and unselected strains of *An. arabiensis*.

3.2. Methods and Materials

3.2.1. Mosquito strains

This study used the insecticide-selected (SENN DDT) and unselected (SENN) laboratory strains of *An. arabiensis*. The profiles of the strains are detailed in sections 2.1 and 3.3.1.

3.2.2. The median lethal concentration (LC_{50}) of plastic additives

The toxicity of the two plastic additives BPA and phthalic acid was determined following the WHO's larviciding protocol (World Health Organization, 2005). Disposable cups were filled with 100 mL of reverse osmosis (RO) water, and between 20 and 25 fourth instar larvae were placed into the cups. Five cups per plastic treatment per strain with a total of three replicates for each strain and plastic treatment was set up. Increasing concentrations of the plastic additives were added to the cups. The increasing concentrations used for BPA were: 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM and 1.0 mM. The increasing concentrations used for phthalic acid were: 9.52 mM, 19.04 mM, 38.08 mM, 76.16 mM and 152.32 mM. The control consisted of 100 mL of RO water and no plastic additives. The mortality of the larvae was assessed 24 hours after establishing the experimental conditions. The median lethal concentration of 50% mortality (LC_{50}) was determined using a Probit analysis (Finney, 1952) performed using MedCalc for Windows, version 23.0.6 (MedCalc Software, Ostend, Belgium). The comparison of mean LC_{50} between the strains for each plastic treatment was compared using an unpaired t-test performed in GraphPad Prism version 10.0.0 for macOS (GraphPad Software, Boston, Massachusetts USA). A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to test for normality. A *p*-value of less than 0.05 was considered statistically significant.

3.2.3. Larval development and success when exposed to plastic treatments

First instar larvae were reared in either untreated water (control) or water treated with plastic and plastic additives (plastic-treated). Containers with the experimental treatments (22 cm x 16 cm x 8 cm) were filled with 1000 mL of RO water and approximately 100 larvae were added to the water. Four plastic treatments were used. The first treatment was artificially degraded disposable nappies that served as a proxy of complex common plastic pollutants found in the larval breeding site. The process of degrading the nappies is described previously in section 2.3. The degraded nappy solution was split and diluted with RO water to bring a total volume of 1000 mL to generate the nappy water solution. Initially, all subsequent experiments used the

LC_{50} calculated for the plastic additives as an initial starting concentration to expose the larvae. However, a lower concentration of the additives was used to ensure survivorship. These concentrations are detailed in section 2.3. In brief, a final concentration of 12.5 µM of BPA per 1000 mL of RO water was used. A final concentration of 59.5 µM of phthalic acid dimethyl ester (phthalic acid) per 1000 mL of RO water was used. Lastly, larvae were exposed to three different sizes (0.5, 1.0 and 2.0 µm) of fluorescently labelled latex beads (as detailed in section 2.3), which served as a proxy of MPs. A final volume of 7 µL of the latex bead solution per 1000 mL of RO water was used for each size of bead.

Three replicates for each treatment and strain were set up. Larvae were fed a standardised amount of food each day. Once the pupal stage was reached, the pupae were removed and placed in Petri dishes in rearing cages and allowed to emerge as adults. The time to pupation was measured as an indication of larval development (Samuel *et al.*, 2020). The data was visualised using boxplots generated using GraphPad Prism version 10.0.0 for macOS (GraphPad Software, Boston, Massachusetts USA). Larval development was analysed using a Kaplan-Meier analysis followed by a log-rank statistical analysis. Additionally, the percent pupation was determined as an indicator of developmental success. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to test for normality. The statistical analysis of developmental success between the treatments was compared using a One-way ANOVA (Gamage and Weerahandi, 1998). A Tukey multiple comparison (Tukey, 1949) *post-hoc* analysis was conducted. A *p*-value of less than 0.05 was considered statistically significant. Analysis and generation of graphs were performed in GraphPad Prism.

3.2.4. Adult longevity when exposed to plastic treatments

Longevity experiments were performed on all adults that emerged from larvae exposed to the plastic treatments mentioned in section 3.2.3. All adults that emerged from each plastic treatment replicate were placed in separate rearing cages. Three replicates per treatment per strain were set up. The larvae exposed to untreated water served as the controls. The mortality of the adults was recorded daily until 100% mortality. The adults were given *ad libitum* access to 10% sugar water. The sugar solution was changed every three days to avoid drying and fungal growth on the sugar pads. Additionally, females were not given a blood meal. A Kaplan-Meier and Log-rank statistical analysis was used to test for changes in survival. A *p*-value of less than 0.05 was considered statistically significant.

3.2.5. Insecticide tolerance when exposed to plastic treatments

In the investigation of the potential of plastic exposure as a selection pressure for insecticide tolerance, larvae were exposed to three plastic treatments: artificially degraded nappies, BPA and phthalic acid. Two hundred first-instar larvae were reared in plastic-treated water, as previously outlined in section 3.2.2. Insecticide tolerance was determined according to a CDC bottle bioassay, as described in Oliver and Brooke (2018b). The three-day-old emergent adults were collected and exposed to known concentrations of the insecticide deltamethrin as described. Deltamethrin is a widely used pyrethroid in IRS (Bhatt *et al.*, 2015). Consequently, if a change in tolerance to deltamethrin following larval exposure to plastic is observed, there could be significant operational implications. Furthermore, laboratory studies indicate that changes in tolerance to deltamethrin are the most responsive change observed, more so than malathion, and therefore, it was selected based on existing precedents.

Since the two *An. arabiensis* strains used have different insecticide resistance profiles, the adults were exposed to deltamethrin at different concentrations and time intervals. The unselected strain, SENN, was exposed to 1 µg/mL of deltamethrin at time intervals of 2, 4, 8, 16 and 32 minutes. The insecticide-selected strain, SENN DDT, was exposed to 10 µg/mL of deltamethrin at time intervals of 10, 20, 40 and 80 minutes. Emergent adults reared in untreated water served as the environmental control, whilst adults exposed to acetone were the handling controls. The median lethal time to 50% mortality (LT_{50}) was determined by a Probit analysis (Finney, 1952) performed using MedCalc for Windows, version 23.0.6 (MedCalc Software, Ostend, Belgium). The statistical analysis of LT_{50} between the treatments was compared using a One-way ANOVA (Gamage and Weerahandi, 1998). A Tukey multiple comparison (Tukey, 1949) *post-hoc* analysis was conducted. A *p*-value of less than 0.05 was considered statistically significant. Analysis was performed in GraphPad Prism version 10.0.0 for macOS (GraphPad Software, Boston, Massachusetts USA), and normality was tested using a Shapiro-Wilk test (Shapiro and Wilk, 1965).

3.3. Results

3.3.1. Resistance profiles of two laboratory strains of *Anopheles arabiensis*

From the insecticide susceptibility tests, it was determined that SENN demonstrated low resistance levels to DDT and permethrin, indicating that this strain was not fully susceptible. The SENN strain was, therefore, classified as an insecticide-unselected strain. The SENN DDT strain exhibited resistance to DDT, deltamethrin, permethrin, and malathion, classifying it as the insecticide-selected strain. The resistance profiles of SENN and SENN DDT are illustrated in Figure 3.1.

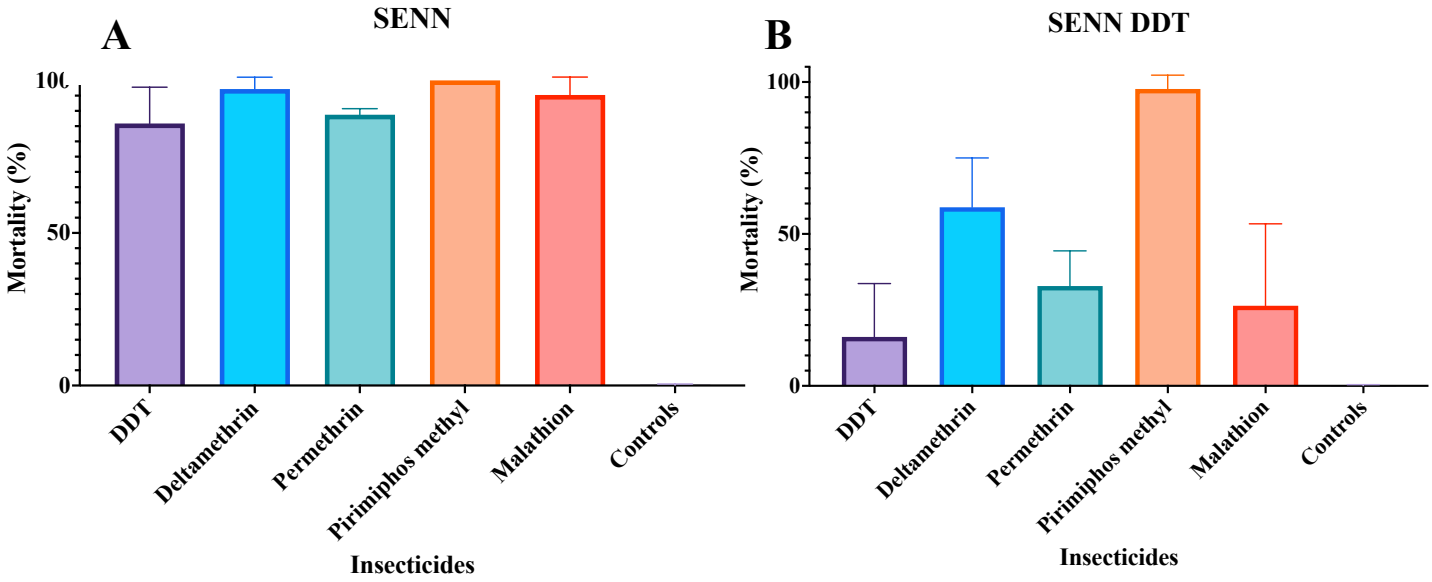


Figure 3.1. Insecticide resistance profile of two laboratory strains of *An. arabiensis*. The percentage mortality of the A) SENN and B) SENN DDT strains exposed to insecticides DDT, deltamethrin, permethrin, pirimiphos methyl and malathion as per the WHO insecticide susceptibility test procedure. The error bars represent the mean ± 1 standard deviation (SD).

3.3.2. The median lethal concentration (LC_{50}) of common plastic additives

There is currently no information regarding the concentrations of plastic chemical additives within larval breeding sites in South Africa. Therefore, the LC_{50} for the two common plastic additives was determined to provide a baseline for subsequent experimentation. The LC_{50} for SENN BPA was determined to be 0.62 mM, whereas SENN DDT BPA was 0.75 mM (Figure 3.2A). Additionally, a comparison of the LC_{50} between the two strains indicated no significant difference (Unpaired t-test: $t_{(4)}$ =0.50 p=0.64). Conversely, the LC_{50} for SENN phthalic acid was determined to be 10.13 mM, and SENN DDT phthalic acid was 11.35 mM (Figure 3.2B).

Similarly, no significant difference in LC_{50} between the strains for phthalic acid was observed (Unpaired t-test: $t_{(4)}$ =0.24 p=0.82). It should be noted that BPA was likely more toxic to SENN larvae than phthalic acid (Unpaired t-test: $t_{(4)}$ =3.23 p=0.03). This was also evident by the lower LC_{50} values calculated for BPA. However, no difference in toxicity was observed in SENN DDT (Unpaired t-test: $t_{(4)}$ =2.48 p=0.07).

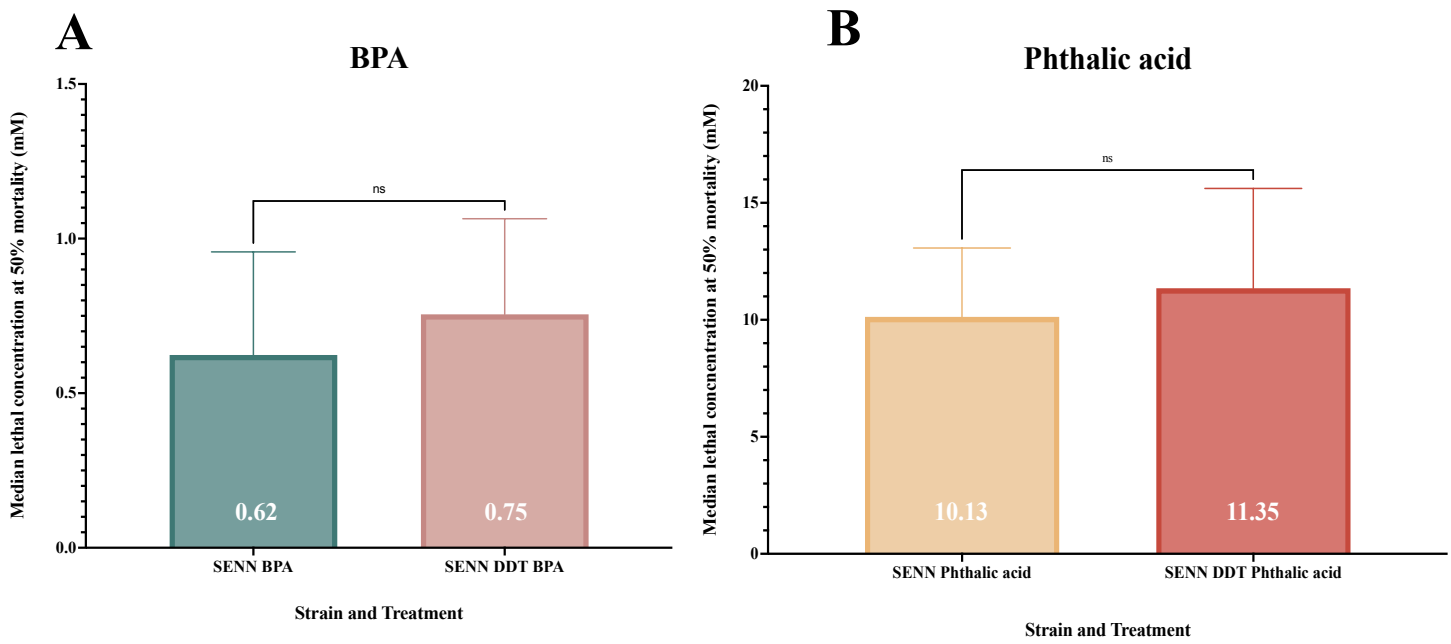


Figure 3.2. The median lethal concentration (LC_{50}) of common plastic additives in insecticide-selected (SENN DDT) and unselected (SENN) strains of *An. arabiensis.* A) LC_{50} determined after exposure to BPA in SENN (left) and SENN DDT (right). B) LC_{50} determined after exposure to phthalic acid in SENN (left) and SENN DDT (right). The error bars represent the mean ± 1 SD. No significant difference between the strains was observed for either treatment.

3.3.3. The effects on larval development and success when exposed to plastic treatments

Figure 3.3A illustrates that SENN and SENN DDT reared in nappy-treated water resulted in a significantly longer time to pupation (nappy-treated SENN: Log-rank test; $\chi^2_{(1,\ N=703)}$ =64.21 p<0.001; nappy-treated SENN DDT: Log-rank test; $\chi^2_{(1,\ N=824)}$ =191.6 p<0.001). This, therefore, indicates that the nappy-treated samples had a significantly slower larval development time in both strains.

A similar pattern of larval development was seen within the latex bead treatments (Figure 3.3D). When compared with the untreated samples all the bead-treated SENN samples had a significantly longer time to pupation with the exception of 2 µm beads-treated SENN (0.5 µm beads-treated SENN: Log-rank test; $\chi^2_{(1, N=865)}$ =152.1 p<0.001, 1.0 µm beads-treated SENN: Log-rank test; $\chi^2_{(1, N=784)}$ =102 p<0.001, 2.0 µm beads-treated SENN: Log-rank test; $\chi^2_{(1, N=869)}$ =0.09 p=0.77). Similarly, in SENN DDT all the beads-treated samples had a significantly longer time to pupation than the untreated samples (0.5 µm beads-treated SENN DDT: Log-rank test; $\chi^2_{(1, N=557)}$ =23.04 p<0.001, 1.0 µm beads-treated SENN DDT: Log-rank test; $\chi^2_{(1, N=474)}$ =15.44 p<0.001, 2.0 µm beads-treated SENN DDT: Log-rank test; $\chi^2_{(1, N=869)}$ =6.29 p=0.01). This, therefore, indicated that upon rearing within water treated with the latex beads, the larvae developed at a slower rate with the exception of SENN 2 µm.

Conversely, rearing within plastic additive-treated water resulted in varying responses between the two strains. The phthalic acid-treated SENN samples (Figure 3.3B) had a significantly faster time to pupation, thus indicating a faster larval development time (Log-rank test; $\chi^2_{(1, N=954)}$ =54.69 p<0.001). Similarly, the BPA-treated SENN larvae had a significantly shorter time to pupation and, therefore, a significantly faster larval development time (Log-rank test: $\chi^2_{(1, N=711)}$ =30.78 p<0.001) (Figure 3.3C). Additionally, it was determined that the phthalic acid-treated SENN DDT samples had a longer time to pupation, thus indicating a longer larval development time than the untreated counterpart (Log-rank test; $\chi^2_{(1, N=479)}$ =11.29 p<0.001). Similarly, BPA-treated SENN DDT samples had a significantly slower time to pupation and thus a longer larval development time (Log-rank test; $\chi^2_{(1, N=440)}$ =63.51 p<0.001). Therefore, this indicates that the plastic additive-treated SENN DDT larvae developed significantly slower when compared to their untreated counterparts.

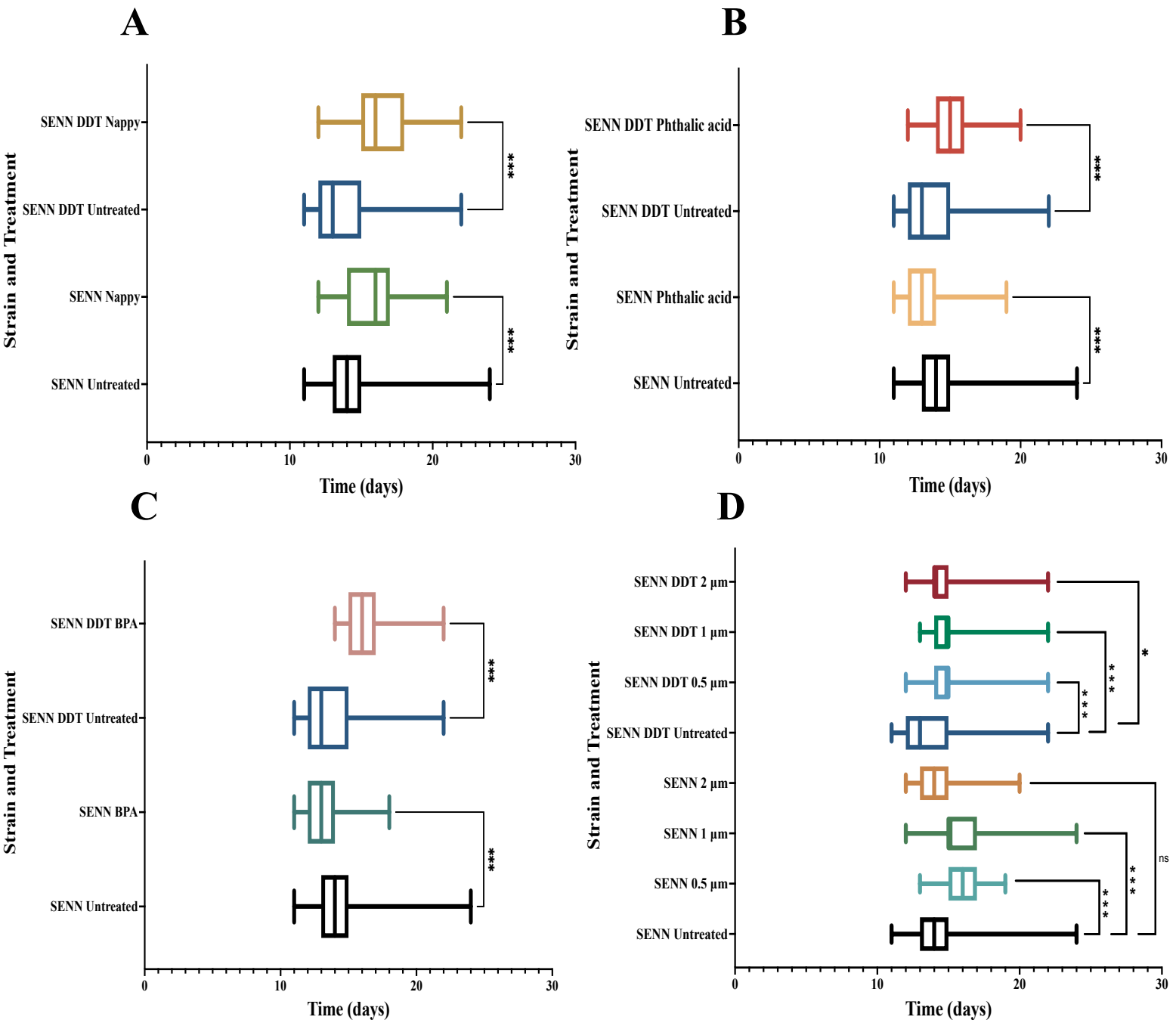


Figure 3.3. Larval development of SENN and SENN DDT strains of *An. arabiensis* when reared in plastic-treated water. A) Larval development of SENN and SENN DDT reared in nappy-treated water. B) Larval development of SENN and SENN DDT reared in phthalic acid-treated water. C) Larval development of SENN and SENN DDT reared in BPA-treated water. D) Larval development of SENN and SENN DDT reared in three different sizes of latex beads (0.5, 1.0 and 2.0 µm). The box plots represent the median (centre line), interquartile range (box), and the minimum/maximum values (whiskers). Significant differences are indicated by an asterisk (*p=0.01, *** p<0.001).

Additionally, to quantify the developmental success, the number of larvae that successfully reached pupation was counted, and the percent pupation was determined (Figure 3.4). The nappy-treated SENN (25.17%) had significantly fewer larvae that reached pupation than the untreated SENN (76.19%) (One-way ANOVA: $p<0.001$, $F_{(3,22)=}$ 8.74. Tukey *post-hoc* multiple comparison: untreated versus nappy-treated SENN, $p=0.002$). By contrast, the nappy-treated SENN DDT (78.13%) had no significant difference in the percentage of pupation compared to their untreated counterparts (46.33%) (Tukey *post-hoc* multiple comparison: untreated versus nappy-treated SENN DDT, $p=0.06$) (Figure 3.4A).

However, no significant difference was observed in percent pupation for both BPA-treated SENN (31.8%) and SENN DDT (25.5%) when compared to their untreated counterparts (One-way ANOVA: $p=0.02$, $F_{(3,21)=}$ 4.29. Tukey *post-hoc* multiple comparisons: untreated SENN compared to BPA-treated SENN, $p=0.06$; untreated SENN DDT compared to BPA-treated SENN DDT $p=0.54$) (Figure 3.4B). Similarly, no significant difference in percent pupation was observed in phthalic acid-treated SENN (49.73%) and SENN DDT (37%) when compared to their untreated counterparts (One-way ANOVA: $p=0.16$, $F_{(3,20)=}$ 1.93. Tukey *post-hoc* multiple comparisons: untreated SENN compared to phthalic acid-treated SENN, $p=0.40$; untreated SENN DDT compared to phthalic acid-treated SENN DDT $p=0.96$) (Figure 3.4C).

Lastly, no significant difference was seen between the percent pupation of all the beads-treated SENN and SENN DDT compared to their untreated counterparts (One-way ANOVA: $p=0.19$, $F_{(7,\ 24)=}$ 1.59. Tukey *post-hoc* multiple comparisons: untreated SENN compared to 0.5 µm beads-treated SENN, $p=0.98$; untreated SENN compared to 1.0 µm beads-treated SENN, $p=0.53$; untreated SENN compared to 2.0 µm beads-treated SENN, $p=0.98$; untreated SENN DDT compared to 0.5 µm beads-treated SENN DDT, $p=0.98$; untreated SENN DDT compared to 1.0 µm beads-treated SENN DDT, $p=0.98$; untreated SENN DDT compared to 2.0 µm beads-treated SENN DDT, $p=0.91$). The percent pupation of 0.5 µm beads-treated SENN was 61.78%, 1.0 µm beads-treated SENN was 46.22% and 2.0 µm beads-treated SENN was 61.89%. The percentage pupation of 0.5 µm beads-treated SENN DDT was 56.89%, 1.0 µm beads-treated SENN DDT was 35.22% and 2.0 µm beads-treated SENN DDT was 65.33%. (Figure 3.4D).

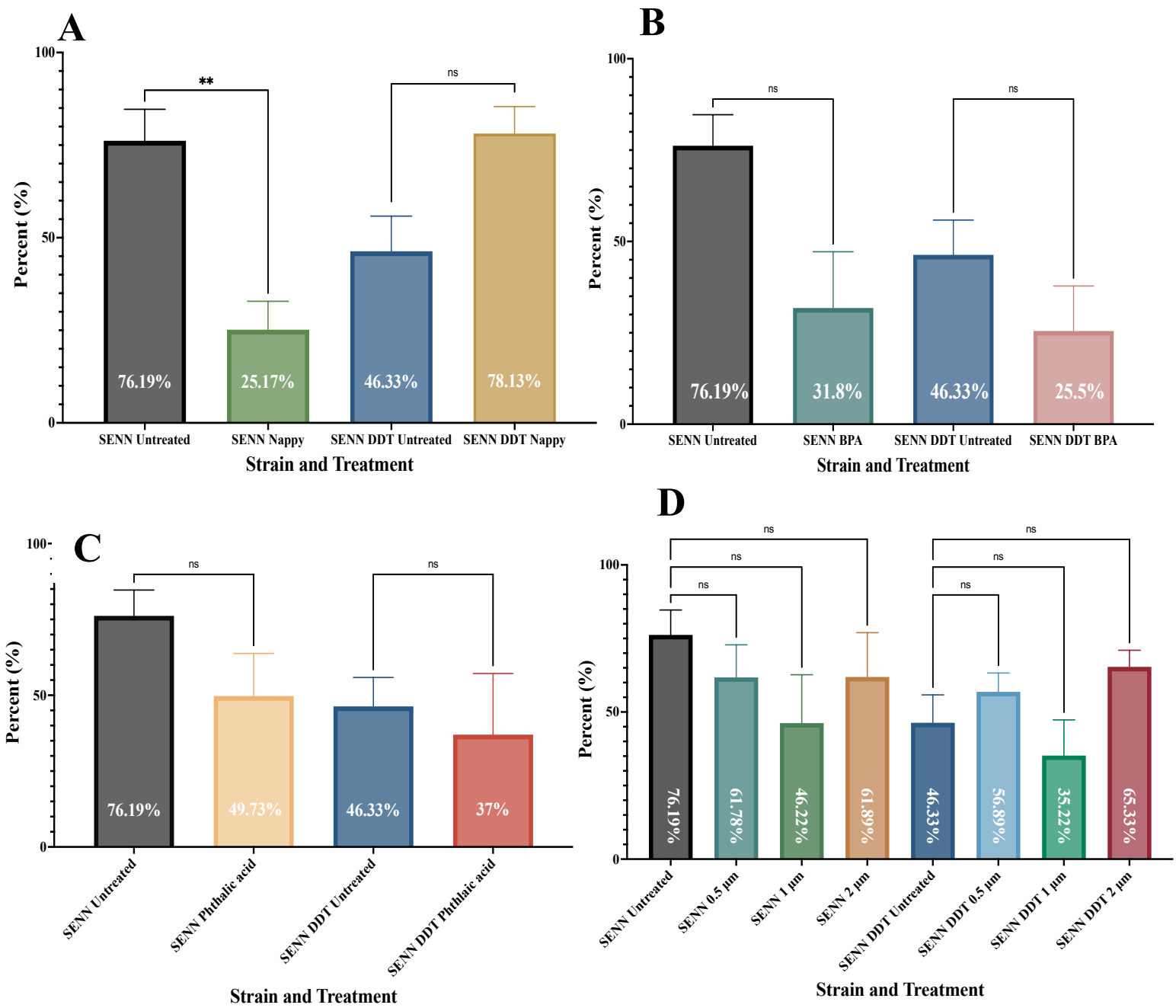


Figure 3.4. The developmental success of SENN and SENN DDT strains of *An. arabiensis* when reared in plastic-treated water. The number of larvae that successfully reached pupation was quantified, and developmental success as a percent pupation was determined. A) Percent pupation of SENN and SENN DDT larvae reared in nappy-treated water. B) Percent pupation of SENN and SENN DDT larvae reared in BPA-treated water. C). Percent pupation of SENN and SENN DDT larvae reared in phthalic acid-treated water. D) Percent pupation of SENN and SENN DDT larvae reared in three different sizes of latex beads (0.5, 1.0 and 2.0 µm). Each error bar represents the standard error of the mean (SEM). Significant differences are indicated by an asterisk (***p*=0.002).

3.3.4. The effect of larval exposure to plastic treatments on adult longevity

After larval exposure to nappy-treated water, it was determined that a significant difference was observed between the longevity of the untreated and nappy-treated SENN females (Log-rank test: $\chi^2_{(1, N=94)}=12.11$; $p<0.001$). The nappy-treated SENN females (43 days) survived significantly longer than the untreated controls (36 days) (Figure 3.5A). However, no significant difference in survival was observed between the longevity of untreated and nappy-treated SENN male samples (Log-rank test: $\chi^2_{(1, N=71)}=0.77$; $p=0.38$) (Figure 3.5B). Compared to SENN, in SENN DDT, the opposite was observed. No significant difference was observed between the survival of the nappy-treated SENN DDT females and their untreated counterparts (Log-rank test: $\chi^2_{(1, N=189)}=0.011$; $p=0.92$) (Figure 3.5C). However, a significant difference was observed between the longevity of untreated and nappy-treated SENN DDT males (Log-rank test: $\chi^2_{(1, N=201)}=5.240$; $p=0.02$). The nappy-treated SENN DDT male survived for a total of 46 days, therefore surviving significantly longer than the untreated males who survived for 38 days (Figure 3.5D).

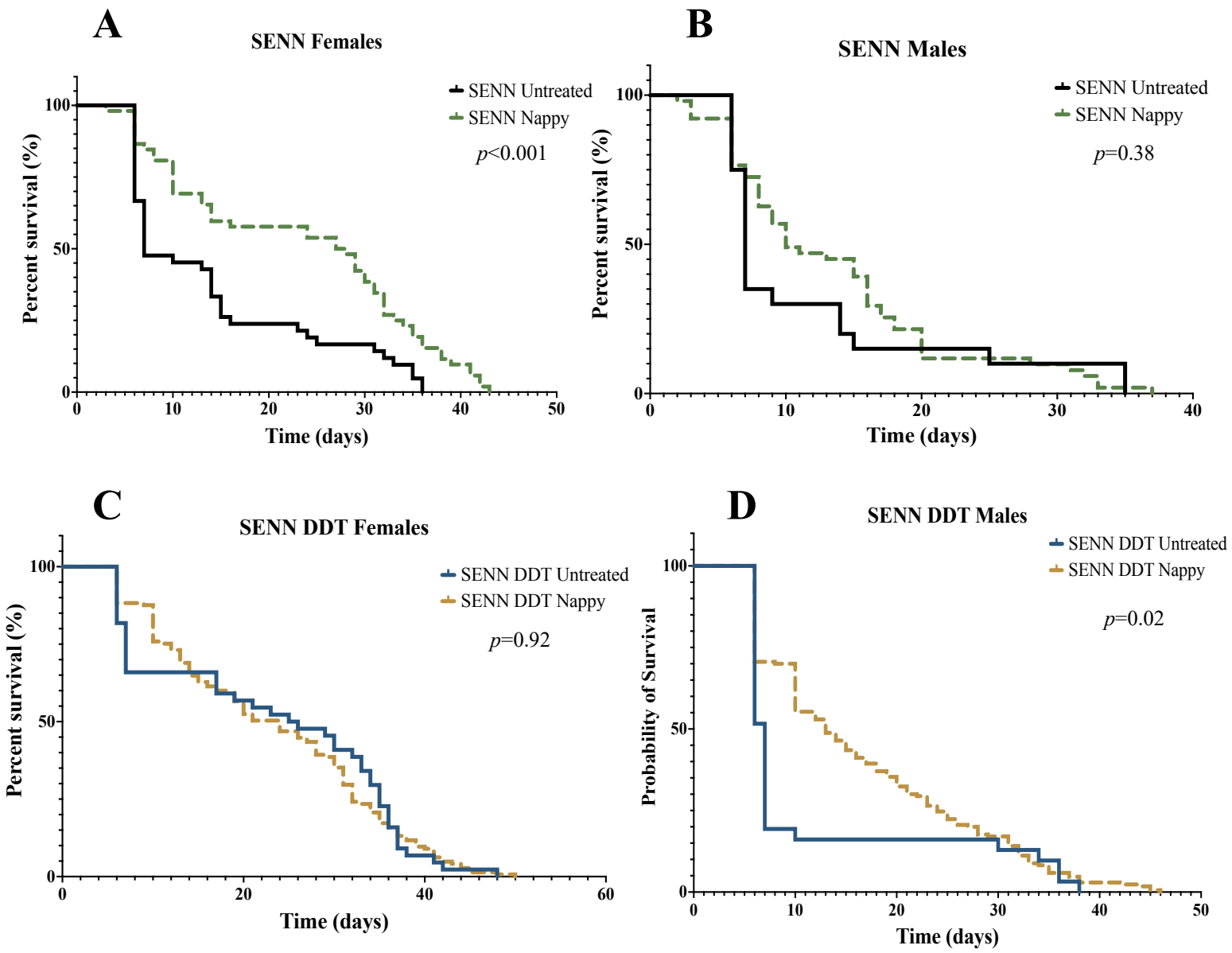


Figure 3.5. A Kaplan-Meier estimator of adult longevity of nappy-treated SENN and SENN DDT females and males. A) Adult survival of nappy-treated SENN females. B) Adult survival of nappy-treated SENN males. C) Adult survival of nappy-treated SENN DDT females. D) Adult survival of nappy-treated SENN DDT males. A Log-rank test was used to compare the adult survival between the plastic treatments, and *p*-values <0.05 were considered significant.

The phthalic acid-treated SENN and SENN DDT females were not significantly different compared to their untreated counterparts (SENN: Log-rank test: $\chi^2_{(1,\ N=290)}$ =0.33; p=0.56. SENN DDT: Log-rank test; $\chi^2_{(1,\ N=124)}$ =0.53; p=0.47) (Figures 3.56A and C). Therefore, no difference in survival of the females after larval exposure to phthalic acid was observed. It was observed that the phthalic acid-treated SENN males survived for a total of 29 days, whereas the untreated males survived for 33 days. Therefore, the phthalic acid-treated males lived for a significantly shorter period than the untreated SENN males (Log-rank test: $\chi^2_{(1,\ N=303)}$ =23.78; p<0.001). (Figure 3.6B). Additionally, no significant difference in the survival of the phthalic acid-treated SENN DDT males was observed (Log-rank test: $\chi^2_{(1,\ N=129)}$ =0.29; p=0.59) (Figure 3.6D).

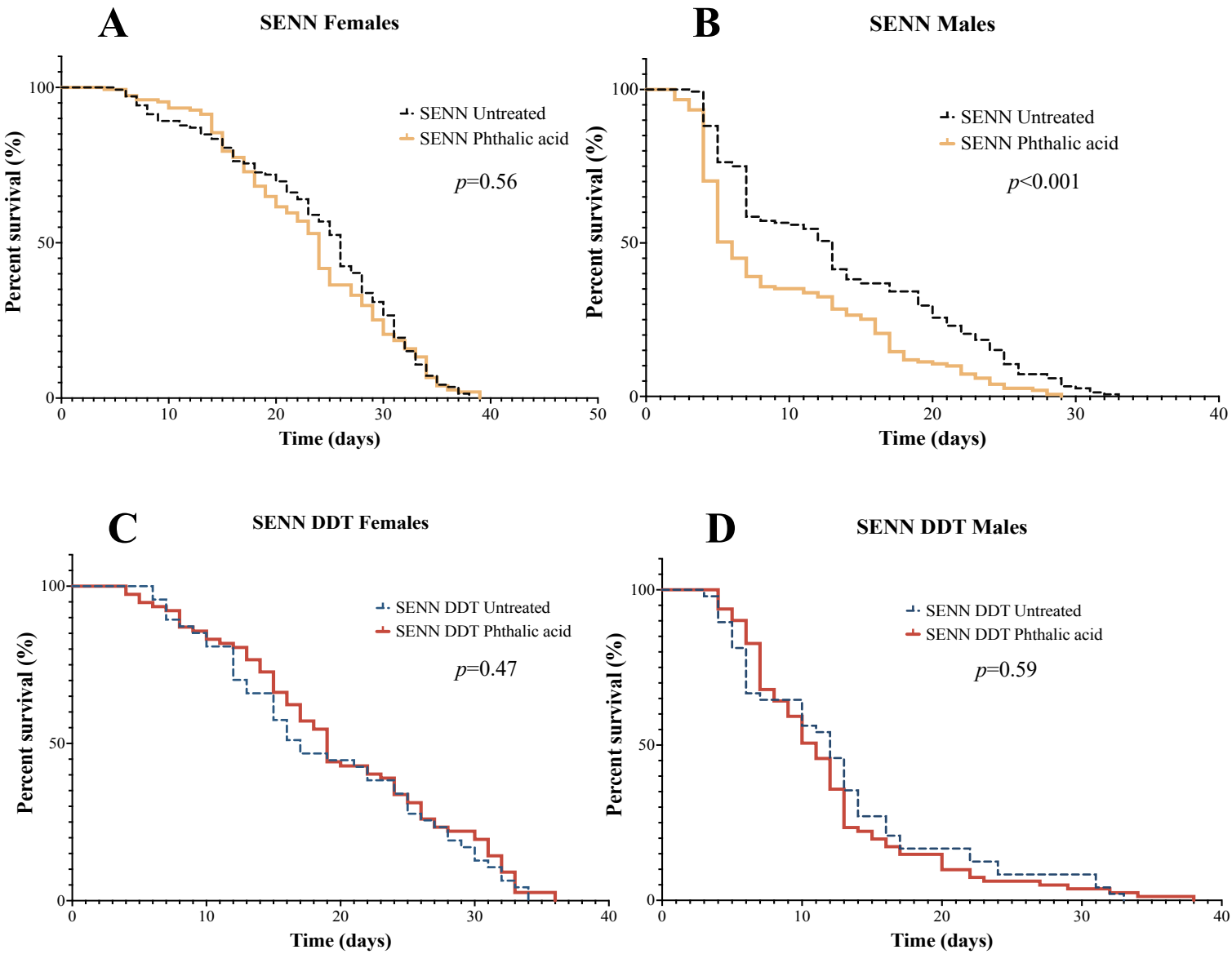


Figure 3.6. A Kaplan-Meier estimator of adult longevity of phthalic acid-treated SENN and SENN DDT. A) Adult survival of phthalic acid-treated SENN females. B) Adult survival of phthalic acid-treated SENN males. C) Adult survival of phthalic acid-treated SENN DDT females. D) Adult survival of phthalic acid-treated SENN DDT males. A Log-rank test was used to compare the adult survival between the plastic treatments, and *p*-values <0.05 were considered significant.

The adult longevity of the BPA-treated SENN significantly differed from the untreated females and males (Figure 3.7). The BPA-treated SENN females survived significantly longer than the untreated SENN females (Log-rank test: $\chi^2_{(1, N=232)}=4.55$; $p=0.03$) (Figure 3.7A). Additionally, a comparison of the survival curves indicated the BPA-treated SENN males survived for a significantly shorter period than the untreated SENN males (Log-rank test: $\chi^2_{(1, N=217)}=8.98$; $p<0.001$) (Figure 3.7B). However, no significant difference in survival was observed between the BPA-treated and untreated SENN DDT for both males and females (SENN DDT females: Log-rank test: $\chi^2_{(1, N=118)}=0.31$; $p=0.58$; SENN DDT males: Log-rank test: $\chi^2_{(1, N=102)}=0.09$; $p=0.76$) (Figures 3.7C and D)

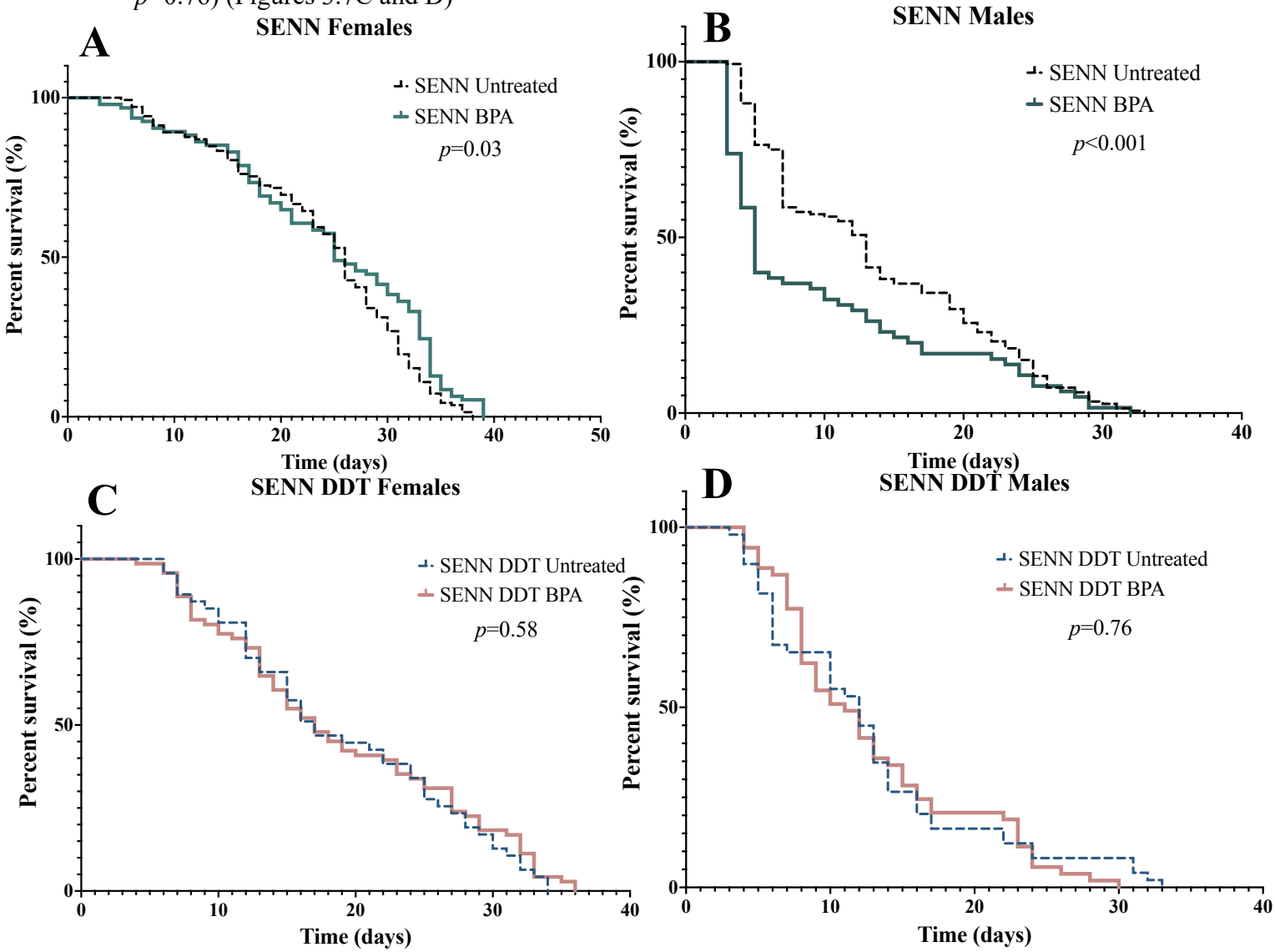


Figure 3.7. A Kaplan-Meier estimator of adult longevity of BPA-treated SENN and SENN DDT. A) Adult survival of BPA-treated SENN females. B) Adult survival of BPA-treated SENN males. C) Adult survival of BPA-treated SENN DDT females. D) Adult survival of BPA-treated SENN DDT males. A Log-rank test was used to compare the adult survival between the plastic treatments, and p-values <0.05 were considered significant.

Analysing the survival of adult SENN and SENN DDT after exposure to latex beads indicates the potential impacts of MPs on adult longevity. In the SENN females, larval exposure to latex beads had a significant effect on adult longevity, with the 0.5 µm beads-treated females surviving significantly shorter time than their untreated counterparts (Log-rank test: $\chi^2_{(1, N=230)}$ =31.87; p<0.001) (Figure 3.8A). Additionally, no significant difference in survival was observed in the 1.0 and 2.0 µm beads-treated SENN females (1.0 µm beads-treated SENN females: Log-rank test: $\chi^2_{(1, N=220)}$ =0.98; p=0.32. 2.0 µm beads-treated SENN females: Log-rank test: $\chi^2_{(1, N=235)}$ =1.38; p=0.24). Similarly, in SENN males, the 0.5 and 1.0 µm beads-treated males survived for a significantly shorter period however, no significant difference was seen in the 2.0 µm beads-treated SENN males (0.5 µm beads-treated SENN males: Log-rank test: $\chi^2_{(1, N=259)}$ =27.64; p<0.001. 1.0 µm beads-treated SENN males: Log-rank test: $\chi^2_{(1, N=216)}$ =27.05; p<0.001. 2.0 µm beads-treated SENN males: Log-rank test: $\chi^2_{(1, N=256)}$ =0.59; p=0.44). (Figure 3.8B).

By contrast, there was no significant difference in survival between the latex beads-treated and untreated SENN DDT females (0.5 µm beads-treated SENN DDT females: Log-rank test: $\chi^2_{(1, N=207)}$ =9.240 × 10^{-6}; p=0.99. 1.0 µm beads-treated SENN DDT females: Log-rank test: $\chi^2_{(1, N=169)}$ =2.08; p=0.15. 2.0 µm beads-treated SENN DDT females: Log-rank test: $\chi^2_{(1, N=198)}$ =0.40; p=0.53). (Figure 3.8C). However, there was a significant difference in the survival of the 0.5 µm beads-treated and untreated SENN DDT males with the 0.5 µm beads-treated SENN DDT males surviving significantly longer (Log-rank test: $\chi^2_{(1, N=195)}$ =11.52; p<0.001). (Figure 3.8D). Additionally, no significant difference was observed between the 1.0 and 2.0 µm beads-treated SENN DDT males with the untreated counterparts (1.0 µm beads-treated SENN DDT males: Log-rank test: $\chi^2_{(1, N=176)}$ =2.13; p=0.14. 2.0 µm beads-treated SENN DDT males: Log-rank test: $\chi^2_{(1, N=220)}$ =2.16; p=0.14).

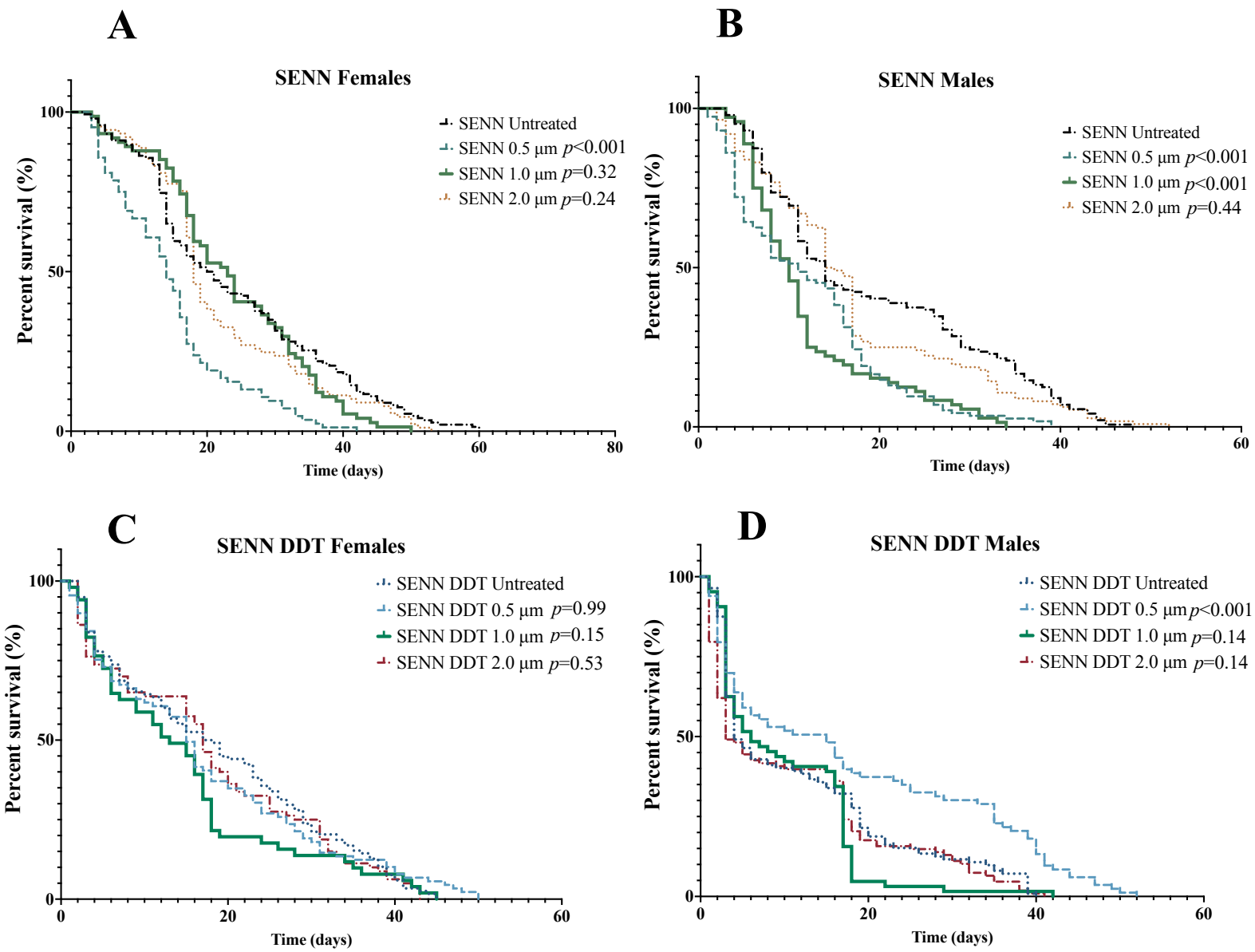


Figure 3.8. A Kaplan-Meier estimator of adult longevity of three different sizes of latex bead-treated SENN and SENN DDT. A) Adult survival of latex beads-treated SENN females. B) Adult survival of latex beads-treated SENN males. C) Adult survival of latex beads-treated SENN DDT females. D) Adult survival of latex beads-treated SENN DDT males. A Log-rank test was used to compare the adult survival between the plastic treatments, and *p*-values <0.05 were considered significant.

3.3.5. The effect of larval exposure to plastic on the insecticide tolerance of emergent adult *Anopheles arabiensis*

The effect of larval plastic exposure on deltamethrin tolerance is illustrated in Figure 3.8. The median lethal time to 50% mortality (LT_{50}) was calculated and assessed. All the plastic treatments resulted in a significant increase in insecticide tolerance of SENN when compared to the untreated SENN control (figure 3.8A) (t-test: untreated vs BPA-treated $t_{(17)}= 3.88$ $p<0.001$, untreated vs nappy-treated $t_{(14)}=2.24$ $p=0.04$, untreated vs phthalic acid-treated $t_{(18)}=3.77$ $p<0.001$).

Conversely, it was determined that a significant increase in insecticide tolerance was present in all the plastic treatments except the nappy-treated SENN DDT (Figure 3.8B) (t-test: untreated vs BPA-treated $t_{(14)}= 5.89$ $p<0.001$, untreated vs nappy-treated $t_{(15)}= 1.86$ $p=0.08$, untreated vs phthalic acid-treated $t_{(17)}= 4.69$ $p<0.001$). Notably, within both SENN and SENN DDT, the plastic additives (BPA and phthalic acid) resulted in the most significant increases in LT_{50}.

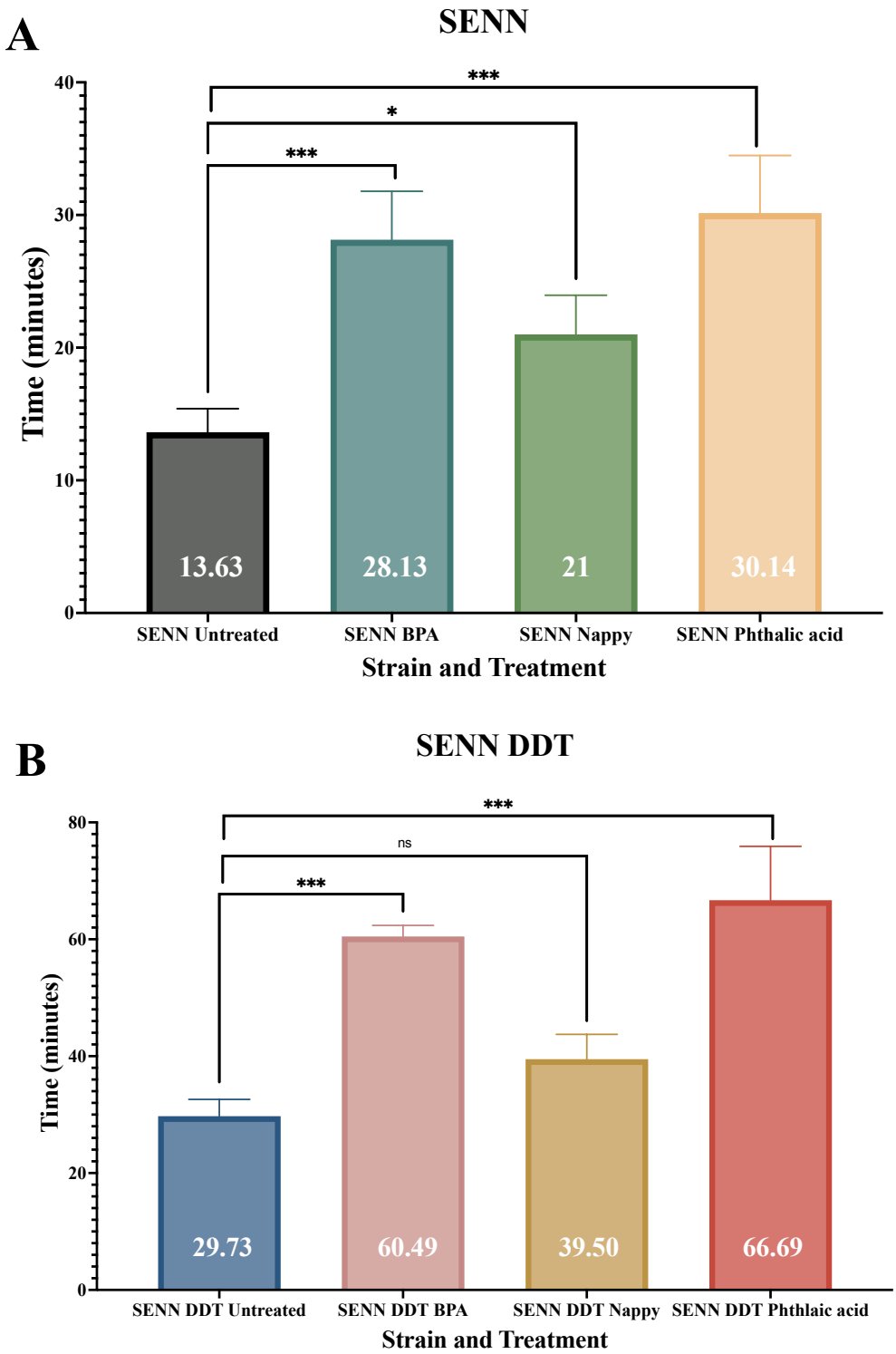


Figure 3.9. The lethal time to 50% mortality (LT_{50}) for adult SENN and SENN DDT after larval plastic treatments. Adults exposed to known concentrations of deltamethrin at different time intervals. A) LT_{50} for adult SENN. B) LT_{50} for adult SENN DDT. A significant increase in LT_{50} was present after all plastic treatments except the SENN DDT nappy treatment. The error bars represent the SEM. Significant differences are indicated by asterisks (*p=0.04, and *** p<0.001).

3.3.6. Summary of life history and insecticide tolerance results

Table 1: Summary of life history and insecticide tolerance results of larval exposure to plastic treatments in the insecticide-selected (SENN DDT) and unselected (SENN) strains of *An. arabiensis*.

	Larval developmental time	Pupal success	Adult longevity		LT_{50}
			♀	♂	
SENN Nappy	↑	↑	↑	0	↑
SENN DDT Nappy	↑	0	0	↑	0
SENN BPA	↓	0	↑	↓	↑
SENN DDT BPA	↑	0	0	0	↑
SENN Phthalic acid	↓	0	0	↓	↑
SENN DDT Phthalic acid	↑	0	0	0	↑
SENN beads 0.5 µm	↑	0	↓	↓	-
SENN beads 1.0 µm	↑	0	0	↓	-
SENN beads 2.0 µm	0	0	0	0	-
SENN DDT beads 0.5 µm	↑	0	0	↑	-
SENN DDT beads 1.0 µm	↑	0	0	0	-
SENN DDT beads 2.0 µm	↑	0	0	0	-

"↑" indicates that a significant increase was observed

"↓" indicates that a significant decrease was observed

"0" indicates that no significant difference was observed

"-" indicates no data was collected

3.4. Discussion

With growing global concern over plastic pollution, it is increasingly important to understand how these pollutants affect mosquito biology. This is particularly relevant since the larval environment of mosquitoes plays a crucial role in the adult life history traits of these insects. Despite various studies on the effects of pollutants on *An. arabiensis*, there is currently, to the best of our knowledge, no data on the impact of plastic on this species. Furthermore, existing research on the effects of microplastics on mosquito development and survival has primarily focused on container-inhabiting species such as *Culex and Aedes* (Al-Jaibachi *et al.*, 2019; Griffin *et al.*, 2023; Thormeyer and Tseng, 2023; McConnel *et al.*, 2024). Additionally, these studies have specifically focused on polystyrene MPs, with only one study examining the effects of polyethylene (Griffin *et al.*, 2023). Currently, the sole published research investigating the effects of MPs on malaria vectors focused on the effect of PET fragments in *An. gambiae s.s* (Shilla *et al.*, 2024). Therefore, there is a paucity of information on plastic additives and the life history of the *An. gambiae* complex. This research, therefore, established a critical foundational understanding of how plastic affects the biology of *An. arabiensis*.

Due to the lack of information regarding the concentration of plastics present within larval breeding sites, the first objective, therefore, was to determine the median lethal concentration (LC_{50}) of common chemical plastic additives. Understanding the LC_{50} is crucial, as there is currently, to the best of our knowledge, no data on the concentrations of these chemicals in larval breeding sites in South Africa, nor is there baseline data on the toxicity of these pollutants to mosquito species. From the results obtained, it was determined that BPA and phthalic acid exhibited no strain-specific differences in toxicity. Therefore, BPA and phthalic acid were as toxic to SENN as to SENN DDT. This finding aligns with what was observed in a study on the effects of acid and detergents on *An. arabiensis* (Chen and Oliver, 2024). Notably, the phenol group in BPA can release protons under specific conditions, thus giving BPA some weak acidic properties (Corrales *et al.*, 2015). Therefore, the acidic properties of BPA and phthalic acid correlate to no significant difference in toxicity observed between the strains for acids (Chen and Oliver, 2024).

However, these findings also contrast with what was previously seen in heavy metal and herbicide larval exposure (Oliver and Brooke, 2018a, 2018b). The studies on larval exposure to pollutants determined that the insecticide-selected strain SENN DDT was more tolerant of

heavy metals and herbicides than the insecticide-unselected strain (Oliver and Brooke, 2018a, 2018b). It was theorised by Chen and Oliver (2024) that the detoxification enzymes in SENN DDT may not play as significant of a role in the tolerance to acids and detergents. In the mulberry pest *Glyphodes pyloalis* Walker, it was determined that exposure to the heavy metal cadmium induced the increased expression of metabolic detoxification enzymes glutathione S-transferases (GSTs) and cytochrome-P450s (Zhao *et al.*, 2024). Similarly, in *An. arabiensis*, metal exposure increased the cytochrome-P450 enzyme activity in both SENN and SENN DDT (Jeanrenraud *et al.*, 2023). This is likely due to detoxification enzymes being unable to use acids as substrates, but metals are suitable, particularly for cytochrome P450s. As such, metals can be metabolised by detoxification enzymes associated with insecticide resistance, while acids cannot. This variability explains the differences in tolerance observed between SENN and SENN DDT regarding heavy metals but not in relation to acids. This could be true for what was observed in the plastic additives as well. However, further research would be needed to confirm this.

An analysis of the life history traits revealed that, overall, larval exposure to plastic was associated with more pronounced differences in SENN. This was not unexpected, as previous studies have indicated that greater responsiveness to larval stressors was observed in SENN rather than SENN DDT. This was observed in SENN's greater responsiveness to epigenetic modulators (Jeanrenaud *et al.*, 2022), acids (Chen and Oliver, 2024) and notably, to metal exposure in the first generation followed by a second-generation reared in untreated water (Jeanrenaud *et al.*, 2020). It is likely that this differential response is related to the insecticide-resistant phenotype. These differences highlight the importance of analysing insecticide-resistant and susceptible mosquitoes when examining responses to stressors.

An important life history trait is the larval developmental rate. A faster developmental rate suggests that the mosquito will spend less time in the vulnerable larval stage. Faster larval development is linked to a greater chance of evading predators by flying away once emerging as adults (Galante *et al.*, 2014). Therefore, it is seen as an advantage to the mosquito populations. However, reduced larval development time could result in a lack of teneral reserves, which could have negative effects on the adult (Barreaux *et al.*, 2018). An increase in adult longevity has been linked to longer larval development time in *An. funestus*. This species generally has a significantly longer larval development time and subsequently has a longer adult lifespan (Oliver *et al.*, 2022). It has been hypothesised that an extended duration

in the larval stage allows mosquitoes to accumulate greater teneral reserves, which translates into increased longevity. Larval exposure of SENN to nappy and bead treatments significantly prolonged developmental time. The longer larval development translated to longer adult longevity in the nappy-treated SENN females. Although there would be a risk of increased predation in the wild, the increased longevity in the adults translates to a significant advantage for this strain.

An additional explanation for the longer larval development time is that in the nappy and latex bead treatments, the larvae consumed the plastic particles, which could lead to a sensation of satiation and thus change the nutritional status of the larvae. This was observed in the lugworm *Arenicola marina* where the ingestion of polystyrene MPs resulted in a reduction in feeding activity and weight loss (Besseling *et al.*, 2013). Additionally, in *An. arabiensis,* it has been shown that nutrient deprivation in larvae resulted in longer development time (Oliver and Brooke, 2013). By contrast, in studies on other mosquito species, it was determined that ingestion of MPs had minimal effects on developmental time in *Aedes* and *Culex* (Thormeyer and Tseng, 2023; McConnel *et al.*, 2024). Therefore, the ingestion of plastic particles or fragments elicits varying responses across different mosquito species, with *An. arabiensis* without a pre-existing insecticide-resistant phenotype exhibiting an extended developmental period as a result.

By contrast, SENN DDT is generally unresponsive to larval effects on adult longevity. This has been observed previously. When compared to SENN, SENN DDT has been observed to survive better within polluted larval conditions such as heavy metals (Oliver and Brooke, 2018b; Jeanrenaud *et al.*, 2020) and herbicides (Oliver and Brooke, 2018a). Adult longevity may be less impacted because of the fitness cost of resistance. The fitness cost of insecticide resistance refers to the trade-off that arises when the physiological or behavioural adaptations conferring resistance reduce the mosquitoes' overall fitness in the absence of insecticides (Suh *et al.*, 2023). These fitness costs can manifest and impact various aspects of the mosquitoes' biology, such as developmental delay and decreased longevity. A study conducted in Cameroon found that pyrethroid resistance in *An. coluzzi* is associated with significant fitness costs, including reduced survival and reproductive success. (Nkahe *et al.*, 2020). Additionally, research from Kenya indicated that insecticide resistance in *An. gambiae* exerts significant fitness costs in immature stages, leading to decreased survival rates and prolonged development (Osoro *et al.*, 2021). Reduced longevity is one of the most common fitness costs

associated with insecticide resistance (Kliot and Ghanim, 2012). SENN-DDT has been demonstrated to generally have a shorter lifespan than SENN (Patel and Oliver, 2024). As such, with a generalised shorter longevity, effects on adult longevity will likely be less easily detected than in a strain that lives longer.

It is noteworthy that exposure to BPA and phthalic acid treatments resulted in significantly faster development in SENN while resulting in a significantly longer developmental time in SENN DDT. This outcome is unexpected, as a faster developmental time in the unselected strain after pollutant exposure has not been documented in previous studies on SENN. However, the observed faster developmental time in SENN after exposure to BPA and phthalic acid aligns with findings from a study investigating the effect of BPA on *Culex* (Valsala and Asirvadam, 2022). Therefore, exposure to BPA and phthalic acid in the unselected strain resulted in a greater advantage in terms of a faster development rate than what was seen in the insecticide-selected strain. Consequently, exposure to the plastic additives resulted in a fitness cost to the insecticide-selected strain in terms of prolonged developmental time. Additionally, a faster developmental time was observed in the exposure to organic fertilisers in the insecticide-unselected strains (Jeanrenaud *et al.*, 2019). There is a range of factors that could affect these outcomes. One of the most likely explanations is the effect of hormesis. Hormesis is a phenomenon whereby a low dose of a stressor or toxicant can have a positive effect but be harmful at higher doses (Jager *et al.*, 2013). Therefore, it is likely that the effects observed may vary by pollutant concentrations. As such, further research is required to further elucidate the underlying mechanisms and provide a more comprehensive understanding of the findings. However, what is evident is that exposure to various plastics and plastic additives can elicit distinct responses in *An. arabiensis,* which is influenced by both the resistant phenotype of the strain and the type of plastic to which they are exposed.

Understanding the fitness costs associated with larval exposure to plastic is crucial for developing effective vector control strategies, as they can influence the dynamics of resistance alleles in mosquito populations and inform the deployment of insecticides to manage resistance. As has previously been observed, metal-polluted environments act as selection pressures for insecticide resistance in *An. arabiensis* (Oliver and Brooke, 2018b). Therefore, it is possible that plastic-polluted environments could also act as a selection pressure for insecticide resistance in vector populations. This is concerning as this could have implications for vector control strategies that rely on insecticides. Consequently, to further investigate the

influence of larval plastic exposure on the resistance phenotype, deltamethrin tolerance was determined. Deltamethrin is an insecticide used in vector control strategies. Therefore, an increase in deltamethrin tolerances is a major concern (Jeanrenaud *et al.*, 2019). Larval exposure to plastic treatments resulted in an overall increase in the deltamethrin tolerance in both SENN and SENN DDT. The most pronounced increase in insecticide tolerance was observed in response to plastic additives.

The plastic additives conferred a twofold increase in insecticide tolerance in both strains. Both BPA and phthalic acid conferred similar increases in deltamethrin tolerance, an outcome that was unexpected given that BPA was found to be more toxic to *An. arabiensis*. However, as both compounds function as plasticizers, it is plausible that they share similar mechanisms of action. This similarity may have resulted in a relatively lower metabolic activity being induced than what was anticipated for BPA. A similar trend was seen in studies on inorganic and organic fertilisers (Jeanrenaud *et al.*, 2019; Samuel *et al.*, 2020), heavy metals (Oliver and Brooke, 2018b) and acids and detergents (Chen and Oliver, 2024) on *An. arabiensis*. Additionally, in the nappy-treated SENN DDT, the increase in insecticide tolerance was insignificant. Similarly, the nappy-treated SENN exhibited what seemed to be a less pronounced increase in insecticide tolerance. A plausible explanation is that the nappies were not fully degraded, as it takes approximately 500 years for a nappy to decompose. Moreover, nappies are composed of various plastic components that have differing rates of decomposition. This incomplete degradation may have limited the bioavailability of the plastic fragments of the nappy, thereby reducing their impact on insecticide tolerance.

A study by Li *et al.* (2024a) determined that exposure to MPs resulted in a greater tolerance to pyrethroid and deltamethrin larvicides. However, the authors determined that the MP acted as sinks for the larvicides, thereby decreasing the concentration to which larvae were exposed. Additionally, the study by Shilla *et al*. (2024) determined that larval exposure to MPs alone and a mixture of MPs and insecticides reduced the efficacy of the insecticide, which subsequently resulted in increased insecticide tolerance in adult *An. gambiae s.s.* This is of concern as the MPs present within larval sites may serve as sinks for the larvicides used. Therefore, this may result in larvae exposure to sublethal concentrations of the larvicide, which may facilitate greater insecticide tolerance. However, the absorption of insecticides by the nappy fragments and the latex beads was not examined in this study. However, it does warrant

further investigation, particularly in understanding the impact of MPs within larval breeding sites and the potential of MPs to increase insecticide tolerance.

The findings of the insecticide tolerance are significant, as females treated with the less toxic phthalic acid did not show any fitness cost in terms of survival changes, however, an increase in insecticide tolerance was observed. This suggests that sublethal concentrations may enhance insecticide tolerance. Additionally, it was theorised that an increase in detoxification activity in the SENN DDT strain contributed to its insecticide tolerance (Oliver and Brooke, 2013). Furthermore, the rise in LT_{50} values, especially in the SENN DDT strain, indicates that exposure to plastic additives may have increased the activity of detoxification enzymes. As a result, it is proposed that SENN DDT can better tolerate plastic exposure due to this enhanced enzyme activity. This pattern was consistent across all plastic treatments. Thus, further supporting the conclusion that plastic exposure appeared to have minimal impact on adult longevity in the insecticide-selected strain of *An. arabiensis*, suggesting limited associated fitness costs. However, further experiments are needed to investigate enzyme activity following larval exposure to plastic.

In conclusion, this study serves as a foundational step in exploring the impact of larval plastic exposure on two laboratory strains of *An. arabiensis* of different resistance profiles. Overall, the study determined that chemical additives and plastic particles had varying impacts on larval development, longevity, and insecticide tolerance. Ingestion of plastic particles has a lesser effect on *An. arabiensis* compared to the chemical additives. It is plausible that various plastic treatments have distinct effects on different physiological systems within the mosquito. Moreover, the resistance phenotype of the strain influences the responsiveness of the mosquito to larval plastic exposure. The insecticide-selected strain SENN DDT demonstrated a greater ability to withstand larval exposure to plastic. Consequently, the insecticide-resistant phenotype may contribute to mitigating the effects of plastic exposure on *An. arabiensis.*

The findings indicate that various types of plastics can produce distinct effects. Thus, further investigation into the influence of other prevalent plastics on the biology of *An. arabiensis* is necessary. While this study focused on larval development and adult longevity, it is crucial to examine additional important life-history traits. Research into the effects of larval plastic exposure on fertility and fecundity is warranted. Furthermore, a study on the transgenerational effects of larval heavy metal exposure revealed that increased insecticide tolerance was passed

down to subsequent generations, even after the removal of the pollutant as a selection pressure (Jeanrenaud *et al.*, 2020). Consequently, it is imperative to investigate the transgenerational effects of larval exposure to plastic, as these findings may carry significant epidemiological implications. Additionally, field studies are necessary to assess the prevalence of plastic pollutants in natural mosquito larval breeding sites. These efforts are essential to fully comprehend the ecological and epidemiological consequences of plastic pollution on malaria vector populations.

Chapter 4: The effect of larval plastic exposure on the midgut microbiota of *Anopheles arabiensis*

4.1. Introduction

Vector control has been the key strategy for combating malaria transmission. However, due to the advent of insecticides and drug resistance, new strategies need to be implemented to combat malaria. Therefore, a greater understanding of the interactions between the *Plasmodium* parasite and the *Anopheles* vectors is required. Understanding the parasite life cycle and its associations with its host will allow for the development of better strategies for transmission-blocking and the overall control of malaria. One area of particular interest is the mosquito midgut. The mosquito midgut has a diverse naturally acquired microbial flora that influences the mosquito's vector competency (Mizushima *et al.*, 2023). The midgut is the major site of parasite escape, and the midgut microflora is important in preventing parasite egress. This microflora, known as the microbiota, consists of a community of microorganisms, including bacteria, fungi, viruses, and archaea, that inhabit a specific environment, such as the mosquito midgut (Berg *et al.*, 2020; Cansado-Utrilla *et al.*, 2021). The midgut microbiota plays a significant role in determining the capacity to transmit *Plasmodium* parasites (Dillon and Dillon, 2004; Poopathi and Tyagi, 2006). Thus, understanding the importance of the mosquito midgut microbiota is critical for developing novel vector control strategies to reduce the transmission of malaria.

The midgut microbiota affects the capacity to transmit parasites through the regulation of immunity (Bäckhed *et al.*, 2005; Round *et al.*, 2011). This is only one of the functions of these crucial bacteria. The midgut microbiota is essential for development (Coon *et al.*, 2014, 2020) reproduction (Jayakrishnan *et al.*, 2018), and aids with nutrition and digestion (Gusmão *et al.*, 2007; Gaio *et al.*, 2011; Guégan *et al.*, 2020). Additionally, insecticide resistance has been associated with changes to gut microbiota (Dada *et al.*, 2018, 2019; Omoke *et al.*, 2021; Pelloquin *et al.*, 2021). Understanding the relationship between the mosquito and the gut microbiota allows for a greater understanding of the overall biology and immunity in particular. These insights can be leveraged for improved vector control.

4.1.1. Influence of microbiota on mosquito development and fitness

The midgut microbiota has been shown to play an essential role in mosquito development, growth, and overall fitness (Liu *et al.*, 2024). During larval development, the presence of certain microbial communities has been linked to faster growth and increased survival rates. It has been demonstrated that mosquitoes reared in axenic (bacteria-free) conditions exhibited delayed development and reduced size compared to those with their natural microbiota (Coon *et al.*, 2014). Notably, Coon *et al*. (2014) determined that in the absence of gut bacteria, the larvae are unable to pupate which poses significant challenges in mosquito development. A study by Ezemuoka *et al.* (2020) demonstrated that upon treatment with antibiotics, the longevity of *An. gambiae* was reduced. However, upon the reintroduction of *Enterobacter cloacae* and *Serratia marcescens*, an increase in larval development was observed (Ezemuoka *et al.*, 2020). The presence of *Klebsiella* and *Aeromonas* was shown to promote the larval development of *Culex pipiens* (Díaz-Nieto *et al.*, 2016). A study by Valzania *et al*. (2018) concluded that continuous exposure of larvae to microbes in aqueous environments may have potentially selected for greater dependence on the gut microbiota for growth and development. These findings all highlight the significance of the midgut microbiota in mosquito development and overall fitness.

Additionally, the longevity of the insecticide-selected *An. arabiensis* was increased after treatment with bactericidal antibiotics (Barnard *et al.*, 2019). However, the longevity of the insecticide unselected *An. arabiensis* was reduced after treatment with a broad-spectrum bacteriostatic erythromycin (Barnard *et al.*, 2019). Therefore, the different microbes present in the midgut result in differing influences on mosquito survival. Similarly, the adult longevity of *An. stephensi* was reduced after antibiotic treatment, further indicating that midgut microbiota plays a role in mosquito development and fitness (Sharma *et al.*, 2013). Furthermore, studies have identified that in *Cx*. *quinquefasciatus* greater longevity and improved survivorship were linked to the presence of *Wolbachia* (Almeida *et al.*, 2011; Alomar *et al.*, 2023).

In addition, mosquitoes require their midgut microbiota to aid with nutrition and digestion. The microbiota assists with the breakdown of complex nutrients from both blood meals and plant-based sugar meals (Gusmão *et al.*, 2007; Gaio *et al.*, 2011; Guégan *et al.*, 2020) thus providing essential metabolites that contribute to mosquito energy needs (Dillon and Dillon, 2004;

Guégan *et al.*, 2020; Romoli *et al.*, 2021). A study by Gusmão *et al.* (2007) identified bacteria present in the gut of *Ae. aegypti* plays a role in the metabolism of sugars. Additionally, upon antibiotic treatment on laboratory-reared female *Ae. aegypti,* a delay in the digestion of blood meals was observed (Gaio *et al.*, 2011). This delay was attributed to the absence of midgut bacteria that exhibit haemolytic activity, such as *Enterobacter* sp. and *Serratia* sp. (Gaio *et al.*, 2011). Mosquitoes require blood meals for reproductive fitness as the nutrients provided by these blood meals are essential for the production of eggs and, thus, successful reproduction (Hansen *et al.*, 2014). A study by Sharma *et al*. (2013) demonstrated that antibiotic treatment resulted in a reduced reproduction potential due to decreased blood meal digestion (Sharma *et al.*, 2013). This was attributed to the effect of the antibiotics diminishing the microbes present in the gut of *An. stephensi* (Sharma *et al.*, 2013). Additionally, *Wolbachia* was linked to the reduction of reproductive fitness (Almeida *et al.*, 2011; Alomar *et al.*, 2023). Upon antibiotic treatment, *Aedes aegypti* survival and fecundity were reduced due to a decrease in microbes present within the midgut (Hill *et al.*, 2014). This further demonstrated the significance of the midgut microbiota in not only nutrition and metabolism but also reproduction.

Furthermore, existing research has demonstrated a strong correlation between the midgut microbiota and insecticide resistance (Soltani *et al.*, 2017; Dada *et al.*, 2018; Sato *et al.*, 2021; Wang *et al.*, 2021c). Research has demonstrated that microbial communities present within the mosquito's midgut may also influence the mosquito's ability to metabolize and detoxify insecticides, thereby contributing to insecticide resistance (Xia *et al.*, 2023; Liu *et al.*, 2025). Alterations to the composition of the midgut microbiota may influence the metabolic capacity of mosquitoes to process insecticides. For example, the presence of *Pseudomonas* spp. and *Serratia* spp. within the microbiota have been associated with the degradation of insecticides as they produce enzymes capable of breaking down xenobiotic compounds (Xia *et al.*, 2023; Liu *et al.*, 2025). Therefore, the presence of these bacteria within the mosquito midgut may aid in enhancing the mosquito's tolerance to insecticides. Research on *An. albimanus* has indicated that variations within the mosquito microbiota corresponded with insecticide resistance (Dada *et al.*, 2018). The insecticide resistance was linked to the selective enrichment of bacteria that possess insecticide-degrading capabilities, as well as the upregulation of detoxification enzymes within the resistant mosquitoes (Dada *et al.*, 2018). Similarly, a study on *An. gambiae* in Western Kenya identified distinct variations in the bacteria that were differentially abundant between the insecticide-resistant and susceptible mosquitoes (Omoke *et al.*, 2021). A noteworthy study on *An. arabiensis* determined that the midgut microbiota is integral to the

mechanisms of insecticide resistance (Barnard *et al.*, 2019). The findings indicated a distinct difference in the composition of the midgut microbiota between resistant and susceptible strains, with the resistant strain exhibiting diminished bacterial diversity and a notable predominance of insecticide-degrading bacterial species (Barnard *et al.*, 2019). These studies suggest that the midgut microbiota plays a vital role in the survival and adaptation of mosquito populations to insecticide exposure. Therefore, understanding the mechanisms by which midgut microbiota modulate insecticide resistance could offer novel insights into vector control strategies. Additionally, identifying and understanding the influences on the midgut microbiota is essential for elucidating its role in development, fitness, and insecticide resistance, thereby contributing to more effective vector control strategies.

4.1.2. The relationship between mosquito immune system, gut microbiota, and vector competence

Mosquitoes, like all insects, rely solely on their innate immunity as they lack an adaptive immune system (Simões *et al.*, 2017). For a blood sucking insect like the mosquito, the midgut plays a part in the immune system and is essential in fighting pathogen infections (Saraiva *et al.*, 2016). Studies on both laboratory-reared and field-caught mosquitoes have revealed that certain bacteria within the mosquito midgut exhibit the capability to control disease transmission (Gonzalez-Ceron *et al.*, 2003; Bai *et al.*, 2019; Santos *et al.*, 2023). There are various ways in which the midgut microbiota controls disease transmission with the midgut bacteria, either causing direct or indirect effects to elicit an immune response.

The direct approach is the inhibition of parasite development by inducing reactive oxygen species (ROS) and activation of immune genes (Gao *et al.*, 2020). Secretions from gut bacteria such as ROS, free radicals, metabolites, small peptides and proteins have the potential to inhibit pathogen development and transmission (Azambuja *et al.*, 2005). Various studies have determined the interplay between the midgut microbiota and the malaria parasite. A study conducted by Dong *et al.* (2009) illustrated that upon antibiotic treatment, the decrease in bacteria resulted in the mosquito being more susceptible to *Plasmodium* infection (Dong *et al.*, 2009). However, it was further observed that these effects are reversible by the reintroduction of bacteria into the midgut (Dong *et al.*, 2009; Gendrin *et al.*, 2015). The authors concluded that the microbiota modulates the anti-*Plasmodium* effects of the immune genes, therefore influencing the mosquito's ability to maintain *Plasmodium* infection. Additionally, Cirimotich

et al. (2011) observed that feeding *An. gambiae* the malaria parasite *P. falciparum* with *Enterobacter* strain *Esp_Z,* resulted in the prevention of parasite development due to the generation of ROS from the bacteria. Furthermore, Capone *et al.* (2013) demonstrated that in *An. stephensi*, *Asaia* sp. induced antimicrobial peptide expression. Additionally, *Serratia* spp. has been shown to produce serralysin proteins and prodigiosin, which have been shown to have pathogen-preventative effects *in vitro* (Castro, 1967; Welch, 1991).

The indirect effect of the microbiota on pathogen infections relates to the microbiota activating the immune response via immune pathways. These pathways include the immune deficiency (Imd), Toll and Janus kinase signal transducer and activator of transcription (JAK/STAT) pathways (Cirimotich *et al.*, 2010; Sim *et al.*, 2014; Kumar *et al.*, 2018; Mukherjee *et al.*, 2019; Tikhe and Dimopoulos, 2021). Additionally, it was observed that acquiring known bacteria such as *Enterobacter* sp. and *Serratia marcescens* resulted in the diminished development of oocysts by stimulating anti-*Plasmodium* immunity (Cirimotich *et al.*, 2011; Bando *et al.*, 2013; Bahia *et al.*, 2014). These findings highlight the potential of manipulating the midgut microbiota to reduce the transmission of malaria.

Paratransgenesis is a novel strategy that eliminates malaria transmission or decreases vector competence by introducing bacterial symbionts that are either native or genetically engineered to produce anti-*Plasmodium* molecules in the mosquito host (Wang *et al.*, 2012; Wilke and Marrelli, 2015; Ratcliffe *et al.*, 2022). However, before any significant strides can be taken in implementing this novel strategy, a better understanding of the factors that influence the midgut microbiota needs to be undertaken. Particularly, a greater understanding of the environmental factors influencing the microbiota needs to be further researched.

4.1.3. Environmental influence on the midgut microbiota

The composition of the mosquito midgut microbiota is influenced by environmental factors such as the larval habitat, diet and exposure to pathogens (Strand, 2018). Studies have shown that field-caught mosquitoes generally have a more diverse midgut microbiota composition than laboratory-reared mosquitoes (Boissière *et al.*, 2012; Osei-Poku *et al.*, 2012). This indicates that different environments can influence the microbiota. Different larval breeding sites harbour different microbial communities, which can result in variations in the midgut microbiota of adult mosquitoes (Gimonneau *et al.*, 2014). Additionally, it has been observed

that a significant portion of bacteria present within mosquito midguts is acquired during their immature aquatic life stages from the surrounding larval habitat (Guégan *et al.*, 2018). A study by Louie and Coffey (2021) indicated that rearing *Ae. aegypti* in different larval environments with different microbial compositions impact the vector competence of the species. Similarly, a study by Duguma *et al*. (2017) observed the correlation between nutrient content and the microbial composition in larval habitats with the adult microbiota of *Culex nigripalpus.* The authors determined that different bacterial communities were associated with mosquitoes emerging from high or low-nutrient habitats (Duguma *et al.*, 2017). These studies all indicate that the larval habitat and the contents present in larval habitats play a significant role in determining the microbiota. Therefore, environmental changes such as pollution and urbanisation can also alter the microbial communities in mosquito breeding sites. This potentially causes variations in the midgut microbiota, which in turn can affect mosquito fitness, immunity and vector competence (Strand, 2018). As such, it is important to understand the effects of adaptation to pollution on mosquito microbial dynamics.

4.1.4. Microplastics and the midgut microbiota

Mosquito larvae often inhabit polluted environments where they can ingest MPs. Microplastics are often present in various freshwater systems where the larvae feed on debris and other organic matter, making them susceptible to unintentional ingestion of MP particles (Eerkes-Medrano *et al.*, 2015; Reynolds and Ryan, 2018). The fact that mosquito larvae are filter feeders means that their exposure to MPs is highly probable. This is particularly true in urban and agricultural areas where plastic pollution is prevalent. Research has demonstrated the dysbiosis of the gut microbiota caused by MPs in a range of animals, including invertebrates. In honeybees, nanoplastics and MPs were seen to cause disruptions to the microbiota (Wang *et al.*, 2022a). Additionally, the study found that exposure to MPs resulted in the stimulation of genes involved in immune inhibition and the suppression of genes related to detoxification (Wang *et al.*, 2022a). Conversely, another study observed that the gut microbiota played a protective role in honeybees against polystyrene MP exposure (Wang *et al.*, 2021b). Research on other invertebrates has demonstrated that MPs can cause gut lesions and inflammation, which can lead to a shift in microbial diversity (Li *et al.*, 2022a; Ghosal *et al.*, 2024). In mosquitoes, it was observed that larval ingestion of MPs in *Ae. aegypti* and *Ae. albopictus* resulted in the perturbation of the midgut microbiota and mycobiota (Edwards *et al.*, 2023). Additionally, the midgut microbiota composition and distribution in *Cx. quinquefasciatus* was

changed upon ingestion of MPs (Li *et al.*, 2024a). The authors found that the changes observed in the microbiota were related to the particle size and concentration of the ingested MPs (Li *et al.*, 2024a).

It has been theorised that MPs affect the gut microbiota of mosquitoes through several mechanisms. Larval exposure to increasing concentrations of polystyrene MPs in *Ae. aegypti* and *Ae. albopictus* resulted in damage to gut tissue (Edwards *et al.*, 2023; Jones *et al.*, 2024). These MPs-induced gut lesions and subsequent inflammation can lead to a shift in microbial diversity (Li *et al.*, 2022a; Ghosal *et al.*, 2024). This is significant as damage to the gut epithelium impacts the vector competence for viruses, which was theorised to occur through alterations to the mosquito gut microbiota and immunity (Jones *et al.*, 2024). However, the authors concluded that there is currently insufficient evidence to definitively state that MPs will impact pathogen transmission in mosquitoes. Therefore, further research is necessary to elucidate the interaction between MPs, mosquito microbiota and vector competency.

Another mechanism by which MPs alter the gut microbiota is through the introduction of plastic-associated bacteria. Microplastics provide surfaces upon which microbial colonisation can occur. This creates what is known as the "plastisphere". (Zettler *et al.*, 2013; Dussud *et al.*, 2018). Therefore, ingestion of MPs by mosquitoes can introduce microbes' part of the plastisphere into the gut, potentially altering the gut microbiota. Studies on marine organisms have found that MPs carry a diverse range of bacteria, including pathogens and antibiotic-resistant bacteria, that could alter the native gut microbiota (Jin *et al.*, 2018; Bowley *et al.*, 2021; Li *et al.*, 2022b). Additionally, MPs act as sinks for other environmental contaminants such as heavy metals, pesticides and organic pollutants (Teuten *et al.*, 2009; Graca *et al.*, 2014; Holmes *et al.*, 2014). The ingestion of MPs, therefore, exposes host organisms to these toxic substances. The exposure may lead to oxidative stress and inflammation in the mosquito gut, therefore creating an environment that favours the growth of certain bacteria and suppresses others (Menéndez-Pedriza and Jaumot, 2020; Song *et al.*, 2022b). When considering all these factors, it is evident that MPs can affect the microbiota. These effects on the microbiota will impact the host biology, particularly immunity and overall fitness (Jones *et al.*, 2024). Therefore, further research needs to be conducted to determine the impacts of plastic on mosquito microbiota.

The ingestion of MPs by mosquitoes may have significant effects on their gut microbiota, with potential implications for mosquito fitness and vector competence. Although research is still ongoing, studies have identified that MPs can act as vectors for chemical contaminants, cause physical damage to the gut and introduce foreign microbes. These interactions, therefore, can lead to alterations of the microbial composition and diversity of the mosquito midgut microbiota. These alterations could impair digestion and immune function and ultimately influence vector competence. Given that plastic pollution is a major global issue, understanding the effects of MPs on mosquito gut microbiota is critical in terms of ecological significance and public health perspectives. However, despite the emerging studies, currently, to the best of our knowledge, there is no information regarding the effect of plastic exposure on the microbiota of malaria vectors. Therefore, this chapter aimed to add to the growing body of knowledge by providing a perspective on the effect of plastic and plastic additives on the midgut microbiota of *An. arabiensis.*

4.2. Materials and Methods

4.2.1. Larval exposure to plastic treatments

The mosquito strains used in this study were insecticide-selected (SENN DDT) and unselected (SENN) strains of *An. arabiensis*. The resistance profiles of the strains are detailed in sections 2.1 and 3.3.1. First instar larvae of SENN and SENN DDT were exposed to four different plastic treatments. Firstly, as a proxy for MPs, larvae were exposed to commercially available fluorescently labelled latex amine-modified polystyrene beads (2 µm). The 2 µm latex beads were selected as they ensured the best survivorship of the mosquitoes, as was observed in Chapter 3. A volume of 7 µL of the latex beads per litre of reverse osmosis water was used. Disposable nappies were used as a representation of complex plastics found in larval breeding sites in South Africa. The process of degrading the nappies is described previously in section 2.3. The degraded nappy solution was split between the replicates and further diluted with 1000 mL of reverse osmosis (RO) water to generate the nappy water the larvae were exposed to. A final concentration of 12.5 µM BPA per 1000 mL of RO water was used. BPA was used as a representative of a plastic breakdown product. Additionally, phthalic acid dimethyl ester (phthalic acid) was used as a representative of a common plasticiser that can leach into the environment. A final concentration of 59.5 µM of phthalic acid per 1000 mL of RO water was used.

The samples needed for sequencing were generated by rearing three replicates of 100 newly hatched first-instar larvae of SENN and SENN DDT in 1000 mL of reverse osmosis water with the plastics treatments as described above. Larvae reared in untreated RO water served as the untreated controls. The larvae were allowed to develop and fed a standardised amount of food. The emergent adults were given *ad libitum* access to 10% sucrose. Once the adults reached the age of 3 days, females were collected for further processing.

4.2.2. Midgut dissection and DNA extraction

Three-day-old non-blood-fed females were collected and freeze-killed in a -80°C freezer. To ensure sterility, the external surface of the mosquitoes, dissecting forceps and surfaces were sterilised using 70% ethanol. The midguts of adult female mosquitoes were dissected using a stereomicroscope (Olympus SZ571). Five midguts were pooled per sample, and five samples were prepared per treatment per strain. A total number of 25 samples per strain were used. The

midguts were pooled directly into a solution of the lysis buffer ATL and proteinase K of the Qiagen DNeasy® Blood & Tissue (Qiagen: 69506, Germany) DNA extraction kit. Total genomic DNA was extracted following the manufacturer's instructions. An initial overnight incubation step was included as recommended per the manufacturer's protocol. A positive extraction control consisting of a commercial Microbial Community DNA Standard (ZymoBIOMICS®, Catalogue no.: D6300) was included. A negative control consisting of the buffers used for the extraction process was included. The concentration and quality of the extracted DNA were quantified using spectrophotometry on a Nanodrop™ spectrophotometer.

4.2.3. DNA amplification and 16S rRNA gene sequencing

The total extracted genomic DNA of the midguts was subjected to PCR to amplify the hypervariable V3-V4 region of the bacterial 16S rRNA gene. (Klindworth *et al.*, 2013). The sequences of the primers used were as follows: forward primer 5′GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3′ and reverse primer 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). A commercial Microbial Community DNA Standard (ZymoBIOMICS®, Catalogue no.: D6300) was also amplified and served as the positive control. The positive control consisted of known bacterial species. Additionally, a non-template sample and an extraction buffer-only sample were prepared and amplified by PCR and served as the negative controls. Following the standard 16S Metagenomic Sequencing Library protocol (Illumina TM, San Diego, CA, USA), the library preparation was conducted. The prepared libraries were subsequently sequenced on the MiSeq platform using the MiSeq Reagent kit v3 (Illumina, USA) and paired-end 2 × 300 bp sequencing was performed (Singh *et al.*, 2022b; Chen *et al.*, 2024a). Sequencing was performed at the Sequencing Core Facility, National Institute for Communicable Diseases (NICD), Johannesburg, South Africa.

4.2.4. Bioinformatic analysis

The bioinformatics analysis performed was as outlined by Chen *et al*. (2024a). Important quality control steps were undertaken to ensure clean reads were used for all downstream analyses. In order to remove the Nextera adapter sequences, the sequences were filtered and pair-end trimmed using trimGalore (v0.6.5-1). The quality of the reads was assessed using

MultiQC (v1.6). The full MultiQC report can be found in Appendix 2. Downstream analysis on the clean reads was performed in R studio (v4.2.1). The DADA2 package (v1.24.0) was used to pre-process the clean reads. The DADA2 pre-processing included further quality inspection, filtering, trimming, dereplication, sample inference, merging pair-end reads and the removal of chimeric sequences (Callahan *et al.*, 2016; Chen *et al.*, 2024a). The amplicon sequence variants (ASVs) were assigned taxonomy, and the ASV abundance estimates were determined using the SILVA reference database (v138; https://zenodo.org/record/4587955#.Y9JGXnZBxPY, accessed 10 February 2024). The phyloseq package (v1.40.0) (McMurdie and Holmes, 2013) was used to generate phyloseq data objects from the DADA2 outputs for further analysis.

Alpha diversity was analysed using the Chao1 and ACE indices to determine species richness. The alpha diversity species diversity was analysed using the Shannon and Simpson diversity indices. The comparison of alpha diversity between the different plastic treatments was determined using a Wilcoxon rank-sum test. A Nonmetric Multidimensional Scaling (NMDS) ordination plot based on the Bray-Curtis distance measurement was constructed to illustrate the beta diversity. A PERMANOVA (permutation test with pseudo-F ratios) was used to statistically assess the data clustering between the different plastic treatments. This was done by using the adonis function in the vegan package (v2.6-4; https://github.com/vegandevs/vegan, accessed 11 February 2024). The relative abundance of the top 5 ranked genera between the different plastic treatments for each strain was visualised using bar plots. The overlapping genera and species were visualised using Upset plots that were generated using Venn Diagram (v1.7.3) and UpsetR (v1.4.0) (Conway *et al.*, 2017). The DESeq2 (v1.24.0) (Love *et al.*, 2014) package was used to analyse the differential abundance between the different plastic treatments for each strain. The construction of all plots except for the Upset plots was done using ggplot2 (v3.4.0) (Wickham, 2011).

4.3. Results

4.3.1. Alpha diversity

The Chao1 and ACE indices are representatives of the bacterial species richness, which is the total number of species in a sample. In SENN, according to both the Chao1 and ACE indices, the highest species richness was seen in the nappy-treated samples (Figure 4.1). Upon comparison of the beads-treated SENN (Chao1: p=0.032, ACE: p=0.032) and nappy-treated SENN (Chao1: p=0.032, ACE: p=0.032), both had significantly higher species richness compared to the untreated samples in SENN (Figure 4.1). However, when the species diversity was compared, only the beads-treated SENN resulted in a significantly higher Shannon diversity index than the untreated SENN (p=0.032). Conversely, the Simpson index indicated that only the BPA-treated SENN samples had a significantly greater species diversity than the untreated samples (p=0.032).

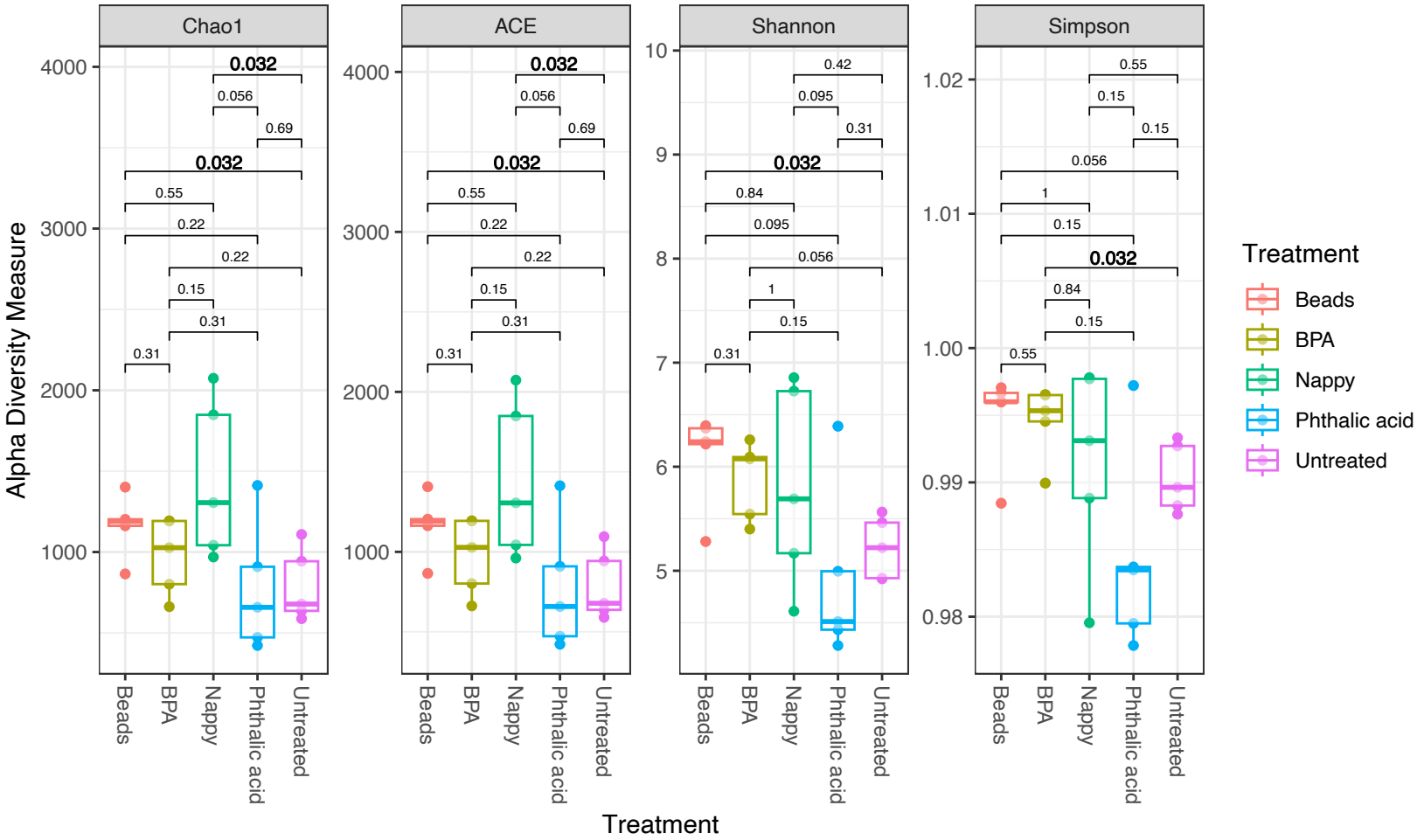


Figure 4.1. Alpha diversity of the gut microbiota of the plastic treatments in SENN. The alpha diversity was measured with the Chao1 and ACE indices for species richness. The Shannon and Simpson indices measure the diversity of the species. A Wilcoxon rank sum test was used to compare the alpha diversity between the plastic treatments, and *p*-values<0.05 were considered significant. The box plots represent the median (line), interquartile range (box), and the minimum/maximum values (whiskers). Significant *p*-values are shown in bold, larger text.

In SENN DDT, differences in species richness were seen between the treatment groups rather than compared to the untreated samples. Significant differences in species richness were present when compared to the nappy-treated SENN. For the Chao1 index, the nappy-treated SENN DDT had a significantly greater species richness than the phthalic acid-treated SENN DDT (p=0.032), BPA-treated SENN DDT (p=0.016) as well as the beads-treated SENN DDT (p=0.032) (Figure 4.2). Similarly, according to the ACE index nappy-treated SENN DDT had a significantly greater species richness than the phthalic acid-treated SENN DDT (p=0.032), BPA-treated SENN DDT (p=0.016) and the bead treatment (p=0.032) (Figure 4.1B). When looking at the species diversity, the Shannon index indicated that only the BPA-treated samples were significantly more diverse than the untreated samples (p=0.032) (Figure 4.2). Upon comparison with the untreated SENN DDT samples, the BPA-treated SENN DDT samples had a significantly greater Simpson index (p=0.008), therefore indicating a higher species diversity. Additionally, the Simpson index also indicated that the species diversity of the BPA-treated SENN DDT was significantly higher than their beads-treated counterparts (p=0.032).

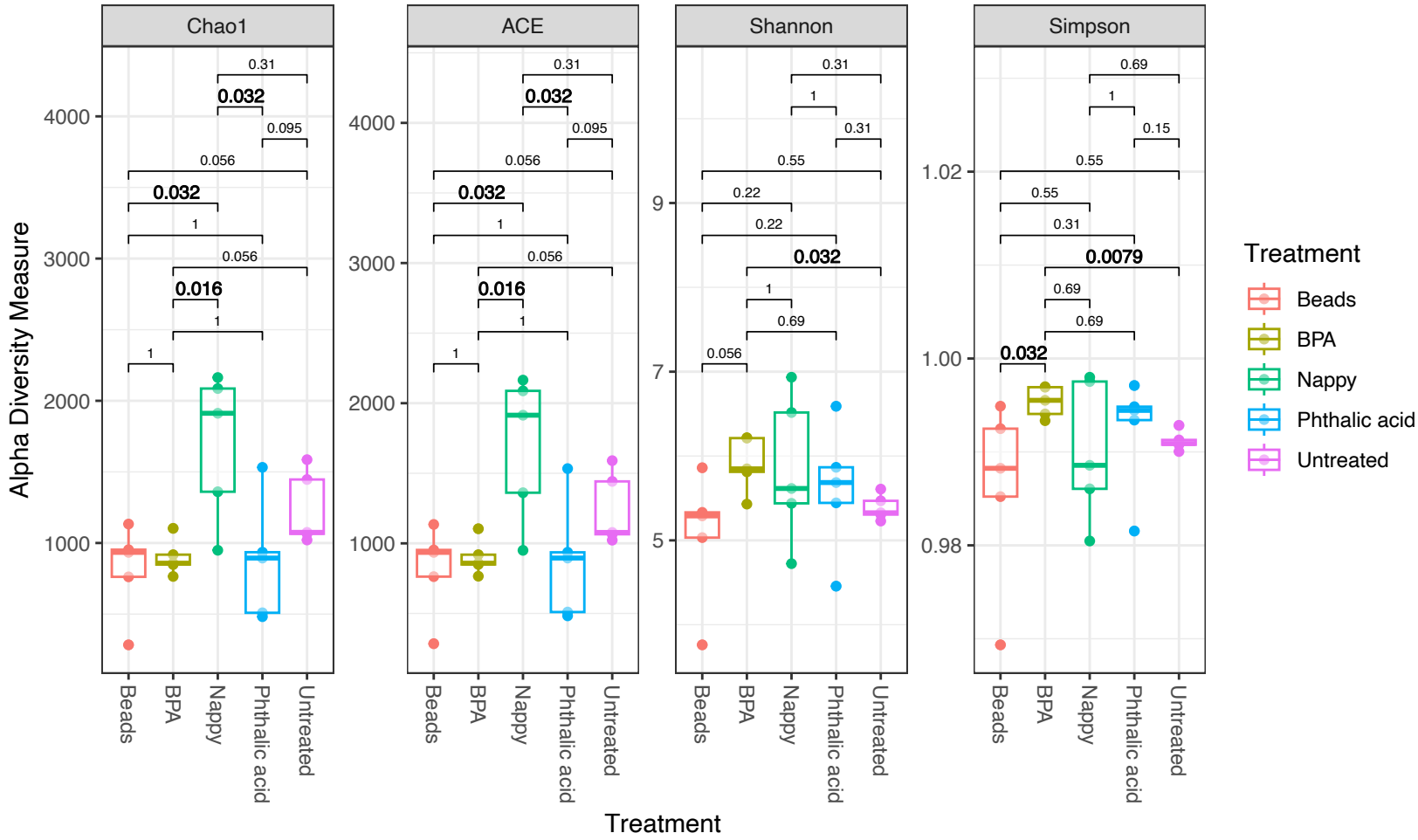


Figure 4.2. Alpha diversity of the gut microbiota of the plastic treatments in SENN DDT. The alpha diversity was measured with the Chao1 and ACE indices for species richness. The Shannon and Simpson indices measure the diversity of the species. A Wilcoxon rank sum test was used to compare the alpha diversity between the plastic treatments and *p*-values<0.05 were considered significant. The box plots represent the median (line), interquartile range (box), and the minimum/maximum values (whiskers). Significant *p*-values are shown in bold, larger text.

4.3.2. Beta diversity

A Nonmetric Multidimensional Scaling (NMDS) ordination plot was used to assess the beta diversity (between-strain diversity) between the two strains of *An. arabiensis* and plastic treatments (Figure 4.3). Based on the PERMANOVA analysis, it was evident that the plastic treatments had a significant effect on the beta diversity (PERMANOVA: $p<0.001$, $R^2=0.26$). However, no significant difference in strains was seen (PERMANOVA: $p=0.05$, $R^2=0.023$), but a significant difference in the interaction between strain and the treatments was observed (PERMANOVA: $p<0.001$, $R^2=0.1$).

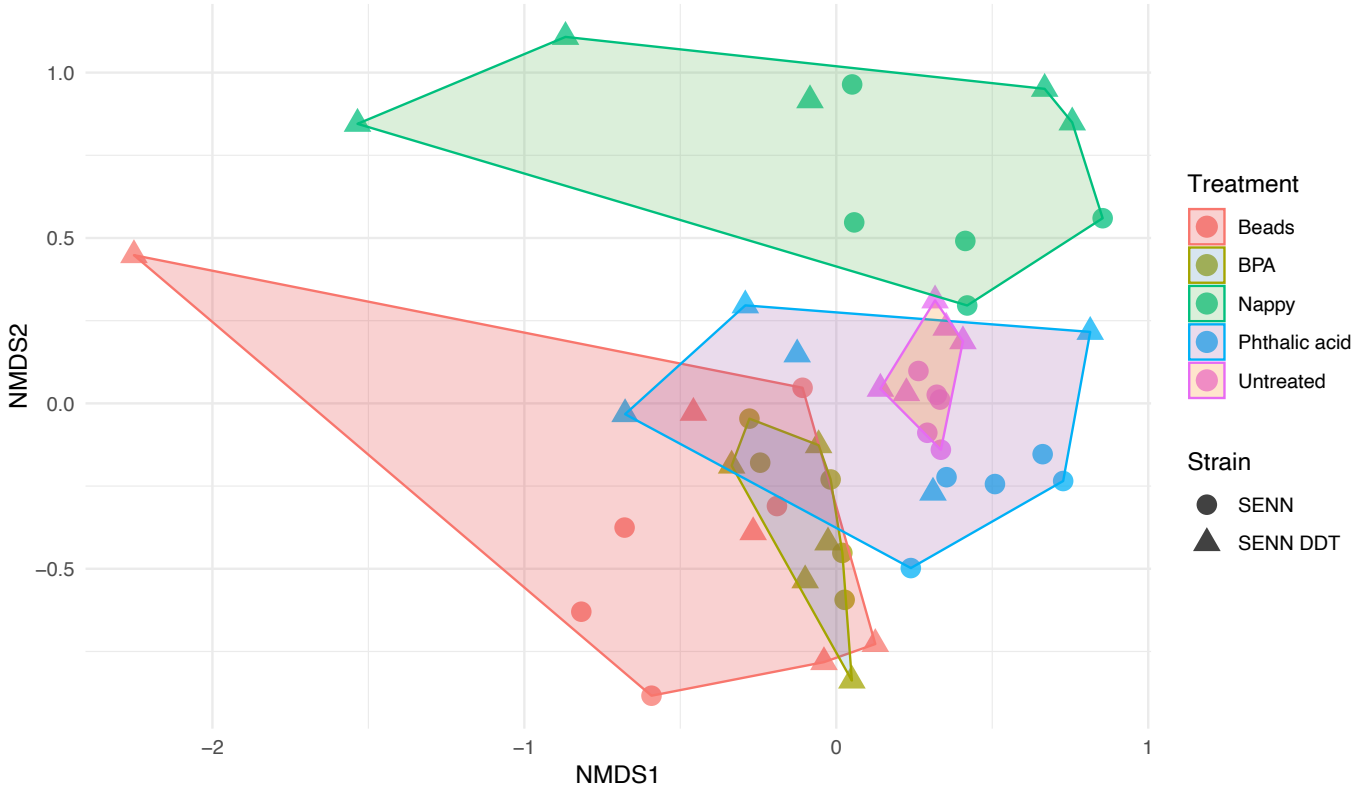


Figure 4.3 Beta diversity of the microbiota of *An. arabiensis* under plastic treatments. A Nonmetric Multidimensional Scaling (NMDS) ordination plot was used based on the Bray-Curtis distance measurement. The stress value was 0.19. A PERMANOVA indicated $p<0.001$ ($R^2=0.26$) for treatments, $p=0.05$ ($R^2=0.023$) for strain and $p<0.001$ ($R^2=0.1$) for the interaction between the treatment and strain. Beta diversity is more significant between treatments than between strains.

4.3.3. Bacterial overlap

Upon analysis of the shared bacterial genera and species between the treatment groups, it was evident that in SENN, there was a total of 26 shared genera between all the treatment groups (Figure 4.4A). The full list of shared and unique bacterial genera and species between SENN exposed to the different plastic treatments can be found in Appendix 2. From the full list of shared and unique bacterial genera, it was determined that there were 15 known shared bacterial genera; however, 11 genera shared between the plastic-treated SENN samples remained unidentified, attributable to the absence of corresponding reference sequences in the existing database. However, the nappy-treated SENN had the highest number of unique genera (42), followed by the phthalic acid-treated SENN (36) (Figure 4.4A). The BPA-treated SENN samples had 21 unique genera, and the beads-treated SENN had 18 unique genera (Figure 4.4A). The untreated SENN samples had the least number of unique genera at 15. Additionally, there were 29 shared bacterial species between SENN exposed to different plastic treatments (Figure 4.4B). Similarly, the nappy-treated SENN had the highest number of unique bacterial species (79), followed by phthalic acid-treated SENN (55) and BPA-treated SENN (43) (Figure 4.4B). The untreated and bead-treated SENN had the least unique species at 33.

In SENN DDT, a different pattern of overlap was observed. Unlike in SENN, the untreated SENN DDT samples had more unique bacterial genera at 59 (Figure 4.4C). The full list of shared and unique bacterial genera and species between the different plastic treatments for SENN DDT can be found in Appendix 2. Twenty-four genera were shared between SENN DDT exposed to the different plastic treatments. Upon analysis of the full list of shared and unique bacterial genera for SENN DDT, it was determined that there were 12 known and 12 unknown shared bacterial genera. The BPA-treated SENN DDT had the second-highest number of unique bacterial genera (25), followed by phthalic acid-treated (22), nappy-treated (21) and lastly, the beads-treated SENN DDT (15). However, SENN DDT had fewer shared species (28) than the unique species across SENN DDT exposed to plastic treatments (Figure 4.4D). The untreated SENN DDT had the highest number of unique bacterial species at 89, followed by phthalic acid-treated (50), BPA-treated (47), nappy-treated (34) and lastly, the beads-treated SENN DDT (33) (Figure 4.4D).

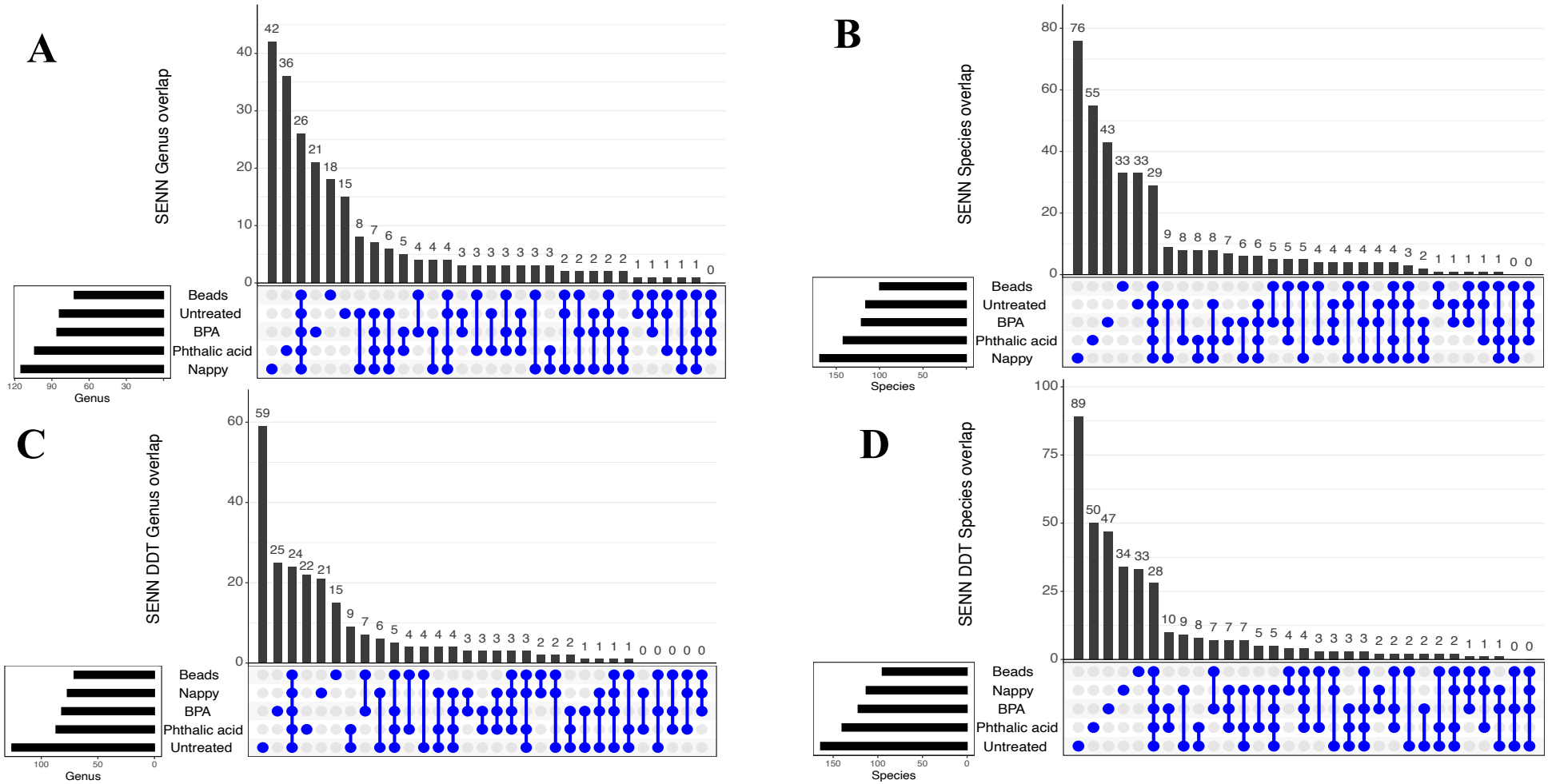


Figure 4.4. Overlapping bacterial genera and species in *An. arabiensis* after larval exposure to plastic treatments. A) Overlapping genera in SENN. B) Overlapping species in SENN. C) Overlapping genera in SENN DDT. D) Overlapping species in SENN DDT. Individual dots represent the numbers of individual bacterial genera or species. Joined dots indicate the number of shared genera and species between the different treatments

4.3.4. Relative abundance

The top five ranked bacterial genera in both strains were *Acinetobacter*, *Citrobacter*, *Elizabethkingia*, *Rahnella* and *Yersinia.* However, it should be noted that *Yersinia* was only relatively abundant in SENN DDT and not SENN. Additionally, the abundance of bacteria differs by plastic treatments. In SENN, the untreated samples were dominated by *Acinetobacter,* followed by *Rahnella* and *Elizabethkingia*. However, *Acinetobacter* was evidently relatively abundant at a higher proportion. In the beads and BPA-treated SENN samples, *Rahnella* was the most relatively abundant genus. However, the nappy-treated SENN samples presented *Elizabethkingia* and *Acinetobact*er as the most relatively abundant genera. The phthalic acid-treated SENN samples were dominated by *Elizabethkingia* (Figure 4.5A).

A similar trend of relative abundance was seen in SENN DDT, with the untreated samples being dominated by *Acinetobacter,* followed by *Elizabethkingia* and *Rahnella.* However, it should be noted that the proportions of genera differ from what was observed in SENN. The abundance of *Acinetobacter* and *Elizabethkingia* is more evenly distributed in SENN DDT than in SENN. Similar to what was observed in SENN, the beads and BPA-treated SENN DDT were dominated by *Rahnella*. However, in the beads-treated SENN DDT samples, *Yersinia* was the second highest-ranked genus. Similarly, in the SENN DDT nappy-treated samples, *Elizabethkingia* was the dominant genus, followed by *Yersinia.* It should be noted that the relative abundance of *Acinetobacter* was lower in nappy-treated SENN DDT than in nappy-treated SENN. However, in the phthalic acid-treated SENN DDT samples, *Elizabethkingia* and *Rahnella* were the dominant genera. Notably, the relative abundance of *Rahnella* in phthalic acid-treated SENN DDT is higher than what was seen in phthalic acid-treated SENN (Figure 4.5B).

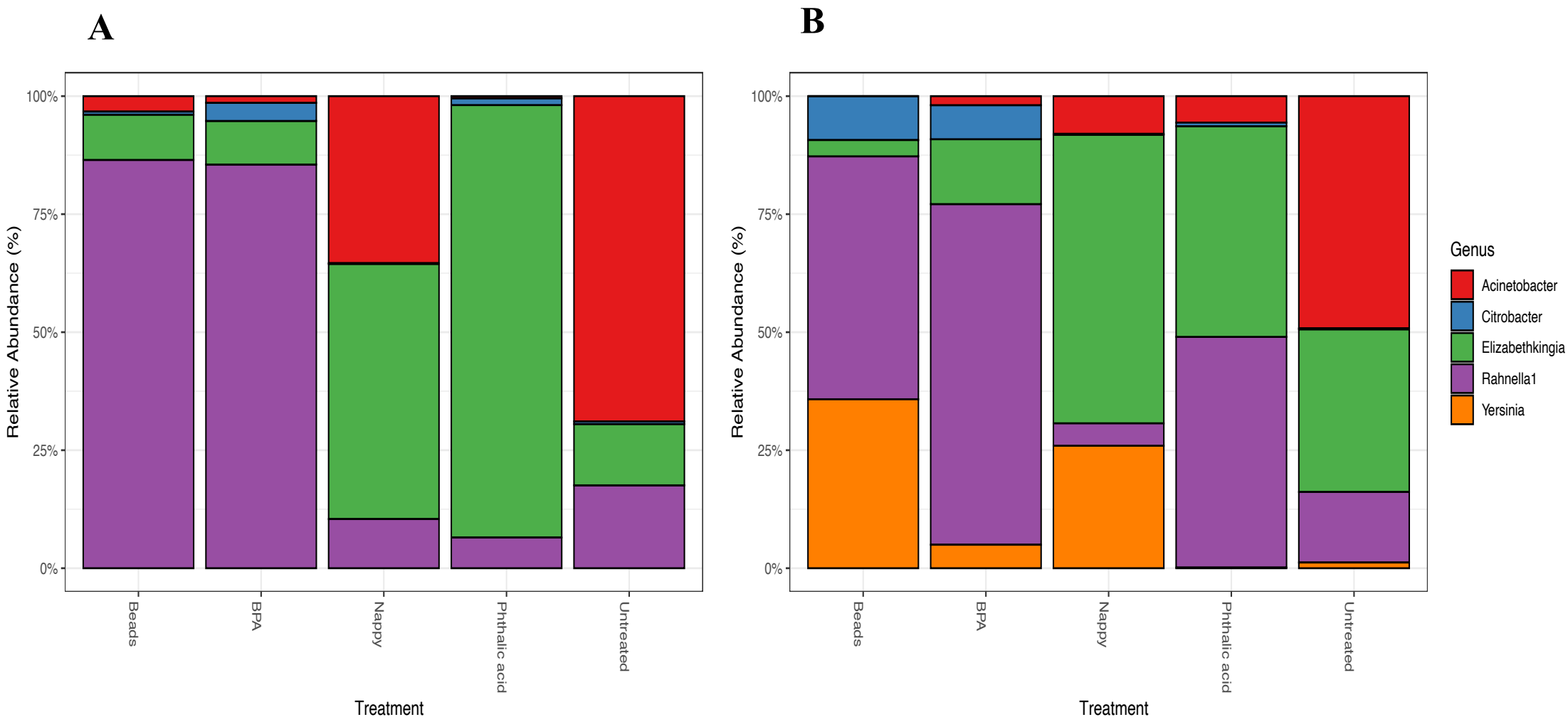


Figure 4.5. Relative abundance of the top five ranked bacterial genera in *An. arabiensis* after exposure to four different plastic treatments.

A) Relative abundance of SENN after plastic treatment. B) Relative abundance of SENN DDT.

4.3.5. Differential abundance

Only the bacterial genera considered differentially abundant with a significant log2fold change (α=0.01) are displayed. From the differential abundance analysis, bacterial genera that were consistently differentially abundant within the untreated samples were observed (Figure 4.6). In the untreated SENN samples, *Duganella, Massilia, Moraxella* and *Roseomonas* emerged as consistently differentially abundant within the untreated SENN samples. However, *Alistipes, Arthrobacter, Bacteroides, Candidatus Soleaferrea, Enterobacter, Incertae Sedis, Kocuria, Novospirillum, Paracoccus,* and *Rhodovarius* emerged as consistently differentially abundant within the untreated SENN DDT samples. Additionally, *Chryseobacterium* was determined to be differentially abundant in all the untreated samples of both SENN and SENN DDT.

Furthermore, a discernible pattern was observed whereby the plastic-treated samples in SENN had more differentially abundant genera compared to their untreated counterparts. Conversely, in SENN DDT, the opposite was observed. The untreated SENN DDT samples generally had more differentially abundant bacteria than the plastic-treated SENN DDT except for BPA-treated SENN DDT. The SENN bead-treated samples presented 13 significantly abundant genera when compared to the 12 in the untreated samples (Figure 4.6A). In the beads-treated SENN DDT, there were 11 differentially abundant genera compared to the 18 in the untreated samples (Figure 4.6E). The BPA-treated SENN consisted of 16 differentially abundant bacteria genera compared to the 9 in the untreated samples (Figure 4.6B). However, in the BPA-treated SENN DDT samples, 19 bacteria genera were differentially abundant compared to the 16 in the untreated samples (Figure 4.6F). The emerging pattern was very clearly observed in nappy-treated samples. The nappy-treated SENN samples consisted of 27 differentially abundant genera compared to only 8 present in the untreated samples (Figure 4.6C). Conversely, in SENN DDT, the nappy-treated samples consisted of only 11 differentially abundant bacteria compared to the 22 in the untreated samples (Figure 4.6G). Additionally, in the SENN phthalic acid-treated samples, 11 bacterial genera were differentially abundant compared to the 8 in the untreated samples (Figure 4.6D). The phthalic acid-treated SENN DDT consisted of 10 differentially abundant bacteria genera compared to the 18 that were present in the untreated samples (Figure 4.6H).

Additionally, a distinction was identified between the bacterial genera preferentially associated with the plastic-treated samples compared to the untreated samples. The plastic-treated SENN

samples exhibited a preference for harbouring the bacterial genera *Cedecea* compared to SENN DDT. However, the plastic-treated SENN DDT samples favoured harbouring *Acinetobacter, Corynebacterium and Gluconobacter.* Additionally, the plastic-treated samples of both SENN and SENN DDT exhibited a preference for harbouring *Asaia Enterococcus, Rahnella*, *Staphylococcus* and *Streptococcus* compared to the untreated samples. Furthermore, plastic-degrading, pesticide-degrading and *Plasmodium*-protective bacterial genera were identified within the plastic-treated samples and will be further discussed in section 4.4.

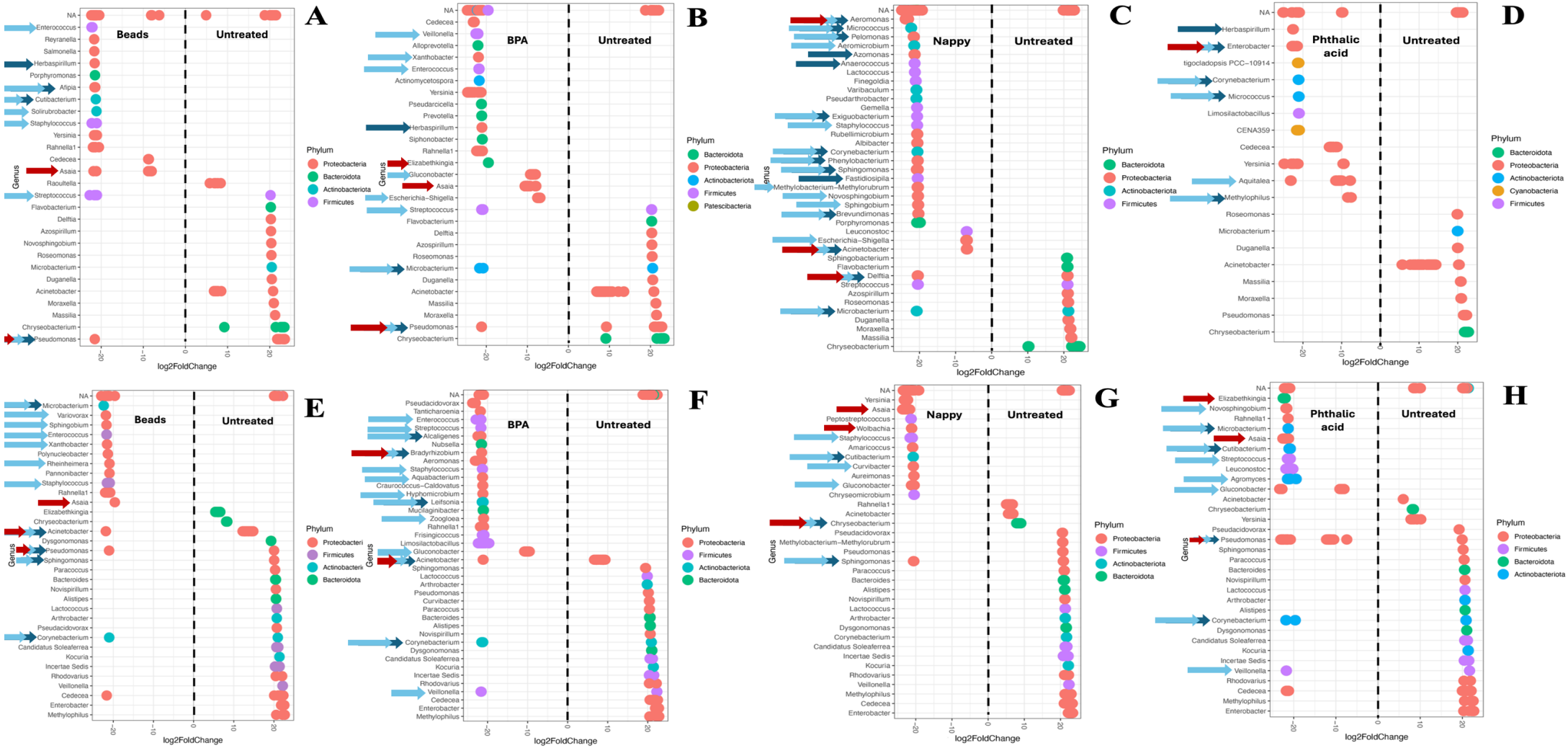


Figure 4.6. Differentially abundant bacterial genera in *An. arabiensis* reared in four different plastic-treated waters. A) Comparison of beads-treated (left) and untreated (right) SENN. B) Comparison of BPA-treated (left) and untreated (right) SENN. C) Comparison of nappy-treated (left) and untreated (right) SENN. D) Comparison of phthalic acid-treated (left) and untreated (right) SENN. E) Comparison of beads-treated (left) and untreated (right) SENN DDT. F) Comparison of BPA-treated (left) and untreated (right) SENN DDT. G) Comparison of nappy-treated (left) and untreated (right) SENN DDT. H) Comparison of phthalic acid-treated (left) and untreated (right) SENN DDT. Dark blue arrows indicate pesticide-degrading bacterial genera. Light blue arrows indicate potentially plastic-degrading bacteria genera. Red arrows indicate *Plasmodium*-protective bacteria genera. Numbers on either side of 0 represent a log2fold change of bacterial genera that are significantly different at α=0.01. Each dot represents a single OTU.

4.4. Discussion

Over the last few years, there have been great strides in documenting the impact of plastic pollution on freshwater organisms, particularly invertebrates. However, studies that have looked at the impact on mosquito populations have focused on *Aedes* and *Culex* mosquitoes (Al-Jaibachi *et al.*, 2018, 2019; Edwards *et al.*, 2023; Li *et al.*, 2024a; McConnel *et al.*, 2024). To the best of our knowledge, there are currently no studies examining the effect of plastic pollution on *Anopheles*. Additionally, these studies document the effect of MPs alone. However, the problem with plastic pollution is more than just MPs but rather the cumulative effects of the plastic fragments, plastic additives (i.e. plasticisers) and the plastic themselves acting as vectors for other pollutants. Therefore, to address the lack of information, this study focused on the major malaria vector *An. arabiensis*. Additionally, this study examined a representative of MP particles (latex beads), plastic additives (BPA, phthalic acid) and a representative of degraded common plastics (disposable nappies). Therefore, this allowed for a more holistic view of plastic pollution's impact on the midgut microbiota of *An. arabiensis*.

Given that the two laboratory strains of *An. arabiensis* used was an insecticide-selected strain with a distinct resistant phenotype (SENN DDT) and an unselected strain (SENN), it, therefore, allowed for the consideration of the insecticide-resistant phenotype. In previous studies, it has been shown that the insecticide resistance phenotype can affect the overall gut microbial composition (Dada *et al.*, 2018; Barnard *et al.*, 2019; Singh *et al.*, 2022a). In this study, it was observed that different plastic treatments caused varying effects on the midgut microbiota of the two mosquito strains. These findings are consistent with prior research examining the impact of pollution on these strains.

The alpha diversity analysis revealed that in SENN the only differences were seen between specific plastic treatments and the untreated samples. The nappy and beads treatments in SENN caused a significant increase in species richness, whereas the beads and BPA treatments caused a greater species diversity. However, in SENN DDT the differences in alpha diversity were more varied. A comparison of the species richness indicated that there was no difference between the untreated and the plastic-treated SENN DDT. Rather, differences were seen between the plastic treatments themselves. The nappy-treated SENN DDT samples, in particular, had greater species richness than the other plastic treatments. Species diversity indicated that in SENN DDT the BPA-treated samples resulted in a greater species diversity

than the untreated and beads treatments. There are conflicting results in the literature with regard to the impact that MPs have on the bacterial diversity of the midgut microbiota. Some studies reported no significant difference in alpha diversity (Shi *et al.*, 2024). The study by Edwards *et al.* (2023) indicated that MP treatment did not cause an overall difference in alpha diversity in both *Ae. aegypti* and *Ae. albopictus*. Conversely, other studies have suggested a reduction in alpha diversity (Qiao *et al.*, 2019b; Hou *et al.*, 2022; Wang *et al.*, 2022b). However, other studies have suggested an increase in alpha diversity (Huang *et al.*, 2022; Hu *et al.*, 2022; Fackelmann *et al.*, 2023). A higher diversity was observed in the 100,000 and 10,000 MP/ml treatments when compared to the untreated control in *Ae. aegypti* (Edwards *et al.*, 2023). Therefore, the increase in species richness caused by the nappy treatment in both SENN and SENN DDT may correlate with what was observed in *Aedes*.

Similarly, the different plastic treatments had a significant effect on the beta diversity, and this was consistent with what was observed in Edwards *et al*. (2023). The authors proposed that the dominance of *Wolbachia* and *Elizabethkingia* within the samples may have influenced the overall diversity observed (Edwards *et al.*, 2023). This could be the case in the present study due to the increased abundance of *Elizabethkingia* observed in the plastic-treated samples. However, the differences in beta diversity observed in the present study were not strain-specific. This observation highlights the possibility that the insecticide-resistant phenotype plays a limited role in influencing the changes to diversity after exposure to plastics. Additionally, the present study is more in line with the studies that showed an increase in bacterial diversity after plastic exposure. A plausible explanation is that upon MP ingestion, the plastisphere plays a role in increasing the microbial communities present in the gut microbiota (Huang *et al.*, 2022; Hu *et al.*, 2022; Fackelmann *et al.*, 2023). Therefore, it is possible that the nappy fragments served as a surface for bacterial growth thus resulting in an increase in species richness within the midgut microbiota. Additionally, BPA is a solid that is poorly soluble in water (Sun *et al.*, 2020). Therefore, as a poorly soluble solid, BPA could be acting as another source of a plastisphere for consumption like the breakdown products of nappies.

When examining the top five ranked genera, it was clear that the untreated samples varied from the plastic-treated samples. This was consistent in both strains. Interestingly, in both strains, the bead and BPA-treated samples were similar in terms of the relatively abundant bacteria. Additionally, the relative abundance of the nappy and phthalic acid treatments was also similar

to each other. In both strains, the dominant genera in the plastic-treated samples were *Rahnella* and *Elizabethkingia* whereas *Acinetobacter* was dominant in the untreated controls. The increased abundance of *Elizabethkingia* observed in this study corresponds to *Elizabethkingia* demonstrated as more abundant in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* after MP ingestion (Edwards *et al.*, 2023; Li *et al.*, 2024a). This is notable as *Elizabethkingia* forms biofilms on plastic surfaces (Jacobs and Chenia, 2011; Tang *et al.*, 2021). Therefore, the increase in abundance of *Elizabethkingia* in the gut microbiota may be a result of the microbial communities present on the surface of plastics. Therefore, this may explain the increase in *Elizabethkingia* observed in the plastic-treated samples regardless of the strain. Additionally, the increased abundance of *Elizabethkingia* after plastic exposure may be of particular interest as *Elizabethkingia anophelis* and *Elizabethkingia meningoseptica* have been shown to negatively impact the development of *Plasmodium* in *Anopheles* mosquitoes (Boissière *et al.*, 2012; Ngwa *et al.*, 2013; Bahia *et al.*, 2014). Therefore, the increase in the abundance of *Elizabethkingia* after plastic exposure indicates that plastic exposure may indirectly influence the transmission of malaria.

Analysis of the bacterial genera and species overlap revealed strain-specific differences. In SENN, the nappy-treated samples had the highest number of unique genera and species, followed by the phthalic acid-treated, BPA-treated, untreated and beads-treated samples. Conversely, in SENN DDT the untreated samples had the highest number of unique genera and species followed by phthalic acid-treated, BPA-treated, nappy-treated and beads-treated samples. This is significant as it indicates that the insecticide-resistant phenotype influences the specific genera and species present rather than alpha and beta diversity. Additionally, fewer bacterial genera and species were shared between the treatments compared to the number that was unique to specific treatments. This implies that the core microbiota was not influenced by the plastic treatments, but rather new bacterial species were acquired. This is similar to what was observed in *Ae. aegypti* and *Ae. albopictus* (Edwards *et al.*, 2023). Moreover, this further emphasises the impact of the plastisphere on the mosquito midgut microbiota.

When analysing the differential abundance, a clear pattern was emerging which further highlighted the influence of the insecticide-resistant phenotype. In the unselected SENN strain, the plastic treatments resulted in more differentially abundant genera than the untreated samples. However, in the insecticide-selected SENN DDT, the untreated samples had more differentially abundant genera compared to the plastic-treated samples. This is consistent with

what was observed in a study that examined the effects of SENN and SENN DDT larval exposure to metal-treated water (Singh *et al.*, 2024). The insecticide-resistant phenotype has been shown to assist mosquitoes with better survivorship when exposed to toxic pollutants. SENN DDT has been shown to cope better than SENN when exposed to toxic pollutants such as heavy metals and herbicides (Oliver and Brooke, 2018a, 2018b). This is due to the increase in detoxification enzymes present in SENN DDT. Therefore, the detoxification enzymes may assist SENN DDT in withstanding plastic treatments. Additionally, an increased abundance of pesticide and plastic-degrading bacteria was seen in the plastic-treated SENN samples. Therefore, plastic exposure may cause an increase in these bacteria genera to assist with managing the resultant effects. Thus, the finding suggests that bacteria could play a role in protecting the larvae from toxic substances as this effect carries over to the adult.

In honeybees, MP ingestion resulted in the accumulation of the plastic in the gut (Wang *et al.*, 2021b). The authors postulated that bacteria present in the gut could facilitate the degradation of MPs and that these bacteria possess specific enzymes for MP degradation (Wang *et al.*, 2021b). In the present study, it was observed that plastic treatments resulted in an increased abundance of bacteria with the potential to degrade MPs. It is important to highlight that the nappy treatment in SENN resulted in the most plastic-degrading bacteria. The most common potential plastic-degrading bacteria were *Acinetobacter*, *Corynebacterium*, *Cutibacterium*, *Enterococcus*, *Gluconobacter*, *Microbacterium*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus*, *Veillonella*.

Acinetobacter and *Sphingomonas* have been implicated in the degradation of polyester, polyethylene terephthalate (PET), polyvinyl and polyhydroxybutyrate (PHB) (Debroas *et al.*, 2017). Additionally, *Acinetobacter* has been seen to degrade untreated polyethylene (PE) (Mohanan *et al.*, 2020) and polystyrene (PS) (Gutierrez, 2019). *Corynebacterium* was found to encode for PETase, which degrades hydrocarbons as well as possesses genes associated with polystyrene degradation (McGowan *et al.*, 2004; Sun *et al.*, 2022a; Helleckes *et al.*, 2023). *Cutibacterium* showed high poly(3-hydroxybutyrate) depolymerase activity and is a hydrocarbon-degrading bacteria (Park *et al.*, 2021; Wang *et al.*, 2023). *Enterococcus* is a potential PS-degrading bacterium (Hou and Majumder, 2021). *Microbacterium* possess genes associated with PS degradation (Sun *et al.*, 2022a). Microbial degradation of untreated PE, low-density PE (LDPE) and the partial degradation of a wide range of petro-plastics including PS, polypropylene (PP), PVC, PET and ester-based polyurethane (PU) has been reported in

Pseudomonas sp (Wilkes and Aristilde, 2017; Mohanan *et al.*, 2020; Nadeem *et al.*, 2021). *Pseudomonas* has also been associated with metabolising BPA as a carbon energy source (Zaborowska *et al.*, 2021). Additionally, BPA promotes the development of *Pseudomonas sp* (Zaborowska *et al.*, 2021). *Staphylococcus* is involved in the degradation of polyester, PET, polyvinyl and PHB (Debroas *et al.*, 2017) as well as PE and PU (Gambarini *et al.*, 2022). *Streptococcus* was seen to degrade PE and encode genes that are associated with PS degradation (Venkatesh *et al.*, 2021; Gambarini *et al.*, 2022; Sun *et al.*, 2022a). Lastly, *Veillonella* is a potential plastic-degrading bacterium (Zhu *et al.*, 2023). Therefore, considering the composition of the disposable nappies mentioned in section 2.3, it is not surprising that an increase in the abundance of plastic-degrading bacteria in the nappy-treated samples was observed. The majority of the plastic components of the nappy have been shown to possibly be degraded by the common plastic-degrading bacteria.

In addition to the increased abundance of plastic-degrading bacteria, an increase in pesticide-degrading bacteria was seen in the plastic treatments. This was also found in the study looking at the effect of SENN and SENN DDT larval exposure to metal-treated water, where an increase in pesticide-degrading bacterial genera in the strains reared in metal-polluted water was found (Singh *et al.*, 2024). This was also observed in the F1 offspring of wild *An. arabiensis,* indicating that the increased effect was present in wild mosquitoes as well (Singh *et al.*, 2024). In the present study, the most common pesticide-degrading genera were *Acinetobacter* (Wang *et al.*, 2021a), *Cutibacterium* (Muturi *et al.*, 2021), *Herbaspirillum* (Venkatachalam *et al.*, 2023), *Microbacterium* (Almeida *et al.*, 2017), *Micrococcus* (Dar and Kaushik, 2022), *Pseudomonas* (Dada *et al.*, 2019; Wang *et al.*, 2021c; Gómez-Govea *et al.*, 2022) and *Sphingomonas* (Lv *et al.*, 2023). Unlike what was seen in terms of the plastic-degrading bacteria, the BPA-treated SENN samples resulted in the most pesticide-degrading bacteria genera. However, it should be noted that there is a clear overlap in terms of bacteria that are considered plastic-degrading and pesticide-degrading. The increase in pesticide-degrading bacteria after the plastic treatment adds to the argument that pollutant exposure could potentially promote insecticide resistance.

Another factor to consider is the effect of plastic pollution on vector competence. Changes to the midgut microbial composition can factor into the vector competence of a species. The presence of *Plasmodium*-protective bacteria prevents transmission by reducing the development of the parasite. Therefore, an increased presence of *Plasmodium*-protective

bacteria potentially correlates with a reduction in malaria transmission. However, examination of the differential abundance indicated that there were seven bacterial genera that are considered *Plasmodium*-protective present in both strains. Notably, the *Plasmodium* protective bacteria *Asaia* was identified to be predominantly abundant within the plastic-treated samples. However, due to the small-scale changes to the abundance of *Plasmodium*-protective bacteria, no clear conclusion was drawn on whether plastic treatment has any significant impact on vector competence. Hence, there is no definite answer as to whether plastic exposure can alter the microbial composition leading to direct alterations to the capacity to alter *Plasmodium* transmission (Jones *et al.*, 2024). Therefore, further investigation into the effect of plastic pollution on vector competence needs to be undertaken.

Previous studies have indicated that MP exposure can lead to intestinal damage (Lei *et al.*, 2018b, 2018a) and stimulate intestinal inflammation (Jin *et al.*, 2018). Studies on zebrafish have proven that MP ingestion results in gut inflammation (Jin *et al.*, 2018; Qiao *et al.*, 2019a). The studies on zebrafish have shown that MP ingestion injured the gut epithelium, increasing intestinal permeability, which subsequently led to an inflammatory response (Qiao *et al.*, 2019a). Studies have also proven that MP exposure causes the generation of ROS and acts as an oxidative stress trigger, initiating and exacerbating the inflammatory responses (von Moos *et al.*, 2012; Jeong *et al.*, 2017). In insects, inflammatory responses are part of innate immunity (Chain and Anderson, 1983). It was theorised that the ingestion and accumulation of MPs in the gut caused damage to the gut epithelium, therefore triggering the activation of phenoloxidase (Silva *et al.*, 2021a). Additionally, MP ingestion in the harlequin fly (*Chironomus riparius*) larvae and the blackworm (*Lumbriculus variegatus*) has been shown to induce oxidative stress, resulting in inflammation and activation of immune responses (Silva *et al.*, 2021c, 2021b). In honeybees, MP ingestion induced gut microbiota dysbiosis, oxidative stress and irregular gene expression in the gut (Wang *et al.*, 2021b). Analysis of the differential abundance in the present study revealed that several bacteria associated with inflammatory responses were abundant after plastic treatment. The most common genera of these bacteria present in the treatments were *Acinetobacter* (Muhammad *et al.*, 2024), *Aeromonas* (Song *et al.*, 2014; Zheng *et al.*, 2022), *Corynebacterium* (Burkovski, 2018; Li *et al.*, 2024b), *Cutibacterium* (Dagnelie *et al.*, 2022; Noh *et al.*, 2022; Li *et al.*, 2024c), *Enterococcus* (Liang *et al.*, 2022), *Gluconobacter* (Kosakamoto *et al.*, 2020; Yamashita *et al.*, 2021), *Pseudomonas* (de Deus *et al.*, 2024), *Sphingomonas* (Higuchi *et al.*, 2021), *Staphylococcus* (Li *et al.*, 2024b), *Streptococcus* (Zhu *et al.*, 2024) and *Veillonella* (Rai *et al.*, 2021). As such, there is a precedent

in insects for MP exposure to modulate the immune system through alterations to the microbiota.

Gluconobacter is particularly interesting in terms of inflammation and immunity in insects. In *Drosophila*, larval Imd activation results in increased *Gluconobacter* prevalence in the gut microbiota and this is transferred to adults (Yamashita *et al.*, 2021). Both the Imd pathway and the Toll pathway can increase the prevalence of *Gluconobacter* in the gut (Kosakamoto *et al.*, 2020). Additionally, *Gluconobacter* works in a positive feedback loop. The increase in *Gluconobacter* stimulates the Imd pathway, which induces *Gluconobacter* proliferation (Kosakamoto *et al.*, 2020). The Imd pathway is an important immune pathway in mosquitoes. It is particularly crucial as this is the most efficient immune pathway against *Plasmodium* infection (Garver *et al.*, 2012; Blumberg *et al.*, 2013). Therefore, *Gluconobacter* potentially plays a role in mosquito immune responses. Subsequently, the proliferation of *Gluconobacter* in the midgut may play a role in the *An. arabiensis* capacity to transmit malaria. An increase in *Gluconobacter* within the mosquito midgut microbiota could increase stimulation of the Imd pathway, which in turn results in a greater immune response in mosquitoes. This, in turn, will increase the likelihood of the elimination of the malaria parasite within the midgut, which is a positive outcome in terms of malaria elimination. Therefore, if inflammatory bacteria that induce immune responses are more prevalent in plastic-treated mosquitoes, this could potentially influence the capacity to transmit parasites. In the present study. *Gluconobacter* was differentially abundant within the plastic-treated samples, particularly BPA-treated SENN and SENN DDT, nappy-treated SENN DDT and phthalic acid-treated SENN DDT. Therefore, the greater abundance of *Gluconobacter* in *An. arabiensis* exposed to these plastics may influence the capacity to transmit malaria.

Another significant finding needs to be highlighted. The differential abundance analysis for the nappy-treated SENN DDT samples indicated the abundance of *Wolbachia* and *Asaia*. This is an uncommon finding as generally, *Anopheles* mosquitoes are not considered natural hosts for *Wolbachia* (Kittayapong *et al.*, 2000; Chrostek and Gerth, 2019). Additionally, the abundance of both *Asaia* and *Wolbachia* in a sample population is uncommon. *Asaia* is known to impede the colonisation of *Wolbachia* since they compete for the same tissue niches (Hughes *et al.*, 2014; Rossi *et al.*, 2015). However, this finding may have positive implications, as both *Asaia* and *Wolbachia* possess characteristics that influence vector competence. Previous studies have demonstrated the application of strains of *Wolbachia* within field samples to inhibit the

transmission of dengue virus by *Aedes aegypti* (Moreira *et al.*, 2009; Frentiu *et al.*, 2014). With regards to malaria, *Wolbachia* has been shown to decrease the amount of sporozoites within the mosquito, thus impeding the transmission of malaria (Gomes *et al.*, 2017). Additionally, in *An. stephensi*, *Wolbachia* was observed to modulate the immunity and, as such, decrease the mosquito's susceptibility to *Plasmodium* (Vandana *et al.*, 2024).

Similarly, *Asaia* is considered a promising paratrasngenesis candidate due to its ability to colonise the reproductive tissues, salivary glands and the midgut as well as their vertical and horizontal transmission within the mosquito population (Favia *et al.*, 2007; Damiani *et al.*, 2010; Mancini *et al.*, 2016). Moreover, *Asaia* has been shown to exhibit anti-*Plasmodium* properties (Tatsinkou Maffo *et al.*, 2024). Previous studies have demonstrated that under experimental conditions, *Asaia* can stimulate the immune response of mosquitoes against parasite infection. *Asaia* acts as an immune modulator, upregulating immune genes and activating the expression of antimicrobial peptides without triggering an innate immune response (Capone *et al.*, 2013). Additionally, research has demonstrated the effectiveness of genetically modified *Asaia* strains, attributed to their capacity to express anti-*Plasmodium* proteins. This expression is driven by a promoter that is activated following a blood meal (Mancini *et al.*, 2016; Shane *et al.*, 2018). The identification of both bacterial genera constitutes a significant finding, especially considering their differential abundance in the nappy-treated SENN DDT. Notably, both bacterial genera exhibit strong anti-*Plasmodium* properties, positioning them as promising paratransgenic candidates. However, further validation of these observations is necessary to establish their significance.

In conclusion, larval exposure to plastic treatments significantly impacted the microbiota of adult *An. arabiensis*. The phenotype associated with insecticide resistance influenced bacterial abundance patterns in mosquito adults. Notably, the insecticide-unselected strain exhibited a higher abundance of bacteria in response to degraded plastic, while the insecticide-selected strain demonstrated a greater variety of genera in the untreated group. This suggests that, in the absence of metabolic activity linked to insecticide resistance, an increased bacterial abundance may help protect mosquitoes from the adverse effects of plastic treatments. Furthermore, larval exposure resulted in a higher abundance of genera capable of degrading plastic and pesticides. There was also a notable presence of bacterial genera associated with inflammation in the plastic treatments; however, no large-scale differences in *Plasmodium*-protective genera were observed. These findings indicate that larval exposure to plastic and its additives can alter

microbial composition in ways that may have epidemiological implications. It is important to note, however, that this study focused on laboratory strains, underscoring the need for further research into the effects of plastic pollutants on wild populations.

Chapter 5: Characterising the transgenerational effects of larval plastic exposure on *Anopheles arabiensis*

5.1. Introduction

Epigenetics refers to the heritable changes in gene expression that do not involve alterations to the DNA sequence itself (Waddlngton, 1942). Epigenetics is a continuously expanding field of study that is key to understanding how organisms adapt to their environments. Environmental factors such as temperature changes, diet and exposure to pollutants have been shown to induce epigenetic changes, hence altering the epigenome (Baccarelli and Bollati, 2009; Weyrich *et al.*, 2018). These alterations may result in advantageous changes gene expression, which can be beneficial as it allows for adaptation to changing environments (Flores *et al.*, 2013) or cause detrimental phenotypic effects (Šrut, 2021). Additionally, the mechanisms underlying epigenetics may moderate the effects of toxic chemicals and substances (Baccarelli and Bollati, 2009). An important consideration is that the epigenetic changes can persist and are heritable. Therefore, these changes can be transferred to subsequent generations (Mirbahai and Chipman, 2014). However, there is a need to understand the underlying mechanisms that allow environmental stressors to induce epigenetic changes.

Plastic pollution is an emerging major environmental concern, affecting ecosystems and human health globally. Microplastic pollution, particularly, is a significant threat to aquatic ecosystems due to its prevalence and impact on organisms (Reid *et al.*, 2019; Corinaldesi *et al.*, 2021). The ingestion of MPs by organisms can lead to potential toxicological effects. Studies have shown that exposure to MPs causes an increase in oxidative stress, developmental toxicity and negative reproductive effects (Besseling *et al.*, 2013; Barboza *et al.*, 2020; Bhagat *et al.*, 2020; Choi *et al.*, 2020). Beyond the physical and chemical toxicity, studies have begun exploring the effect of MPs on molecular processes and gene regulation, particularly epigenetic changes (Patra *et al.*, 2022). Most studies on epigenetic mechanisms have focused on vertebrates, primarily on human health and less on the implications on invertebrate species (Jeremias *et al.*, 2020). Understanding the relationship between plastic exposure and the resultant epigenetic changes allows for a better understanding of the underlying mechanisms governing phenotypic and overall population changes in a species (Vandegehuchte and Janssen, 2014). In mosquitoes, epigenetic changes have been shown to play a role in cold temperature and insecticide tolerance in *Aedes albopictus* (Oppold *et al.*, 2015; Kreß *et al.*,

2017). In *An. gambiae*, it was determined that *Plasmodium* infection-induced chromatin changes (Ruiz *et al.*, 2019). Therefore, in order to better understand the effects of plastic pollution on *An. arabiensis*, a deeper understanding of the epigenetic changes that plastic may induce is vital. The two key mechanisms of epigenetic regulation in mosquitoes are DNA methylation and histone modifications (Dupont *et al.*, 2009; Gibney and Nolan, 2010).

5.1.1. DNA Methylation

DNA methylation involves the reversible covalent modification of DNA at the 5′ position of cytosine residues, therefore resulting in 5-methyl-cytosine (5mC) (Newell-Price *et al.*, 2000). In mammals, this modification typically occurs next to a guanine nucleotide that is separated by a phosphodiester bond linking cytosine and guanine-containing nucleotides and is referred to as CpG (Razin and Cedar, 1991; Weber and Schübeler, 2007). The addition of a methyl group causes changes to the biophysical characteristics of DNA (Gibney and Nolan, 2010). The addition of methyl groups can inhibit the recognition of DNA by some proteins, or they can aid the binding of proteins to the DNA (Prokhortchouk and Defossez, 2008). DNA methylation of a promoter region facilitates the binding of gene-repressor proteins that induce heterochromatin (tightly coiled DNA) formation (Grewal and Moazed, 2003; Goll and Bestor, 2005). Transcription factors bind to unmethylated regions, which causes the remodelling of chromatin structures, therefore leading to gene expression and thus preventing methylation occurring in that region (Li and Zhang, 2014).

Different types of DNA methylation can occur. In addition to 5mC methylation, 5-hydroxymethyl cytosine (5hmC) can occur (Li and Zhang, 2014). The removal of a methyl group by the ten-eleven translocation (TET) dioxygenase enzymes results in the oxidisation of 5mC nucleotides to 5hmC (Tahiliani *et al.*, 2009; Williams *et al.*, 2011). Therefore, 5hmC acts in an opposite manner than 5mC. The removal of a methyl group from a promoter region results in increased gene expression due to the formation of euchromatin (lightly packed DNA) (Kangaspeska *et al.*, 2008; Guo *et al.*, 2011; Mellén *et al.*, 2017).

While DNA methylation is a well-studied epigenetic mechanism in mammals, its role in insects has been less defined, with studies suggesting significant variation across species. Unlike in mammals, the genome of invertebrates is sparsely methylated and rather presents with an assortment of methylation patterns (de Mendoza *et al.*, 2019; Šrut, 2021). The DNA

methylation present in invertebrates is restricted to the transcriptional regions or gene bodies (de Mendoza *et al.*, 2019). Low levels of DNA methylation were seen in *Caenorhabditis elegans* and dipteran insects (Mukherjee *et al.*, 2015; Schübeler, 2015; Noordhoek *et al.*, 2018). In mammals, four DNA methyltransferases are present: DNMT1, DNMT2, DNMT3a, and DNMT3b (Robertson *et al.*, 1999). However, in *Bombyx mori* (silkworms), *Tribolium castaneum* (beetle), and *Schistocerca gregaria* (locust), only DNMT1 are present (Xu *et al.*, 2021). Additionally, dipteran species such as *Drosophila melanogaster* and *An. gambiae* do not have DNMT1 or DNMT3 (Zemach *et al.*, 2010). In a study on *Nilaparvata lugens* (brown planthopper), it was seen that the DNA methylation patterns vary depending on environmental signals and insect life stage (Gupta and Nair, 2022). In the examination of the methylation patterns in species of the *An. gambiae* complex, it was seen that there were fundamental species-specific variations in the level of methylation, and this was consistent post immune stimulation as well (Patel *et al.*, 2024).

Determining the changes in methylation patterns across organisms, particularly mosquito species, can have major implications for understanding the effects of environmental stressors such as chronic exposure to pollutants (Weinhold, 2006). Significantly, in a study examining the effect of larval exposure to heavy metals on the epigenetic architecture of *An. arabiensis*, altered DNA methylation patterns were observed in the adults (Jeanrenaud *et al.*, 2022). The study observed that in the insecticide-selected strain of *An. arabiensis* (SENN DDT), a decrease in 5mC and an increase in 5hmC were present. Therefore, suggesting larval exposure to heavy metals increased gene expression (Jeanrenaud *et al.*, 2022). However, the aforementioned study demonstrated that in *An. arabiensis*, histone acetyltransferase activity was more responsive to larval metal exposure. Furthermore, the lack of methylation and a study showing a lack of activity of DNA methylating enzymes in *An. gambiae s.s*. (Djihinto *et al.*, 2023) suggests that DNA methylation plays a minor role compared to histone modifications.

5.1.2. Histone modifications

In addition to the covalent modifications of DNA, histones and their posttranslational modifications have also been implicated in the chromatin structure and regulation of gene expression (Dupont *et al.*, 2009). Histone proteins are important for the packaging of DNA within the nucleus. DNA in eukaryotic cells is packaged into a highly organised and compact structure known as chromatin. The chromatin is made of the nucleosome, which consists of

DNA that is wound around an octamer of histone proteins. Each octamer consists of a two-unit central heterotetramer consisting of histones H3 and H4 and a heterodimer consisting of histones H2A and H2B (Kornberg, 1974). The linker DNA, folded DNA and histones are held together with the linker histone H1. The histone-modifying enzymes are responsible for histone post-translation modifications that affect the histone tails and include methylation, acetylation, phosphorylation and ubiquitination (Kouzarides, 2007; Marmorstein and Trievel, 2009). Histone modifications impact gene expression, DNA repair, DNA replication and chromatin folding due to alteration of the DNA-histone interaction in the nucleosome (Esteller, 2008; Alaskhar Alhamwe *et al.*, 2018).

The most well-studied histone modification is acetylation and methylation of lysine (K) residues on histones H3 and H4. Histone acetylation is generally associated with transcriptional activation, whereas deacetylation is correlated with transcriptional repression (Hamilton, 2011). Histone acetylation weakens the DNA-histone interactions, therefore changing the chromatin structure to euchromatin, hence enabling transcription (Gibney and Nolan, 2010; Bannister and Kouzarides, 2011). Histone methylation is a more complex process as it can result in both transcription activation and repression (Esteller, 2008; Bannister and Kouzarides, 2011). There is less information regarding the effects of histone phosphorylation. What is known is that histone phosphorylation encompasses the addition of a negatively charged phosphate group to the histone tail (Rossetto *et al.*, 2012). Another type of histone modification is histone ubiquitylation. This histone modification involves the addition of large ubiquitin molecules to lysine residues, which can result in gene silencing and transcriptional activation (Bannister and Kouzarides, 2011). Additionally, it is important to consider the effect of mutations on histones and what this means for gene expression. For example, the mutation of H2A and H2B sites can alter the transcription of various genes (Parra *et al.*, 2006; Parra and Wyrick, 2007).

Histone modifications are catalysed by enzyme activity. The three primary enzymes responsible for these modifications are histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases (HMTs). Histone acetylation is controlled by HATs and HDACs. HATs catalyse the addition of an acetyl group to the N-terminal of a lysine residue in the histone tail. This results in reducing the strength of the bond between the histone and DNA, therefore resulting in the euchromatin formation and transcriptional activation (Fierz and Muir, 2012; Swygert and Peterson, 2014; Alaskhar Alhamwe *et al.*, 2018). Conversely,

histone deacetylation occurs via HDACs, which removes the acetyl group, resulting in the repression of gene expression (Ceccacci and Minucci, 2016; Alaskhar Alhamwe *et al.*, 2018). Histone methylation is catalysed by the addition of a methyl group by HMTs (Rea *et al.*, 2000; Bedford and Clarke, 2009). Unlike in histone acetylation, the addition of a methyl group results in the blocking of binding of transcriptional factors and regulatory proteins employing steric hindrance (Bannister and Kouzarides, 2011).

In insects, histone modifications are associated with processes such as development, behaviour and responses to environmental stressors (Simola *et al.*, 2016; Olivares-Castro *et al.*, 2021; Palli, 2021). Histone acetylation has been shown to influence the caste development between worker and soldier ants (Simola *et al.*, 2016). Additionally, histone acetylation has been linked to the regulation of gene expression during insect diapause. Diapause is a form of dormancy that allows insects to survive unfavourable environmental conditions. Epigenetic regulation, particularly through histone modifications, is critical for the induction and maintenance of diapause. In the beet webworm (*Loxostege sticticalis* L.), it was determined that HDAC was involved in diapause by altering the juvenile hormone levels (Cui *et al.*, 2022a). Additionally, in a study on the flesh fly (*Sarcophaga bullata*), histone deacetylation was proposed as a regulator of diapause (Reynolds *et al.*, 2016). In *Culex pipiens*, it was observed that in the fat body, the H3K27me2 levels decrease in diapausing mosquitoes relative to non-diapausing mosquitoes (Wei *et al.*, 2024).

In the study on species from the *An. gambiae* complex, it was determined that there was no significant difference in HAT activity between the four species (Patel *et al.*, 2024). However, upon blood feeding and immune stimulation, a decrease in HAT activity was observed (Patel *et al.*, 2024). In *An. arabiensis*, larval metal exposure resulted in noticeable changes to HAT activity in the adults. It was determined that in the insecticide selected *An. arabiensis* strain, an increase in HAT activity was observed, whereas in the insecticide unselected strain, a decrease in HAT activity was seen (Jeanrenaud *et al.*, 2022). Additionally, the authors noted that in the insecticide-selected strain, an increase in HAT activity corresponded to a decrease in 5mC methylation and a decrease in HAT activity related to an increase in 5mC (Jeanrenaud *et al.*, 2022). The aforementioned studies demonstrated that HAT activity exhibits significantly heightened responsiveness to environmental stimuli, as observed in larval exposure to metals, immune stimulation and blood feeding. This, therefore, underscores the importance of investigating the effects of larval exposure on HAT activity rather than methylation activity.

5.1.3. Epigenetics and plastic exposure

To date, very few studies have investigated the epigenetic effects of exposure to plastic pollutants. Moreover, even fewer studies have investigated the epigenetic effects of plastic pollution on insects. In a study on male rats, it was determined that polyethylene MP exposure resulted in an increase in DNA methylation in a dose-dependent manner (Farag *et al.*, 2023). However, in *Daphnia magna*, exposure to polyethylene MPs resulted in no marked effect on global DNA methylation (Song *et al.*, 2022a). The majority of studies on plastic pollution and epigenetics have focused on endocrine-disrupting chemicals present in plastic, such as BPA and phthalates. These chemicals can interfere with hormonal signalling pathways and have been shown to induce epigenetic changes. In zebrafish (*Danio rerio*), BPA was seen to cause a reduced expression of DNMT1, thus resulting in a decrease in the global methylation DNA patterns (Laing *et al.*, 2016). Studies have shown that BPA exposure can cause alterations in DNA methylation patterns, histone modifications and miRNA expression (Senyildiz *et al.*, 2017; Cheong *et al.*, 2018; Cariati *et al.*, 2020; Qin *et al.*, 2021). Research has proven that exposure to phthalates can lead to epigenetic changes by altering DNA methylation and histone acetylation. A study on rats that were exposed to di-(2-ethtlhexyl) phthalate (DEHP) linked changes in DNA methylation patterns in genes related to reproductive health in male rats (Sekaran and Jagadeesan, 2015). Similar studies found that prenatal exposure to phthalates could lead to altered DNA methylation in the placenta and potentially affect foetal development (Martinez-Arguelles *et al.*, 2009; Manikkam *et al.*, 2013).

It is important to note that these epigenetic modifications can persist through subsequent generations. In *D. magna,* exposure to MPs in the parent generation resulted in transgenerational effects on growth and reproduction (Song *et al.*, 2022a). Similarly, studies on aquatic organisms such as intertidal barnacles (*Amphibalanus amphitrite*), oysters (*Crassostrea gigas*) and freshwater prawns have demonstrated that parental MP exposure resulted in decreased survival and increased developmental rates, alterations to immunity, and reduced locomotor activity in the subsequent generations (Yu and Chan, 2020; Bringer *et al.*, 2022; Sun *et al.*, 2022b). These studies highlight the significance of generational impacts due to MP exposure. However, there is a lack of information regarding the mechanisms underlying these changes. It is proposed that investigation into the MP-induced epigenetic changes will help elucidate the mechanisms of the transgenerational effects of plastic pollutants.

Notably, the interaction between plastic pollution and epigenetics is an emerging area of research that has critical implications for understanding the long-term health effects of plastic-associated chemicals and particles. Although research on the direct epigenetic effects of MPs is still limited, existing evidence suggests that MP exposure may also influence gene regulation. However, there is a severe lack of information regarding the epigenetic effects of plastic pollutants on mosquito species, particularly within malaria vector species. Therefore, this study seeks to bridge this knowledge gap. Therefore, this chapter aimed to determine the transgenerational effects of *An. arabiensis* larval exposure to plastic and plastic additives.

5.2. Methods

5.2.1. Mosquito strains

This study used insecticide-selected (SENN DDT) and unselected (SENN) laboratory strains of *An. arabiensis*. The resistance profiles of the strains are detailed in sections 2.1 and 3.3.1.

5.2.2. Tracing the fate of MPs

In order to determine the fate of the MPs in the adults, third instar SENN and SENN DDT larvae were exposed to three different sizes of commercially available fluorescently labelled latex beads. The three sizes used were 0.5 µm, 1 µm and 2 µm. The third instar larvae were placed in containers and exposed to 10 µL of the latex bead aqueous solutions per 250 mL of RO water. The larvae were fed a standardised amount of food to supplement their development. The presence of the latex beads was then examined in fourth instar larvae using a Leica fluorescent microscope (Leica M165 FC). The larvae were allowed to pupate and emerge as adults. The emergent adult midgut, salivary glands and reproductive tissues were dissected using a stereomicroscope (Olympus SZ571). The presence of the latex beads in the adult midguts, salivary glands, and reproductive tissues was determined using a fluorescent microscope (Leica M165 FC). Fluorescence of the beads were λ_{ex} ~520 nm; λ_{em} ~540 nm (0.5 µm beads), λ_{ex} ~575 nm; λ_{em} ~610 nm (1 µm beads) and λ_{ex} ~360 nm; λ_{em} ~420 nm (2 µm beads) and was detected using a Yellow-fluorescent protein (YFP) and DAPI filters. Images were captured using an iPhone XS.

5.2.3. Generation of transgenerational samples

The first (F1) generation was produced by exposing 200 first-instar SENN and SENN DDT larvae (less than 24 hours old) from the parent colony to three plastic treatments: artificially degraded nappies, BPA and phthalic acid. The larvae were reared as per the concentrations described in section 2.3. The emergent adults were placed into cages and given access to 10% sucrose. Once the adults reached three days old, a portion was collected and stored in a -80 °C freezer until required. The remaining adults were allowed to mate, and females were provided two blood meals. Two days after the final blood meal, egg plates were placed in the cages. The resultant eggs from the F1 generation were placed in untreated water. The eggs were allowed to hatch and develop. The emergent adults served as the second (F2) generation. The three-

day-old adults were collected and stored in a -80 ℃ freezer until needed. Three-day-old adults from the parent colony served as the control.

5.2.4. Histone Extraction

Before HAT activity was determined, the histone proteins needed to be extracted. Therefore, histones were extracted using the EpiXtract™ Total Histone Extraction Kit (Enzo Life Sciences, Catalogue no.: ENZ-45014). The kit exclusively isolates and extracts histones from tissues and cells by treatment with pre-lysis, lysis and balance buffers. However, the kit was designed for the extraction of histones from mammalian cells and tissues. Therefore, adjustments to the protocol were made to accommodate the mosquito specimens used. Three mosquitoes were placed in a 1.5 mL tube for each strain, treatment, sex and generation. Three samples for each treatment, generation, sex and strain, were prepared. A total of 92 samples were prepared. A total of 40 µL of the 1X Pre-lysis buffer was added to the tubes, and a homogenisation bead was added to assist with the homogenisation. Mosquitoes were homogenised using a Qiagen TissueLyser II (Qiagen, Germany) for four minutes at 25 Hz. The homogenised mixture was transferred to a new 1.5 mL tube and centrifuged at 9170 rcf for one minute at 4 ℃. The supernatant was removed, and the pellet resuspended in 120 µL of the Lysis buffer and left on ice for 30 minutes. The samples were then centrifuged for five minutes at 10950 rcf at 4 ℃. The supernatant was then transferred into a new 1.5 mL tube, and 36 µL of the Balance-DTT buffer was added. The protein concentrations were quantified using a Bradford assay (Bradford, 1976), as detailed in Appendix 3. The extracted histones were stored in a -20 ℃ freezer until needed.

5.2.5. HAT activity colorimetric assay

The histone extracts were used to determine the HAT activity using a HAT Activity Colorimetric Assay Kit (Enzo Life Science, catalogue no.: ALX-850-326). This kit uses acetyl-CoA as a cofactor. The acetylation of acetyl-CoA by active HAT releases the free form of CoA, which then serves as an essential coenzyme for producing nicotinamide adenine dinucleotide (NADH). NADH can easily be detected spectrophotometrically upon reacting with a soluble tetrazolium dye. The 50 µg of extracted histones was added to individual wells of a 96-well plate. The kit-provided nuclear extract served as the positive control, whereas RO water was used as a negative control. A 2X HAT buffer, HAT substrate I, HAT substrate II and NADH-

generating coenzyme was provided by the kit. The assay reagents were mixed and placed in the 96-well plate. The plate was incubated at 37 ℃ for 4 hours, and absorbance was read at 440 nm with a plate reader (SpectraMax® ABS Plus: Molecular Devices). HAT activity was reported as relative absorbance after the background reading was removed.

5.2.6. Statistical analysis

GraphPad Prism version 10.0.0 for macOS (GraphPad Software, Boston, Massachusetts USA) was used for statistical analysis. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was used to test for normality. Normally distributed data was analysed with a One-way ANOVA (Gamage and Weerahandi, 1998). Tukey multiple comparison test was used as a *post-hoc* analysis (Tukey, 1949). A *p*-value of less than 0.05 was considered statistically significant.

5.3.Results

5.3.1. Detection of fluorescent latex beads in *Anopheles arabiensis* larvae and adults

Based on the fluorescence results it was evident that the SENN and SENN DDT larvae readily fed on the 0.5 and 1 µm fluorescently labelled latex beads. The 0.5 µm beads were detected within the gut of the fourth instar larvae of both SENN (Figure 5.1 A) and SENN DDT (Figure 5.1 B). In the adults, the fluorescence was only detected within the testes, therefore indicating the presence of the 0.5 µm beads in the testes of SENN (Figure 5.1 C) and SENN DDT (Figure 5.1 D).

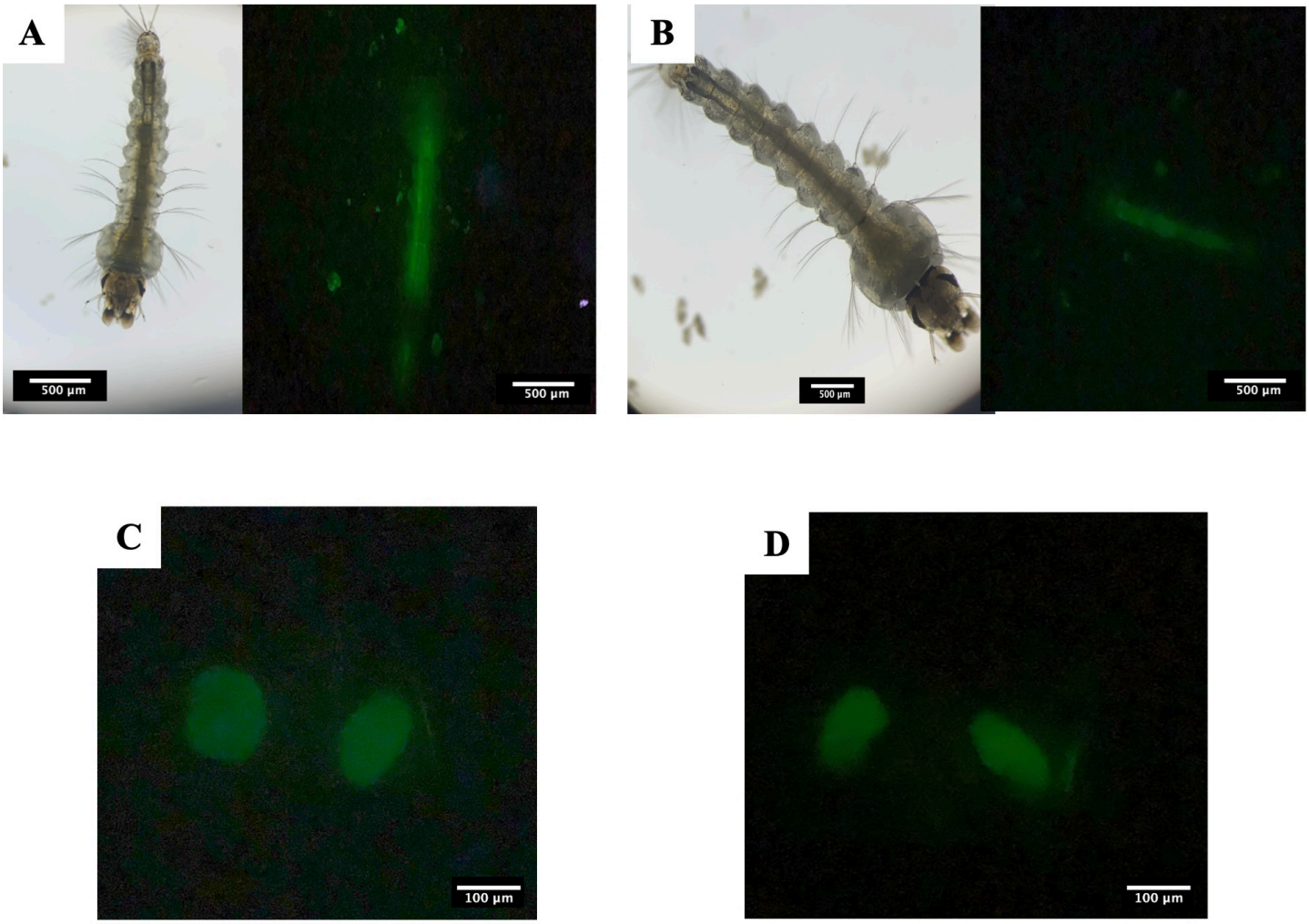


Figure 5.1. Detection of 0.5 µm fluorescent latex beads in SENN and SENN DDT. A) SENN fourth instar larvae ingestion of 0.5 µm latex beads. B) SENN DDT fourth instar larvae ingestion of 0.5 µm latex beads. C) Detection of 0.5 µm latex beads in adult SENN testes. D) Detection of 0.5 µm latex beads in adult SENN DDT testes.

Similarly, the 1 µm beads were detected within the gut of the fourth instar larvae of SENN (Figure 5.2 A) and SENN DDT (Figure 5.2 B). In the adult SENN, the 1 µm beads were only seen within the testes (Figure 5.2 C). However, in SENN DDT, the 1 µm beads were detected within both ovaries and testes (Figure 5.2 D).

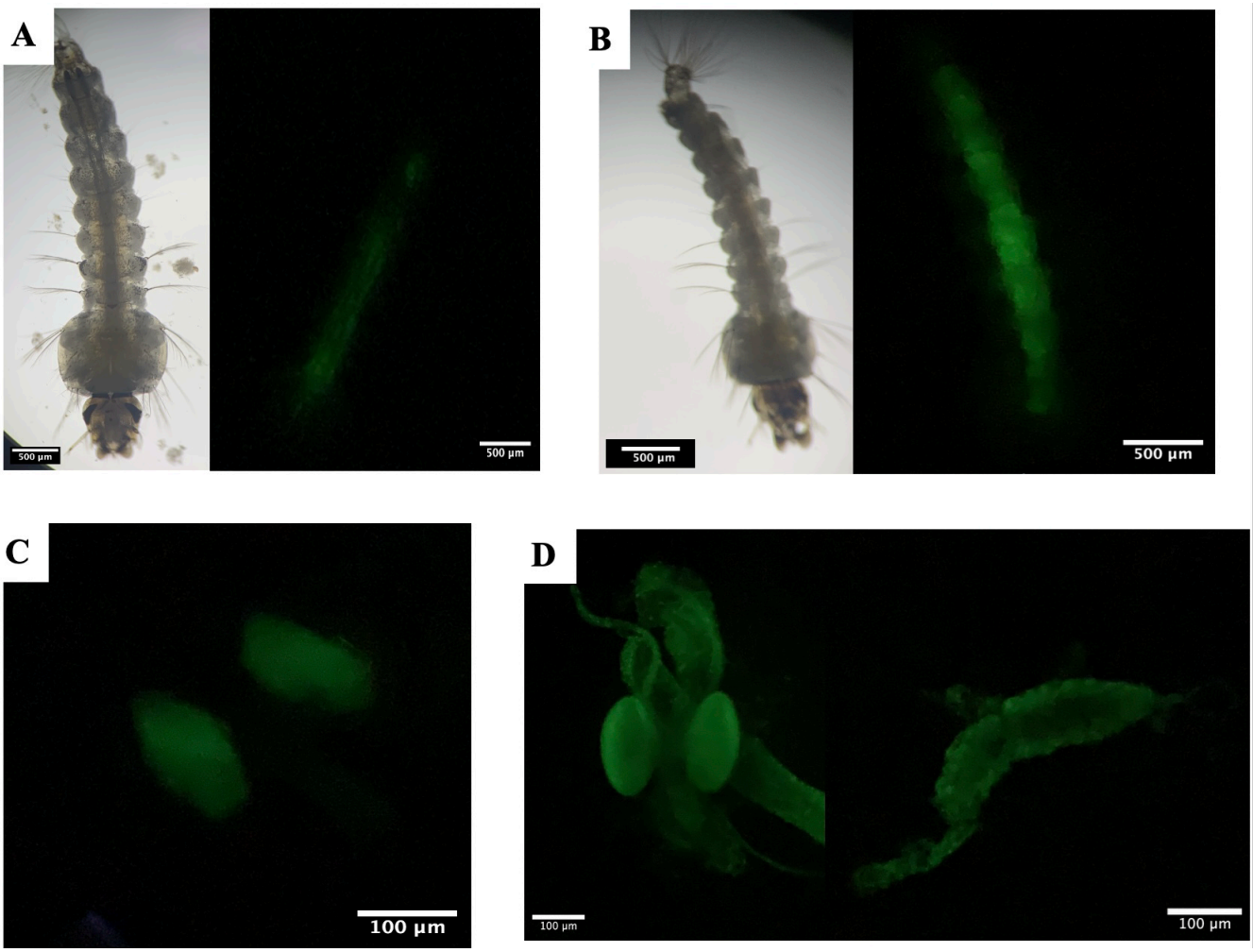


Figure 5.2. Detection of 1 µm fluorescent latex beads in SENN and SENN DDT. A) SENN fourth instar larvae ingestion of 1 µm latex beads. B) SENN DDT fourth instar larvae ingestion of 1 µm latex beads. C) Detection of 1 µm latex beads in adult SENN testes. D) Detection of 1 µm latex beads in adult SENN DDT testes (left) and ovaries (right).

However, the 2 µm beads were only faintly detected within the fourth instar larvae of SENN (Figure 5.3 A) and SENN DDT (Figure 5.3 B). Additionally, the 2 µm beads were faintly detected within the midgut (Figure 5.3 D) in addition to the ovaries and testes (Figure 5.3 C) of SENN. It should be noted that the presence of the 2 µm beads was not found within any tissues of adult SENN DDT. Additionally, the salivary glands were dissected, but no positive detection of any size beads was seen within both strains of *An. arabiensis*. The presence of latex beads in the reproductive tissues is important as it may have a significant impact on the offspring.

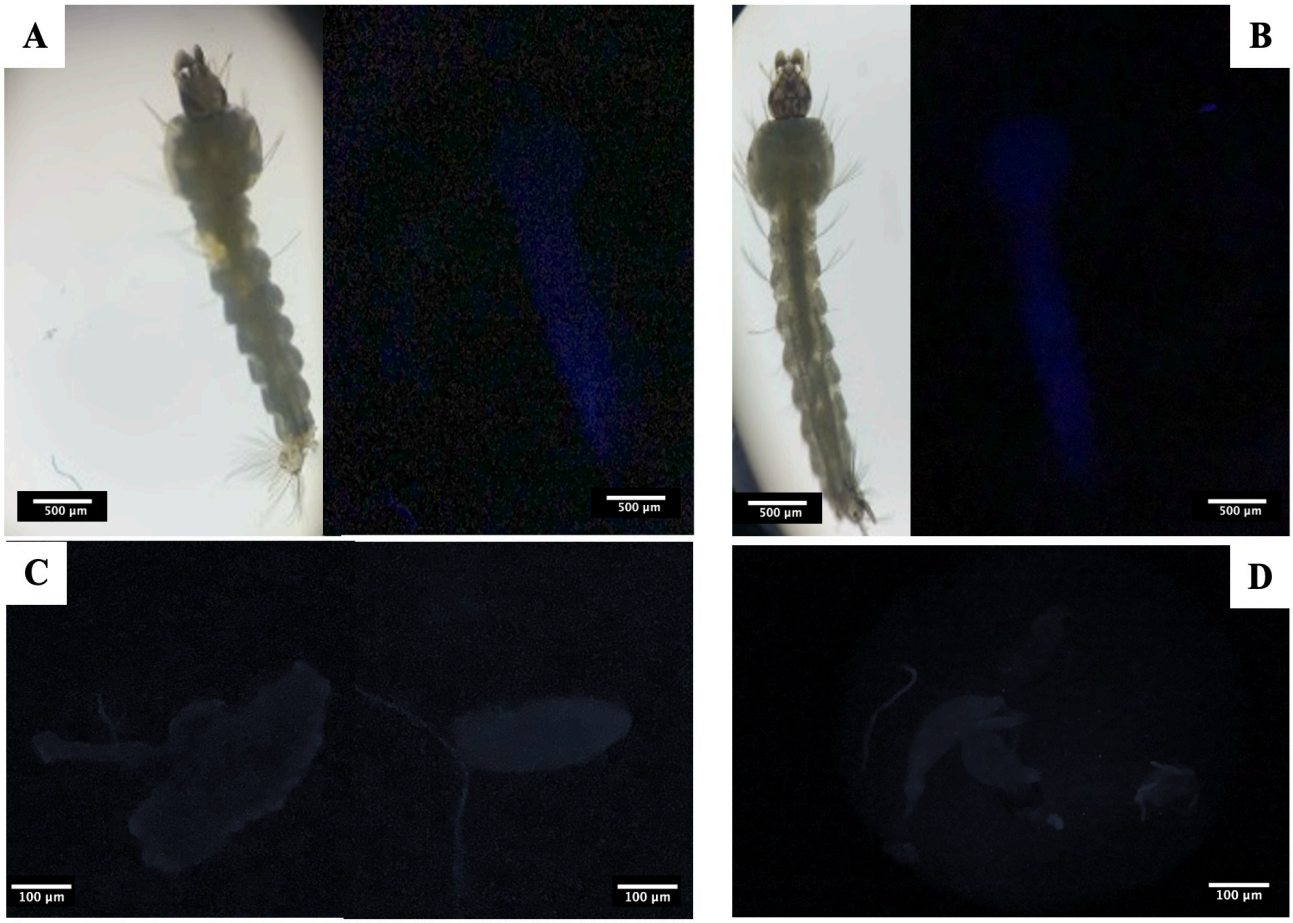


Figure 5.3. Detection of 2 µm fluorescent latex beads in SENN and SENN DDT. A) SENN fourth instar larvae ingestion of 2 µm latex beads. B) SENN DDT fourth instar larvae ingestion of 2 µm latex beads. C) Detection of 2 µm latex beads in adult SENN testes (right) and ovaries (left). D) Detection of 2 µm latex beads in adult SENN midgut. No clear detection of the latex beads was seen in adult SENN DDT reproductive organs or midgut.

5.3.2. The effect of plastic exposure on HAT activity

Figure 5.4 indicates the HAT activity after larval exposure to plastic treatments. When HAT activity was compared in SENN females, a significant difference was observed (One-way ANOVA: p=0.02, $F_{(6,15)=}$ 3.91). Upon *post-hoc* multiple comparison analysis, a significant difference between the untreated and the SENN F2 nappy-treated was seen (Tukey multiple comparison: p=0.04) (Figure 5.4A). A significant difference was seen after analysis of the HAT activity within the SENN males (One-way ANOVA: p=0.001, $F_{(6,15)=}$ 6.92). *Post-hoc* analysis revealed significant differences between the untreated and SENN F1 BPA (p=0.002), untreated and SENN F1 PAE (p=0.04) and the untreated and SENN F2 nappy (p=0.04) (Figure 5.4B). However, a comparison of the HAT activity in SENN DDT showed no significant difference in either females (Figure 5.4C) or males (Figure 5.4D). These results indicate that the most significant changes in HAT activity after larval plastic exposure were within SENN. Notably, the most significant differences were seen within the SENN males, particularly the SENN F1 BPA-treated males. It should also be emphasised that significant differences were seen within both the F2 nappy-treated male and female SENN despite no significant difference observed in the F1 nappy-treated SENN.

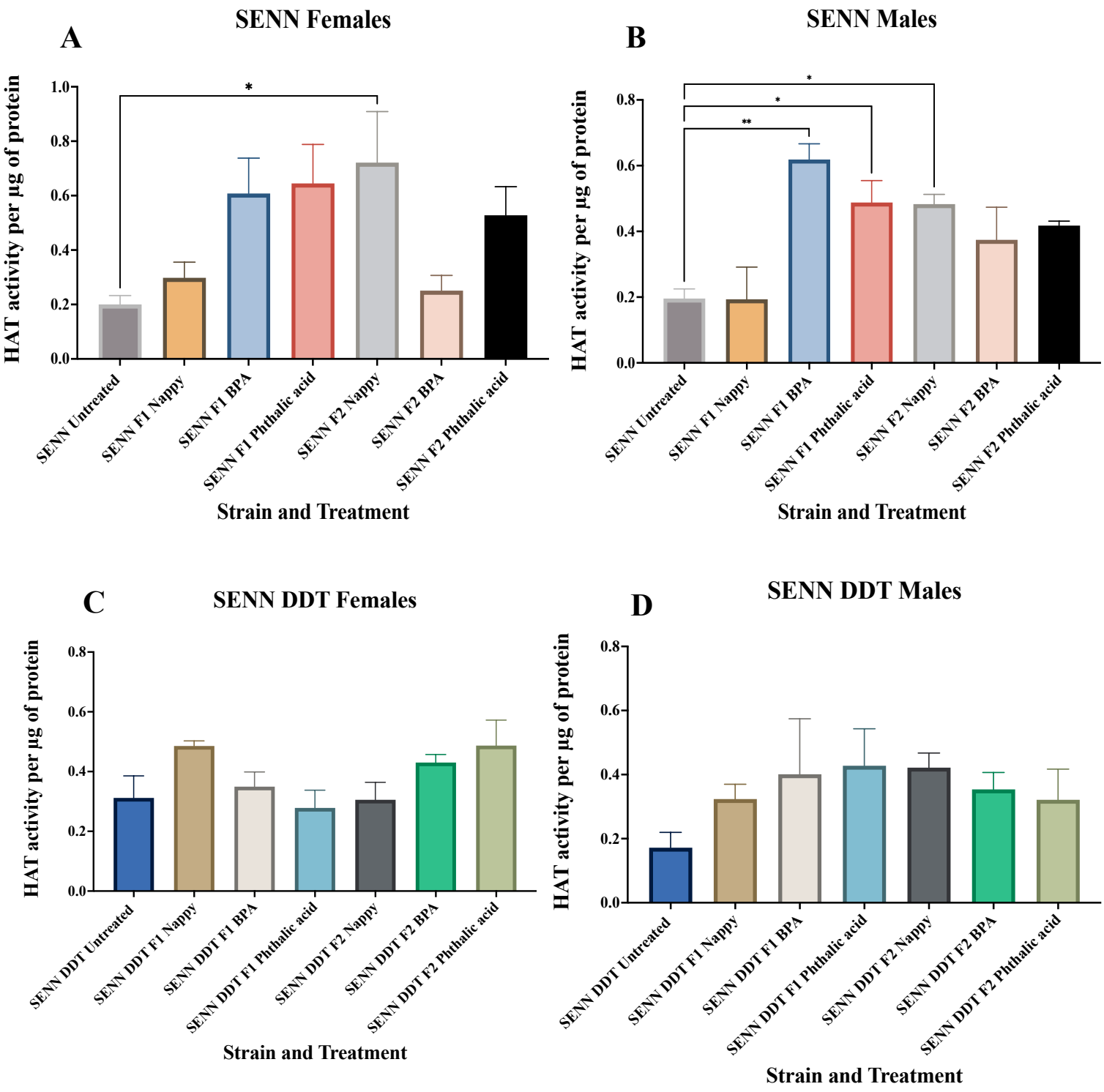


Figure 5.4. Global change in HAT activity after larval exposure to plastic treatments in *An. arabiensis*. HAT activity is reported as the relative optical density per µg of protein. A) HAT activity in SENN females. B) HAT activity in SENN males. C) HAT activity in SENN DDT females. D) HAT activity in SENN DDT males. The error bars represent the SEM. Significant differences are indicated with an asterisk (*p=0.04, **p=0.002).

Despite the limited significant differences seen between the different generations and treatments, there was an obvious increase in HAT activity, particularly in SENN. Therefore, to account for these differences, the fold change was determined. The fold change was calculated by normalising the HAT activity of the treatments to the untreated controls. The SENN F1 nappy-treated females had a 1.5-fold change. Whereas the SENN F2 nappy-treated females had the highest fold change of 3.6. The SENN F1 BPA-treated females had a fold change of 3. However, SENN F2 BPA-treated females had the lowest fold change of 1.3. SENN F1 PAE-treated females had a fold change of 3.2, and the SENN F2 phthalic acid-treated females had a fold change of 2.6 (Figure 5.5A). Conversely, SENN F1 nappy-treated males presented with a low-fold change of 1. SENN F1 BPA-treated males presented with the highest fold change of 3.2. SENN F1 phthalic acid-treated and SENN F2 nappy-treated males had a fold change of 2.5. SENN F2 BPA-treated males had a fold change of 1.9. SENN F2 phthalic acid-treated males had a fold change of 2.1 (Figure 5.5B). These results indicate that despite no significant difference observed in some treatments, there was an increase of between one and three times the HAT activity after the treatments. The results also indicate that larval plastic exposure resulted in an increase in HAT activity within both males and females in SENN. Notably, the plastic additives had a greater fold change in the F1 generation, whereas the nappy treatment had a greater fold change within the F2 generation.

In SENN DDT, the fold changes observed were not as prominent as what was seen in SENN. The SENN DDT F1 nappy-treated females had a fold change of 1.6, but the F2 nappy-treated females had a fold change of 1. The SENN DDT F1 BPA-treated females had a fold change of 1.1, and the F2 BPA-treated females had a 1.4-fold change. SENN DDT F1 phthalic acid-treated females had a fold change of 0.8, whereas the F2 phthalic acid-treated females had a fold change of 1.6 (Figure 5.5C). However, in the SENN DDT males, higher fold changes were observed than what was observed in the females (Figure 5.5.D). SENN DDT F1 nappy-treated males had a fold change of 1.9, and the F2 nappy-treated males had a fold change of 2.5. The SENN DDT F1 BPA-treated males had a fold change of 2.3, whereas F2 BPA-treated males had a fold change of 2.1. The SENN DDT F1 phthalic acid-treated males had a fold change of 2.5, and the F2 phthalic acid-treated males had a fold change of 1.9. These results highlight that in SENN DDT, there was a greater increase in HAT activity after plastic treatment within the males. Notably, in SENN DDT, the opposite of what was seen in SENN was observed. The SENN DDT females indicated that the F1 nappy treatment had a higher fold change than the

F2 nappy treatment. Additionally, in the females, the plastic additives resulted in a higher fold change in the F1 generation rather than in the F2.

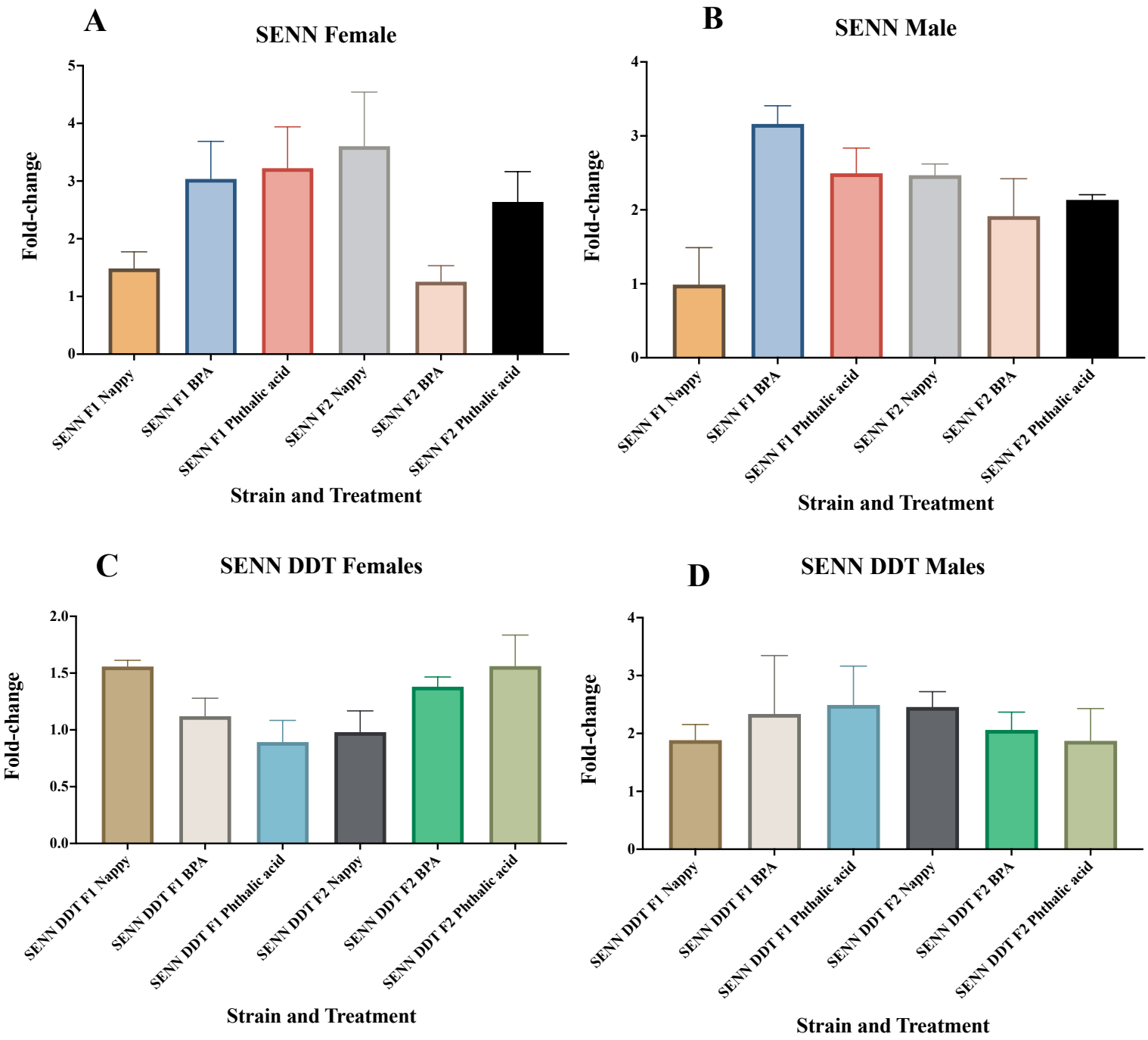


Figure 5.5. The fold change of the HAT activity after larval exposure to plastic treatments in *An. arabiensis.* A) Fold change in SENN females. B) Fold change in SENN males. C) Fold change in SENN DDT females. D) Fold change in SENN DDT males. Fold change was calculated by normalising the relative HAT activity of the treatments to the untreated controls. Each error bar represents the SEM.

5.4. Discussion

Plastic pollution has emerged as a serious global environmental issue, with its impact spanning several ecosystems and organisms. The fragmentation of plastic into micro- and nanoplastics has provided an additional challenge regarding plastic pollution, as organisms can ingest plastic fragments. Therefore, plastic can be transferred across the different trophic levels (Cui *et al.*, 2022b). The ubiquitous nature of plastics indicated that exposure to plastics was inescapable for insects, particularly mosquitoes. The ingestion and transference of MPs by *Culex* and *Aedes* mosquitoes have been studied extensively (Al-Jaibachi *et al.*, 2018, 2019; Cui *et al.*, 2022b; Simakova *et al.*, 2022). The study by Al-Jaibachi *et al.* (2018) identified that the smaller 2 µm MPs were transferred to the pupae and larvae of *Cx. pipiens* and were accumulated within the Malpighian tubules. Similarly, in *Ae. aegypti,* the ontogenic transference of MPs was observed (Simakova *et al.*, 2022). Ontogenic transference refers to the transfer of substances or traits from one developmental stage to another within the mosquito's life cycle. Additionally, Gopinath *et al.* (2022) observed the accumulation of nanoplastic residues within the ventral diverticulum, Malpighian tubules, intestine, rectum and notably the salivary glands of *Aedes*. However, there have been no reports on the potential for malaria vectors to ingest and transfer MPs.

Therefore, to contribute to the current literature, this study examined the ingestion and transfer of three different sizes of commercially available fluorescently labelled latex beads in insecticide-selected (SENN DDT) and unselected (SENN) strains of *An. arabiensis*. The fluorescence of the smaller latex beads (0.5 µm and 1 µm) was easily detected within the fourth instar larvae of both strains. However, the 2 µm was only faintly detected within the larvae. Therefore, indicating that *An. arabiensis* has the propensity to ingest smaller MP particles. Additionally, latex beads were detected within the emergent adults. This highlights the potential of MPs to be transferred to adult *An. arabiensis,* as was seen in other mosquito species. However, unlike previous studies, this study detected the presence of latex beads within the reproductive tissues. Previously, the accumulation of MPs was seen within the Malpighian tubes (Al-Jaibachi *et al.*, 2018). However, in a recent study, Gopinath *et al*. (2022) identified that the smaller nanoplastics translocated to other tissues. However, these studies did not examine the reproductive tissues. Therefore, it is entirely possible that the smaller latex beads can be redistributed within the reproductive tissue in adult *An. arabiensis.* This is a significant finding as MPs within reproductive tissues may have downstream implications on

subsequent offspring. This finding also raises the question of whether the presence of MPs within the reproductive tissue affects the germline and if these effects are transgenerational.

An emerging area of research is how plastic pollution affects gene regulation through epigenetic mechanisms. Environmental stressors such as plastic pollution can be modulated by these epigenetic mechanisms. Histone acetylation functions to regulate gene expression by altering the chromatin structure and, therefore, is an important mechanism employed under environmental stress. In insects, including mosquitoes, HAT activity plays a fundamental role in various biological processes such as development, immune response and adaptations to environmental stresses (Simola *et al.*, 2016; Olivares-Castro *et al.*, 2021; Palli, 2021). Therefore, this study focused on the effect of larval plastic exposure on the HAT activity of *An. arabiensis* as an epigenetic marker. In a previous study that examined the effect of larval metal exposure on *An. arabiensis,* it was determined that HAT activity was decreased in SENN and increased in SENN-DDT (Jeanrenaud *et al.*, 2022). However, in the present study, a different pattern emerged. It was determined that no significant difference in the HAT activity was observed in both males and females of SENN DDT. Conversely, the most significant increase in HAT activity was present within the males, particularly the SENN F1 BPA-treated males. This is interesting as BPA is an endocrine-disrupting chemical and has been implicated in various epigenetic regulatory mechanisms.

Studies have shown that BPA exposure can alter HAT activity, leading to changes in gene expression and hormone regulation, stress response and development (Hong *et al.*, 2024). Additionally, BPA exposure resulted in increased histone acetylation in brain tissues, therefore affecting neural development and behaviour (Kumar and Thakur, 2017; Bi *et al.*, 2022). Furthermore, studies have demonstrated that different doses of BPA affected histone methylation and acetylation (Hong *et al.*, 2024). A study indicated that chronic exposure to non-toxic doses of BPA reduced histone acetylation levels in the testis of adult male rats (Chen *et al.*, 2017). A decrease in histone acetylation levels is linked to a decrease in HAT activity. Additionally, chronic exposure to BPA affected the expression of histone methylation in the germ cells and decreased sperm fertilisation in adult male minnows (Zhu *et al.*, 2020). However, studies have determined that exposure to high doses of BPA increased HAT activity in the testes of male zebrafish (González-Rojo *et al.*, 2019) and impaired embryonic development (Lombó *et al.*, 2019). The results from the study by González-Rojo *et al*. (2019) are in line with the results obtained in the present study. However, the concentration of BPA

used in this study is not necessarily environmentally relevant. Therefore, the BPA concentration may be higher than environmental doses. Hence, differing HAT activity responses to lower or higher concentrations of BPA in *An. arabiensis* is likely.

Although the direct impact of MPs on HAT activity has not been extensively studied, there is evidence that suggests that exposure to MPs can induce oxidative stress and inflammation, which are known to influence histone acetylation (Anbumani and Kakkar, 2018). Oxidative stress can lead to the activation of signalling pathways that affect HAT activity, potentially altering gene expression patterns that regulate cellular stress responses (Rahman *et al.*, 2021). Therefore, MP exposure may lead to epigenetic modifications including alteration to histone acetylation as a response to environmental stress. In the present study, the artificially degraded nappies were used as a proxy for complex common plastic pollutants. The nappy treatment resulted in no significant increase in HAT activity in the F1 generation. However, in SENN the F2 generation resulted in an increase in HAT activity in both males and females. Therefore, this indicates that plastic fragments have a greater potential to cause epigenetic alteration in the germline of *An. arabiensis*.

Transgenerational inheritance refers to the transmission of these epigenetic modifications via meiosis in the germ cell line, ultimately affecting offspring that have not been exposed to environmental stressors (McCarrey, 2014). Transgenerational effects were observed within fathead minnows exposed to MPs (Wade *et al.*, 2024). Similarly, a study on *An. arabiensis* determined the transgenerational effects of larval exposure to metal pollution. (Jeanrenaud *et al.*, 2020). The study determined that exposure to heavy metals in a single generation can have effects on subsequent generations, even if they are bred in clean water. Additionally, the transgenerational effects included an increase in insecticide tolerance in the subsequent generations (Jeanrenaud *et al.*, 2020). Consequently, in the present study, the increase in HAT activity in SENN after BPA and phthalic acid exposure was not conserved in the subsequent generations that were not exposed to plastic treatments. However, notably no change in HAT activity in SENN males and females of the F1 generation exposed to nappy-treated water was observed but the subsequent F2 generations exposed to untreated water resulted in a significant increase in HAT activity. This is a significant finding as it indicates that complex plastics have a greater impact on the offspring and may have implications on population dynamics.

However, to better understand the implications of the transgenerational effects of plastic exposure, further research needs to be conducted. Deeper insight into the phenotypic differences is necessary. Understanding the transgenerational effects of plastic exposure on longevity, fertility, and fecundity will allow for a greater understanding of the epidemiological significance of exposure to plastic. Additionally, a crucial consideration is the transgenerational effect of plastic exposure on insecticide tolerance. This is particularly important as based on the current study the two strains of *An. arabiensis* responded differently to plastic exposure. The insecticide-selected strain had no significant difference in HAT activity. However, the most noticeable increases in HAT activity were seen in the unselected strain. Therefore, these findings suggest that the insecticide-resistant phenotype is not only implicated in the different detoxification enzyme activities but also the variable epigenetic responses to environmental stressors.

The limited significant differences seen between the different generations and treatments did not seem to correlate with the increase in HAT activity particularly in SENN. Therefore, to account for these differences, the fold change was determined. The fold change in SENN DDT was not as prominent as SENN. Additionally, the SENN DDT males had an overall higher fold change than the females therefore indicating a greater HAT activity in males. In SENN, the plastic additives had a greater fold change in the F1 generation. However, the nappy treatment resulted in a greater fold change within the F2 generation. A possible reason for this is that in the nappy treatment, the larvae ingest the plastic particles which can be redistributed to different tissues. This theory correlated to the previous results that detected the fluorescent latex beads within the reproductive tissues. Additionally, the chemical additives are known endocrine-disrupting compounds, interfering with hormonal signalling pathways that have been shown to induce epigenetic changes. Additionally, the plastic additives can be absorbed by the larval body thus causing a contrasting response to the ingestion of plastic. However, there is no definitive reasoning for the different responses observed therefore further investigation is needed.

This study has several limitations that need to be highlighted. Firstly, due to the limited studies on insect epigenetics, the commercial histone extraction and HAT activity kits used are developed for mammalian cell and tissue studies. Therefore, these kits may not be as sensitive to the HAT activity present within mosquitoes. Optimisation and validation of the kits used should be considered to ensure that the results obtained are valid. A major limitation of this

study is the focus solely on HAT activity. Previous studies on *An. arabiensis* have shown marked changes to HAT activity upon immune stimulation and exposure to metals (Jeanrenaud *et al.*, 2022; Patel *et al.*, 2024). Therefore, there exists a precedent that histone modifications play a crucial role in the epigenetic regulation of *An. arabiensis*. However, in order to obtain a deeper understanding, the exact histone modifications need to be elucidated. In studies on invertebrate species such as the gall fly (*Eurosta solidaginis*), histone H3 and H4 modifications have been implicated in the freeze tolerance of the species (Bloskie and Storey, 2023). Therefore, although there are limited studies on histone H3 and H4 modifications in mosquito species, there exists research from other organisms that indicates that these epigenetic modifications are integral to the regulation of gene expression in response to environmental stress. Furthermore, further research is needed to elucidate the specific role and mechanisms of the different histone modifications in mosquito biology, particularly in response to environmental stress such as plastic pollution.

In conclusion, the presence of fluorescent latex beads was detected within fourth instar larvae and the reproductive tissues of *An. arabiensis*. The results of this study indicated that larval plastic exposure resulted in strain-specific changes in histone acetylation with the SENN males, conferring a greater HAT activity. Additionally, the complex plastic and plastic additives resulted in differing responses. The nappy treatment resulted in greater HAT activity within the F2 generation, indicating the potential transgenerational effect of plastic exposure. However, further studies on the transgenerational effects of plastic exposure particularly on insecticide tolerance are required. This is of particular importance, as in Chapter 3 larval plastic exposure resulted in significant increases in insecticide tolerance; however, the transgenerational implications were not investigated. Therefore, further studies on the effects of plastic exposure on epigenetic changes in malaria vectors are required to bridge the lack of knowledge surrounding this topic.

Chapter 6: General discussion and conclusion

The escalation of anthropogenic activities on a global scale is substantially exacerbating the persistent challenges associated with environmental pollution. Among the principal contributors to this problem is plastic pollution. Notably, until recently, research has predominantly focused on the impacts of plastic pollution within marine environments. As global plastic production continues to increase, tackling plastic pollution necessitates comprehensive strategies that address not only marine environments but also freshwater and terrestrial ecosystems. Moreover, MPs often carry absorbed chemicals, such as pesticides, heavy metals and other pollutants, which can have additional toxicological effects on mosquitoes and the organisms that consume them (Graca *et al.*, 2014; Holmes *et al.*, 2014).

Due to their widespread presence in diverse habitats and their adaptability, mosquitoes can serve as valuable biological indicators for evaluating the extent of MP contamination. Ingesting MPs may impact larval feeding efficiency, development, and survival rates, which can subsequently alter population dynamics. Variations in mosquito populations can have a ripple effect on aquatic environments, as mosquito larvae are a vital food source for various predators (Cui *et al.*, 2022b). Additionally, mosquitoes are holometabolous, transitioning from aquatic larvae to flying adults. This distinctive life cycle allows any MPs ingested by the larvae to be transferred to the adult stage. This transference enables the movement of MPs from aquatic environments to terrestrial ones (Al-Jaibachi *et al.*, 2018, 2019; Simakova *et al.*, 2022). As a result, it can lead to the distribution of MPs across various habitats, effectively linking aquatic ecosystems to terrestrial ones (Jones *et al.*, 2024). In addition to their environmental significance, mosquitoes are well-known as disease vectors. The relationship between plastic pollution and mosquito biology may have important implications for disease transmission. Furthermore, the consumption of MPs may impact adult mosquitoes' fitness and behaviour, which in turn influences disease transmission dynamics.

Research has shown that mosquito larvae can ingest MP particles suspended in water, which can lead to physical and physiological impacts (Al-Jaibachi *et al.*, 2018). However, studies on MP in mosquitoes have focused on *Aedes* (which transmit viruses) and *Culex* (which are nuisance mosquitoes and can transmit filarial worms) (Al-Jaibachi *et al.*, 2018, 2019; Edwards *et al.*, 2023; Griffin *et al.*, 2023; McConnel *et al.*, 2024). Therefore, there is a lack of research on the impact of plastic pollution on malaria vectors (anophelines). Consequently, this

necessitates the need to investigate the effect of plastic pollutants on malaria vectors, especially on *Anopheles* species. Therefore, this study examined the major malaria vector, *An. arabiensis*, and the impact of various types of plastic pollutants.

Previous research has mainly concentrated on the effects of polystyrene as an MP. Therefore, to enhance the current knowledge, this study focused on complex plastics commonly found in larval breeding sites in South Africa, such as disposable nappies (Kordecki *et al.*, 2022; Schenck *et al.*, 2023). It also investigated plastic additives like BPA and phthalic acid, as well as representative of MP particles such as latex beads. Furthermore, the study explored various aspects of mosquito biology and physiology, including life history, midgut microbiota, and epigenetics. Additionally, one of the biggest challenges in malaria control is the emergence of insecticide resistance, which can be significantly influenced by exposure to pollutants (Antonio-Nkondjio *et al.*, 2011; Kabula *et al.*, 2011; Jeanrenaud *et al.*, 2019). Various studies have shown that larval exposure to polluted waters has resulted in increased tolerance to insecticides in *An. arabiensis* (Oliver and Brooke, 2018b; Jeanrenaud *et al.*, 2019; Samuel *et al.*, 2020; Chen and Oliver, 2024). However, no studies, to the best of our knowledge, have examined the effect of plastics on insecticide tolerance in *An. arabiensis*. Thus, the impact of MPs on insecticide tolerance was also evaluated. Moreover, the two strains utilised in this study allowed for the examination of plastic effects on mosquitoes of different insecticide-resistant profiles, which, to the best of our knowledge, has never been undertaken before. This research, therefore, establishes a foundation for understanding the effects of plastic on the biology of *An. arabiensis* and enhances knowledge about its impact on insecticide tolerance.

A significant finding in this study is the differential response of two distinct strains of *An. arabiensis* to plastics exposure. This is a major finding as the differing resistance profiles of the two strains may influence the mosquitoes' adaptive capacity when exposed to plastic-polluted environments. The overall analysis revealed that the insecticide-selected strain, SENN DDT, was not considerably affected by exposure to these plastic treatments. Notably, this strain demonstrated minimal impacts on adult longevity and epigenetic alterations. This finding is crucial, as the insecticide resistance phenotype may help mitigate the effects of plastic treatments on the mosquitoes' biology. Consequently, the insecticide-selected strain appeared to be more adept at managing the impacts of plastic exposure compared to the unselected strain, underscoring the differences between the strain responses.

The selection pressure exerted by plastic pollution on mosquito strains exhibits notable variability. This suggests that the influence of plastic pollution in wild populations is dependent upon the specific insecticide-resistance phenotype present within those populations. Particularly, this variability may manifest differentially in regions characterised by mosquito populations with active resistance as opposed to areas dominated by insecticide-susceptible strains. The resistant phenotype identified in the insecticide-unselected SENN strain reflects the characteristics found within wild populations of *An. arabiensis* in South Africa. Importantly, these wild populations are not fully resistant to insecticides (Munhenga *et al.*, 2022). Consequently, it is plausible that exposure to plastic pollution may elicit responses in wild *An. arabiensis* populations in South Africa that are consistent with those observed in the SENN strain, as outlined in this study. Conversely, in West Africa, where documented instances of active insecticide resistance in wild *An. arabiensis* populations have been reported (Antonio-Nkondjio *et al.*, 2011, 2015; Kabula *et al.*, 2011); it is plausible that exposure to plastic pollutants could trigger responses comparable to those observed in the insecticide-selected SENN DDT strain utilised in this study.

Therefore, the role of plastic pollution as a differential selection pressure is influenced by both the type of mosquito population and the specific regional context. The principal conclusion to be drawn from this analysis is the critical importance of considering the pre-existing insecticide resistance phenotypes present within mosquito populations when evaluating the effects of plastic pollution. It is not feasible to make broad generalisations regarding the precise impact of plastic pollution across diverse mosquito populations. The responsiveness of mosquitoes to such pollutants is inherently linked to the resistant phenotypes they exhibit. Consequently, the resistance phenotype will ultimately dictate the direction of the selection pressure imposed by pollutants. The unselected SENN strain, while not fully susceptible to insecticides, does not exhibit the characteristics of a resistant strain, thus leading to differing responses to pollution compared to a resistant strain. Notably, increased responsiveness was recorded in the SENN strain following larval exposure to plastics. Therefore, in South Africa, it is hypothesised that the influence of plastic pollution will promote the attributes associated with plastic exposure as outlined in this study more swiftly than in regions where established insecticide-resistant phenotypes already exist. In contrast, in countries such as Nigeria and Cameroon, where insecticide resistance is well-established, the selection pressure from plastic pollution may be less pronounced. Therefore, the implications of plastic pollution on fundamental physiological

and biological aspects of *An. arabiensis,* such as life history traits, are likely to vary by region, shaped significantly by the pre-existing insecticide resistance phenotypes.

Furthermore, the significant increase in LT_{50} values (a measure of insecticide tolerance), particularly within the insecticide-selected strain, suggests that exposure to plastic resulted in decreased sensitivity to the insecticide deltamethrin. The implication of this finding is profoundly concerning within the context of vector control. Deltamethrin, a widely utilised pyrethroid in vector control strategies, particularly IRS, plays a critical role in the suppression of vector populations (Tangena *et al.*, 2020; Gimnig *et al.*, 2024). Consequently, an observed increase in tolerance to deltamethrin upon larval plastic exposure poses a significant threat to the efficacy of existing vector control strategies. The observed increase in insecticide tolerance can be attributed to the heightened activity of detoxification enzymes within the insecticide-selected strain. The insecticide resistance phenotype assisted the mosquito with better survivorship when exposed to toxic pollution. This adaptation is associated with an increased activity of detoxification enzymes, which facilitates the breakdown of harmful substances (Oliver and Brooke, 2018b). While it is postulated that SENN DDT's ability to cope with plastic exposure is due to enhanced enzyme activity, this remains unconfirmed, as the study did not specifically analyse detoxification enzyme activity in relation to plastic exposure. Additionally, this study only examined a tolerance against a single pyrethroid and warrants further investigation of other insecticides. Therefore, further research is necessary to clarify the effects of plastic exposure on detoxification enzymes and insecticide tolerance in different insecticide classes.

Moreover, studies on plastic pollution in mosquito populations have greatly focused on MPs and the damaging physical effects of plastics. However, there is a complexity in understanding the impact of MPs due to the varying physical and chemical properties associated with plastic (Campanale *et al.*, 2020). The effects of plastic pollution are not limited to only the physical effects, but also the chemical additives associated with the plastics that may leach into the environment. The present study determined that these plastic additives impose a more substantial selection pressure than the plastic fragments and MPs themselves. Significantly, the plastic additives resulted in a greater influence on insecticide tolerance. This corresponded to previous studies on pollutant effects on insecticide tolerance (Chen and Oliver, 2024; Li *et al.*, 2024a). Particularly, phthalic acid exposure presents a significant challenge to vector control strategies. Notably, this chemical additive, which demonstrated lower toxicity levels, has been

identified to confer greater insecticide tolerance. Consequently, exposure to phthalic acid could undermine the efficacy of the currently utilised insecticides in vector control strategies such as IRS. Furthermore, phthalic acid did not impose any additional fitness cost regarding longevity. Stability in adult longevity corresponds to the persistence of mosquito populations, facilitation of effective disease transmission, and promotion of adaptations to environmental stressors without additional pressures shortening mosquito lifespans. Additionally, the concentration of phthalic acids in the environment is higher than BPA, attributed to their widespread use and greater tendency to leach into the environment. Consequently, it is highly probable that *An. arabiensis* encounters higher concentrations of phthalic acid in their larval habitats. Given that this chemical additive is less toxic towards *An. arabiensis*, it is more likely that these mosquitoes may adapt to the changes observed in the present study when exposed to phthalic acid. Therefore, given that phthalic acid does not pose a significant threat to *An. arabiensis*, it is plausible that these mosquitoes may exhibit adaptive responses over time in relation to the presence of phthalic acid. This potential adaptation could pose considerable obstacles to the efficacy of vector control strategies.

Furthermore, there exists the potential for MPs to exert transgenerational effects on mosquito populations. Transgenerational effects tend to be mediated by epigenetic modulation. However, insect epigenetic studies tend to lag behind those of mammals, and this study attempted to address this paucity of information. The present study determined that plastic fragments have the potential to cause epigenetic alteration in *An. arabiensis*. Additionally, plastic fragments are more likely to induce epigenetic alterations in the offspring of the exposed mosquitoes rather than in the first generation directly exposed to the plastics. These alterations are primarily modulated by histone activity rather than DNA methylation (Djihinto *et al.*, 2023). This study examined the transgenerational effect on histone acetyltransferase activity. Understanding the effect of plastic exposure on specific histone modifications would give a greater understanding of the mechanisms of the transgenerational effects of plastic pollution.

The present study focused on the effect of larval plastic exposure on insecticide tolerance within a single generation. However, a previous study has identified a significant increase in insecticide tolerance in multiple generations following exposure to MPs in *An. gambiae s.s.* (Shilla *et al.*, 2024). Additionally, prolonged larval exposure to MPs was suggested to enhance tolerance to MPs in addition to increase insecticide resistance in both larvae and adult stages across multiple generations of *An. gambiae s.s* (Shilla *et al.*, 2024). Thus, it is of utmost

importance that we elucidate the transgenerational effects of larval plastic exposure on insecticide tolerance in *An. arabiensis* to understand the significance of the challenges posed by plastic pollution on subsequent generations of species.

Additionally, it should be emphasised that despite previous studies identifying MPs within the larvae, pupae, gut, Malpighian tubes and salivary glands of mosquitoes (Al-Jaibachi *et al.*, 2018, 2019; Cui *et al.*, 2022b; Simakova *et al.*, 2022), this study was the first, to the best of our knowledge, to identify the presence of fluorescent MPs within the reproductive tissues in *An. arabiensis*. In contrast to previous studies on *Culex* and *Aedes,* which successfully identified MPs within the salivary glands, this study was unable to positively identify the presence of MPs within the salivary glands. Therefore, it is less likely that *An. arabiensis* will transmit MPs through their bite. *Culex* and *Aedes* are considered cosmopolitan mosquito species and have demonstrated an ability to adapt to breeding within polluted environments. Consequently, it is reasonable to hypothesise that these species may possess more effective capabilities for processing plastic pollutants. Therefore, this raises two possible explanations for the absence of MPs within the salivary glands of *An. arabiensis*. Firstly, it is entirely plausible that this study failed to identify MPs in the salivary glands but cannot conclusively state that *An. arabiensis* is will not transmit MPs. This would need to be confirmed by feeding studies tracing the potential passage of MPs from the mosquito to the host being bitten. Secondly, *Anopheles* is still adapting to breed within polluted environments and thus are less adept at processing MPs and thus cannot transmit MPs. This is a reasonable explanation as generally *An. arabiensis* prefer breeding within clear freshwater bodies of water. However, in recent years, *An. arabiensis* has begun adapting to breed within polluted environments as a result of anthropogenic activities (Mwangangi *et al.*, 2006; Antonio-Nkondjio *et al.*, 2011; Kabula *et al.*, 2011). However, this opens up an avenue for further investigation into the possibility of *An. arabiensis* transmitting MPs.

Additionally, analysis of the alteration to the midgut microbiota upon larval exposure to plastic indicated that a more pronounced effect was observed within the unselected strain. This was particularly evident in the analyses of bacterial overlap and differential abundance. The results showed that the unselected strain had a greater number of bacterial genera and species after the nappy treatment. By contrast, the insecticide-selected strain exhibited a higher diversity of bacterial genera and species in the untreated samples. This finding aligns with previous observations regarding *An. arabiensis* when exposed to heavy metal pollutants (Singh *et al.*,

2024). Therefore, it was postulated that the microbiota was altered through the plastisphere. It has been theorised that the plastisphere plays a significant role in increasing the microbial communities present in the gut microbiota (Huang *et al.*, 2022; Hu *et al.*, 2022; Fackelmann *et al.*, 2023). Consequently, the presence of nappy fragments provided a suitable surface for the proliferation of bacterial colonies. Furthermore, the lower solubility of BPA may contribute to its role as an additional component of the plastisphere, potentially leading to an increase in species richness within the midgut microbiota.

In the context of the differing midgut microbiota, the observed differences may represent an added challenge regarding both *Plasmodium* resistance and insecticide resistance. The presence of bacterial genera in the plastic treatments that resemble those in the untreated samples may serve as potential targets for use in paratransgenesis. However, the variability of bacterial genera between samples after exposure to the plastic treatments is more likely to compound the issues related to increased insecticide resistance and refractoriness. A differential increase in bacterial genera associated with pesticide tolerance was observed within plastic-treated mosquitoes. Therefore, in the absence of significant detoxification enzyme activity, it is plausible that the acquisition of such bacteria may serve a protective function. Furthermore, the presence of these specific bacterial genera may contribute to a decrease in sensitivity to the insecticides employed in vector control, an occurrence that could have detrimental implications for the efficacy of vector control strategies.

Additionally, consideration of the effect of plastic treatments on the immune system of the mosquito is crucial. Although the best way to examine this would be to examine *Plasmodium* infection after larval plastic exposure, this study design allowed examination of the prevalence of *Plasmodium*-protective bacteria. The presence of *Plasmodium*-protective bacteria plays a crucial role in preventing the transmission of malaria by inhibiting the development of the parasite (Gonzalez-Ceron *et al.*, 2003; Bai *et al.*, 2019; Santos *et al.*, 2023). Consequently, a higher abundance of *Plasmodium*-protective bacterial genera may be associated with fewer incidences of malaria transmission. Seven bacterial genera that are considered *Plasmodium*-protective were identified across the different plastic-treated samples. However, no strain-specific differences in the abundance of *Plasmodium*-protective bacteria were observed. This is significant as it suggests that the presence of *Plasmodium*-protective bacteria within the midgut microbiota is not influenced by the resistance profile of the mosquito but rather the environmental stimuli.

Notably, variations in the abundance of *Plasmodium*-protective bacterial genera were observed between the different plastic-treated samples. Specifically, an increased prevalence of *Plasmodium*-protective bacteria in the plastic-treated samples was observed, suggesting a decreased potential for the *Plasmodium* parasite to evade host defences. *Asaia* was identified to be differentially abundant within the plastic-treated rather than untreated samples. *Asaia* is considered a promising paratransgenesis candidate due to its anti-*Plasmodium* properties (Tatsinkou Maffo *et al.*, 2024). Moreover, *Asaia* has been shown to enhance mosquito immune responses against parasite infection by upregulating immune genes and stimulating antimicrobial peptide expression without triggering an innate immune response (Capone *et al.*, 2013). Given the increased abundance of *Asaia* in plastic-treated samples, it raises important questions about how plastic pollution may influence the prevalence of *Plasmodium*-protective bacteria. Understanding this relationship could provide valuable insights for malaria control strategies, potentially turning an environmental challenge into an opportunity for disease prevention.

Importantly, an in-depth analysis of the midgut microbiota is crucial in the context of paratransgenesis. It is imperative to recognise that environmental factors, particularly pollution, significantly impact the composition of the midgut microbiota (Akorli *et al.*, 2024). Consequently, there exists an urgent need to identify symbionts that are not only prevalent across various local breeding habitats but also resilient to the differing levels of pollution characteristic of these environments. In the context of South Africa, it is imperative to identify natural symbionts that do not require transformation, as this approach may prove to be more cost-effective. Furthermore, the characterisation of the local larval breeding sites, particularly regarding their pollution status, is a critical factor influencing the suitability of specific bacteria for use as paratransgenic symbionts. Therefore, it is essential to collect wild populations from these local larval habitats and subsequently breed the F1 generations in controlled, clean water environments. This method will facilitate the identification of potential candidates for paratransgenesis, thereby enhancing the efficacy of the symbiotic relationship in the relevant and differing environments.

Despite the significant findings, this study had several limitations. The concentration and presence of the plastic additives used in this study are not characterised in larval breeding sites. Therefore, it is uncertain if the concentrations used in this study are environmentally relevant.

However, this is a common issue faced in studies on MPs and highlights the need for the current characterisation of larval breeding sites (Jones *et al.*, 2024). Additionally, the insectary conditions for the duration of this study were unstable. Although this was mitigated by multiple replicates, it should be noted that these results may potentially vary under stable insectary conditions. Furthermore, this study focused solely on HAT activity, however, various epigenetic mechanisms may be impacted by plastic exposure. Therefore, further studies on the interplay between the epigenetic mechanisms with a particular emphasis on the different histone modifications are necessary. Moreover, further investigation into the effect of plastic exposure on the immune responses is required. The primary limitation of this study was the use of laboratory strains. In order to gather a more comprehensive understanding of the effect of plastic exposure on malaria vectors, wild mosquitoes should be examined.

In conclusion, this study serves as an initial step in exploring the impact of larval plastic exposure on *An. arabiensis.* Overall, the study found that plastic additives and fragments impact mosquito biology differently, with notable variations between insecticide-selected and unselected strains. Consequently, the insecticide-resistant phenotype appears to confer some resilience against plastic exposure, suggesting that resistance traits may mitigate adverse impacts on mosquito biology. However, the presence of plastic-induced epigenetic alterations, particularly in complex treatments like disposable nappies, underscores the need for further research to understand the long-term transgenerational effects of these pollutants on disease vectors. Additionally, various types of plastics can produce distinct effects, therefore, further investigation into the influence of other prevalent plastics on the biology of *An. arabiensis* is necessary. Significantly, larval exposure to plastic and plastic additives has profound effects on life history, midgut microbiota composition and epigenetic alterations. Importantly, these effects are contingent upon the specific insecticide resistance phenotypes present in *An. arabiensis*. Consequently, it is imperative to characterise these impacts at both regional and local levels for a comprehensive understanding of their ecological implications.

Given the ecological and epidemiological implications, future studies should expand to examine wild mosquito populations, as this will deepen our understanding of the real-world impact of plastic pollution on mosquito biology, insecticide tolerance, and vector-borne disease dynamics. Ultimately, this research highlights the critical intersection of pollution, vector biology, and public health, underlining the urgency of addressing and mitigating the challenges of pollution.

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Appendix

Appendix 1: Ethics waiver

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 2: Microbiota Supplementary documents

Table 2: Links to the MultiQC report and the Excel documents of the OTU table, overlapping family, genus and species for plastic-treated SENN and SENN DDT.

File type	Link
MultiQC report	https://1drv.ms/u/c/c5e72196c1517dc4/EaYB7c4-M9FLhN6VTDV-Qx4BAolA3kJYaUU0OuaSr2TTbA?e=enUrXO
OTU tables	https://1drv.ms/f/c/c5e72196c1517dc4/EvGsXRgYLnhOoMWkXywAQfABu7ObLvry3pmncOMx3I_K5A?e=cLjx1D
SENN overlap	https://1drv.ms/f/c/c5e72196c1517dc4/EkESx-eipg5PgFm8PLtzyIQB0IqX6dnJ17N3ATXH-vm0Ug?e=CWgwAy
SENN DDT overlap	https://1drv.ms/f/c/c5e72196c1517dc4/Es2nr8omk4JLkG0fOcDb7eEBEM_TfT3q1pw4gUduz4Cz-w?e=bZJXGc

Appendix 3: Bradford Assay for quantifying protein concentrations

Standard concentrations of bovine protein (BSA) were prepared by serial dilutions. The concentrations used were 0, 10, 20, 30, 40, 50, 100, 200, 300, 400 and 500 µg/mL. Ten µL of the standard BSA and unknown samples were aliquoted into the individual wells of a 96-well plate. 200 µL of the QuickStart dye was added to the wells. The plate was incubated at room temperature for five minutes after shaking for 30 seconds. The absorbance was measured using a plate reader (SpectraMax® ABS Plus: Molecular Devices) at 595 nm. A standard curve was generated by plotting the average blank corrected 595 nm measurements for each standard BSA versus the concentration (µg/mL).

Appendix 4: Proof of manuscripts arising from this study

ENVIRONMENTAL MICROBIOLOGY REPORTS
Open Access

The effect of larval exposure to plastic pollution on the gut microbiota of the major malaria vector Anopheles arabiensis Patton (Diptera: Culicidae).

Journal:	*Environmental Microbiology Reports*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Misser, Shristi; University of the Witwatersrand Johannesburg Wits Research Institute for Malaria; National Institute for Communicable Diseases, Centre for Emerging Zoonotic and Parasitic Diseases Chen, Chia-Yu; University of the Witwatersrand Johannesburg Wits Research Institute for Malaria; National Institute for Communicable Diseases, Centre for Emerging Zoonotic and Parasitic Diseases Ismail, Arshad; National Institute for Communicable Diseases, Sequencing Core Facility; University of Venda, Department of Biochemistry and Microbiology, Faculty of Science, Engineering and Agriculture,; Durban University of Technology, Institute for Water and Wastewater Technology Oliver, Shüné V.; University of the Witwatersrand Johannesburg Wits Research Institute for Malaria; National Institute for Communicable Diseases, Centre for Emerging Zoonotic and Parasitic Diseases
Keywords:	microbiome, pollution microbiology, bioinformatics, environmental signal/stress responses
Keyword List:	
Abstract:	Plastic pollution is prevalent in water bodies. However, most studies on plastic pollution focus on marine environments, with limited knowledge about its impact on freshwater ecosystems. This paucity of information extends to the effect on aquatic insects, with little reported data on the effect of plastic on malaria vectors. This is concerning as microplastics are reported to perturb the gut microbiota of culicine mosquitoes. This study examines how larval exposure to degraded plastic, plastic additives (phthalic acid, Bisphenol-A), and latex beads affects the gut microbiota of adult *Anopheles arabiensis*, with a comparison of the insecticide-unselected (SENN) and insecticide-selected (SENN-DDT) strains. The larval exposure had a minimal effect on alpha-diversity, but each plastic stressor altered beta-diversity in a non-strain-specific manner. Plastic-treated SENN showed an increase in unique bacterial genera. In contrast, untreated SENN-DDT displayed the highest abundance of unique genera, suggesting gut bacteria may play a role in mitigating the effect of plastic exposure in unselected strains. Additionally, larval plastic exposure increased bacteria associated with plastic degradation and pesticide metabolism. Although there was no significant change in *Plasmodium*-

	protective bacterial genera, inflammation-associated bacterial genera increased in both strains after treatment, suggesting potential immune modulation.

SCHOLARONE™
Manuscripts

The effect of larval exposure to plastic pollution on the gut microbiota of the major malaria vector *Anopheles arabiensis* Patton (Diptera: Culicidae).

Shristi Misser[1,2], Chia-Yu Chen[1,2], Arshad Ismail[3,4,5], Shüné V. Oliver[2,1]

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5: Institute for Water and Wastewater Technology, Durban University of Technology, Durban 4000, South Africa.

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Abstract

Plastic pollution is prevalent in water bodies. However, most studies on plastic pollution focus on marine environments, with limited knowledge about its impact on freshwater ecosystems. This paucity of information extends to the effect on aquatic insects, with little reported data on the effect of plastic on malaria vectors. This is concerning as microplastics are reported to perturb the gut microbiota of culicine mosquitoes. This study examines how larval exposure to degraded plastic, plastic additives (phthalic acid, Bisphenol-A), and latex beads affects the gut microbiota of adult *Anopheles arabiensis*, with a comparison of the insecticide-unselected (SENN) and insecticide-selected (SENN-DDT) strains. The larval exposure had a minimal effect on alpha-diversity, but each plastic stressor altered beta-diversity in a non-strain-specific manner. Plastic-treated SENN showed an increase in unique bacterial genera. In contrast, untreated SENN-DDT displayed the highest abundance of unique genera, suggesting gut bacteria may play a role in mitigating the effect of plastic exposure in unselected strains. Additionally, larval plastic exposure increased bacteria associated with plastic degradation and pesticide metabolism. Although there was no significant change in *Plasmodium*-protective

bacterial genera, inflammation-associated bacterial genera increased in both strains after treatment, suggesting potential immune modulation.

Keywords: microbiome, pollution microbiology, bioinformatics, environmental signal/stress responses

Introduction

Plastics are polymers that can be easily shaped and moulded. Plastics were initially developed from natural materials like latex from trees and natural rubber. Since the invention of the first synthetic plastic, Bakelite, in 1907, synthetic polymers have been used as the primary forms of plastic. Plastics were considered safe, clean and inexpensive materials which led to large-scale use of these products (Meikle, 1995)

A 2017 review estimated the total amount of plastic produced to date was 8.3 billion tons. Furthermore, it is estimated that 60% of plastic ever produced has been discarded and remains in landfills (Geyer *et al.*, 2017). As such, waste plastic is subject to degradation, usually by hydrolysis, photodegradation, thermooxidative degradation or biodegradation by microbes. The biodegradation of plastic waste by microbes results in the breakdown of these polymers into mesoplastics (between 5mm and 2.5cm), microplastics (<5mm) and nanoplastics (<1μm) (Andrady, 2011). These breakdown products, however, are not the only source of microplastics. Primary microplastics arise from plastics that are purposefully manufactured to be small-sized to be added to products such as scrubbing agents. As such, domestic waste is a major source of primary microplastic pollution (Boucher and Friot, 2017).

The most common plastic pollutants come from packing materials. These include polyethylene (38% of plastic production), Polypropylene (24%), Polystyrene (6%), Polyethylene terephthalate and Polyvinyl chloride (19%). Nylon and cellulose acetate also contribute substantially to plastic pollution (Andrady, 2011). However, it is not only these polymers themselves that contribute to the pollution associated with plastic production. Additives such as plasticizers contribute to the pollution associated with plastics as well, often through the breakdown of the plastics. These include Bisphenol-A (BPA) and phthalates (Nayanathara Thathsarani Pilapitiya and Ratnayake, 2024).

Plastic pollution, whether microplastics, plastic components or plastic additives, has a range of health and environmental effects. There has been a major focus on the effect of marine plastic pollution (Napper and Thompson, 2023; Nayanathara Thathsarani Pilapitiya and Ratnayake,

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2024). However, despite the ubiquity of plastic pollution, there is far less understanding of the effect of this type of pollution on freshwater sources, particularly on freshwater invertebrates. Most research on freshwater invertebrates has been performed on planktonic and benthic organisms, usually model organisms such as *Daphnia* (Jones *et al.*, 2024). Furthermore, even recent reviews of literature on the effects of plastic pollution on freshwater invertebrates did not have a large focus on insects (Pirillo and Baranzini, 2022). However, there have been reports of the effect of microplastics on the life history of mosquitoes, due to their importance as disease vectors.

The probability of exposure to plastic pollution varies depending on the distribution of mosquitoes. Mosquitoes associated with urban areas such as *Culex*, *Aedes* and *Anopheles stephensi* are more likely to be exposed to plastic pollution due to their association with human activity. Most *Anopheles* species would be exposed through agricultural sources and at lower rates (Jones *et al.*, 2024). It is therefore likely that there is less research on the effects of plastic exposure on *Anopheles*. The effects of microplastic exposure on mosquitoes are largely dependent on the size of the plastic particle, concentration and mosquito species. Variable effects have been seen on larval development (neutral to negative), and ontogenic transfer of microplastics is not guaranteed. This was observed by a comparison of the effect of plastic in *Ae. aegypti*, *Ae. albopictus*, *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. tarsalis* (Jones *et al.*, 2024). Where ontogenic transfer does occur, mosquitoes can transfer the microplastics through bites (Li *et al.*, 2024). If microplastics are present in adults, mosquitoes can be potential mechanical vectors of these products and could potentially transfer them to new environments or trophic levels (Al-Jaibachi *et al.*, 2018).

In addition to the variability in the effects of microplastics on mosquito life history, there are two clear effects that exposure to microplastics can have on the mosquito. Microplastics can serve as sinks for persistent organic pollutants (Steinman *et al.*, 2020; Napper and Thompson, 2023). This can perturb the metabolome (Wang *et al.*, 2022) and has been reported to reduce the efficacy of insecticides (Li *et al.*, 2024). As some plastic additives are known endocrine disruptors, the chemical disruption is unsurprising (Napper and Thompson, 2023).

Furthermore, there have been recent reports of microplastics affecting the mosquito microbiome (Edwards *et al.*, 2023; Li *et al.*, 2024). Factors that affect the mosquito microbiome are important due to their critical role in the organisms' life history. The mosquito gut microbiome is critical for mosquito development (Coon *et al.*, 2014; Coon *et al.,* 2017),

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egg production (Coon *et al.*, 2016) , digestion and immunity (Gao *et al.*, 2020). As such, larval exposure to plastic could have large-scale effects on mosquito life history through the modulation of the microbiome.

Although there have been recent reports of microplastic effects on mosquitoes, to the best of our knowledge there have been no reports on the effects on African malaria vectors. This is a knowledge gap as anthropophilic members of the *An. gambiae* complex is adapting to breeding in polluted waters which facilitates their spread to new habitats (Antonio-Nkondjio *et al.*, 2011). This adaptation is particularly true for *An. arabiensis*, which is increasingly adapting to urban environments (Azrag and Mohammed, 2018; Bimbilé Somda *et al.*, 2018; Fournet *et al.*, 2022). For *An. arabiensis*, this is problematic because this vector is extremely adaptable and highly flexible in feeding habits (Sinka *et al.*, 2010). Moreover, due to *An. arabiensis* predominantly biting outdoors, this species is difficult to control by conventional vector control activities which typically focus on indoor biting mosquitoes (Kitau *et al.*, 2012). As such, it is critical to understand the effect of plastic exposure on *An. arabiensis*.

Experimental Procedure

Strains used in this study

Two laboratory strains of *An. arabiensis* were used in this study. The SENN strain was colonised in 1980 from a strain originating from Gezira in Sudan (Hemingway, 1983). SENN had low-level malathion resistance but at present, the strain is primarily, but not fully, insecticide-susceptible. This strain therefore represents an insecticide unselected strain. From SENN, the SENN-DDT strain was selected by continuous exposure to 4% DDT since 1995 which continues to the present day. SENN-DDT displays resistance to DDT, permethrin, deltamethrin, λ-cyhalothrin, and malathion (Oliver and Brooke, 2017). Resistance in SENN-DDT is mediated by fixation of the L1014F mutation in the para-sodium channel gene as well as enhanced metabolic detoxification enzymes (Oliver and Brooke, 2013). Both strains were reared according to the methods described in Hunt *et al.* (2005). In brief, mosquitoes were reared at 25 ^{o}C (± 2 ^{o}C), and 75% humidity (± 5%). Mosquitoes were exposed to a 12:12 hour light: dark photoperiod with a 30-minute dawn/dusk cycle. Larvae were fed a diet of Beano™ dog biscuits and brewer's yeast (3:1) ratio and adults were allowed ad libitum access to 10% sucrose.

Larval pollutant exposure

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A range of plastic pollutants were used in this study. Fluorescent latex beads (2 µm, Sigma Aldrich: catalogue no: L0280) were used as a proxy for microplastics. A volume of 7 µl of this commercial solution was used per litre of water. Disposable nappies (Brand name Huggies ® extra care disposable nappies: Kimberly Clarke; Serial number: 5029053548647) were used as complex plastic pollutants. This product consisted of wood pulp, sodium polyacrylate, polypropylene, polyethylene, adhesives, polyester, polyurethane elastics, and polyolefin elastic. In order to produce a degraded product, a single nappy was placed in 2000 ml of reverse osmosis water and allowed to degrade for 3 months under the insectary conditions described earlier. The degraded sample was further split into two and diluted with an additional 1000 ml to produce the water that constituted the nappy sample.

The diphenylmethane derivative, Bisphenol-A (BPA) (Sigma Aldrich: catalogue no: 80-05-7) is used in the production of polycarbonate plastics and would therefore be a common plastic pollutant and serves as a representative of plastic breakdown products. A final concentration of 12.5 µM was used per 1000 ml of reverse osmosis water.

Phthalic acid dimethyl ester (1,2-benzenedicarboxylic acid) (Sigma-Aldrich: catalogue no: 131-11-3) is used as a plasticizer and is a common pollutant associated with the production of plastic and does leach into the environment. Although not a plastic breakdown product, it does serve as a pollutant associated with plastics. A final concentration of 59.5 µM was used per 1000 ml of reverse osmosis water.

In order to generate samples for sequencing, 100 first instar larvae (<24 hours after hatching) of each strain were reared in 1000 ml of each of the described plastic pollutants. Samples reared in untreated reverse osmosis water served as an untreated control. Larvae were fed a standardised amount of food and adults were collected upon emergence. The adults were allowed *ad libitum* access to 10% sucrose until the age of 3 days when females were then collected for processing.

Sample processing and DNA extraction

Three-day-old non-blood fed females were cold-killed and the external surface ethanol-sterilised. The midguts were dissected under sterile conditions. Three midguts were pooled per sample and 5 samples were used per treatment. The samples were then subjected to DNA extraction using a Qiagen DNeasy blood and tissue kit (Qiagen: Catalogue number 69506) according to the manufacturer's directions. The samples were not physically homogenised, but an overnight incubation was performed to allow full tissue dissolution. A commercial microbial

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DNA extraction control was included as an extraction positive control (ZymoBiomics ®: Catalogue D6300). A buffer-only negative control was also included.

Next Generation Sequencing and Bioinformatics

Total genomic DNA was used as a template to amplify the V3–V4 hypervariable region of the bacterial 16S rRNA gene. The primer sequences were as follows: forward primer 5′ GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3′ and reverse primer 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). A positive control ™ consisting of a commercial Microbial Community DNA Standard (ZymoBIOMICS®: D6300) and two negative controls (a non-template and an extraction buffer-only) were also amplified by PCR. Samples were sequenced at the Core Sequencing Unit at the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa, as described in Chen *et al*. (2024)

Bioinformatic analysis was performed as per (Chen *et al.*, 2024). In brief, the Nextera adapter sequences were removed by trimGalore (v0.6.5-1) and MultiQC (v1.6) was used for quality control. Clean reads were pre-processed using the DADA2 package (v1.24.0) (Callahan *et al.*, 2016), and all downstream analysis was performed in R studio (v4.2.1). The obtained amplicon sequence variants (ASVs) were assigned taxonomy and the ASV abundance estimates were determined using the SILVA reference database (v138; https://zenodo.org/record/4587955#.Y9JGXnZBxPY, accessed 10 February 2024). The phyloseq package (v1.40.0) (McMurdie and Holmes, 2013) was used to create phyloseq data objectives from the DADA2 outputs for further analysis. Alpha diversity was analysed using the Chao1 and ACE indices to assess species richness, while species diversity was assessed by the Shannon and Simpson diversity indices. The alpha diversity between the treatments was compared using the Wilcoxon rank-sum test. Ordination plots to assess beta diversity were constructed using the Nonmetric Multidimensional Scaling (NMDS) method. The data clustering for the NMDS plot was statistically assessed using a PERMANOVA (permutation test with pseudo-F ratios). Relative abundance for the top 5 genera was displayed in bar plots. UpSet plots to display shared microbial communities were generated using Venn Diagram (v1.7.3) and UpsetR (v1.4.0) (Conway *et al.*, 2017). Differential abundance analysis between sample groups was performed using DESeq2 (v1.24.0) (Love *et al.*, 2014).

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Results

Alpha diversity

The Chao1 and ACE indices represent bacterial species richness (the total number of species in a given sample). Both of these indices are sensitive to rare OTUs. For the SENN strain, the nappy and bead treatment had a significantly higher species richness than the untreated samples. This was true for the Chao1 index (p=0.032) (Figure 1A) and ACE (p=0.032) (Figure 1B). Species diversity was assessed using the Shannon and Simpson indices. In SENN, only the bead treatment had a higher Shannon diversity index than the untreated SENN (p=0.032), with no further significant differences between the treatments (Figure 1C). For the Simpson index, the BPA treatment was significantly more diverse than the untreated sample (p=0.032) (Figure 1D). This was the only difference in the treatment.

For the SENN-DDT samples, there was a slight difference in the bacterial richness indices compared to SENN. The differences were between the treatment groups rather than compared to the untreated control. For the Chao1 index, the nappy treatment had a significantly greater species richness than the phthalic acid treatment (p=0.032), BPA treatment (p=0.016) as well as the bead treatment (p=0.032) (Figure 1E). Similarly, for the ACE index, the nappy treatment was significantly higher than phthalic acid (p=0.032), beads (p=0.032) and BPA (p=0.016) (Figure 1F). In terms of species diversity, the only significant difference in the Shannon index was an increased diversity in the BPA treatment compared to the untreated samples (p=0.032) (Figure 1G). In the Simpson index, again, the BPA treatment had a significantly higher diversity than the untreated sample (p=0.008) (Figure 1H). BPA treatment was also significantly more diverse than the bead treatment in terms of the Simpson index (p=0.032) (Figure 1H)

Beta diversity

Beta diversity, assessed here by NMDS analysis, was a measure of diversity between different *An. arabiensis* strains and plastic treatments. Larval exposure to plastic treatments had a significant effect on the beta diversity of the adults (PERMANOVA: p<0.001, R^2= 0.256). Although there was not a significant effect of strain (p=0.050, R^2=0.023), there was a significant effect of interaction between strain and treatment (p<0.001, R^2=0.104).

Relative abundance

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The five most abundant bacterial genera in both strains were *Acinetobacter*, *Citrobacter*, *Elizabethkingia*, *Rahnella*, and *Yersinia*. However, the proportions of the bacteria differ by treatment. In untreated SENN, *Acinetobacter*, *Rahnella* and *Elizabethkingia* dominated. In SENN treated with beads and BPA, *Rahnella* and *Elizabethkingia* were the dominant genera. *Elizabethkingia* dominated in nappy- and phthalic acid- treated SENN, particularly in the latter. However, the next most dominant genera in the nappy-treated SENN was *Acinetobacter*, and *Rahnella* in the phthalic acid treatment (Figure 3A).

Although the patterns were similar in SENN-DDT, the proportions differed. Notably, *Yersinia* was more present in SENN-DDT than SENN. Untreated SENN-DDT, like SENN, was dominated by *Acinetobacter*, *Rahnella* and *Elizabethkingia* but in different proportions. *Acinetobacter* and *Rahnella* were more evenly distributed in untreated SENN-DDT than in SENN Like in SENN, the beads- and BPA-treated SENN-DDT were more similar to each other than other treatments where they were both dominated by *Rahnella*. However, the next most abundant genera in the bead- treated SENN-DDT was *Yersinia* while for BPA-treated SENN-DDT it was *Elizabethkingia*. Similar proportions of *Citrobacter* were present in these two treatments. *Elizabethkingia* dominated the nappy- and phthalic acid-treated SENN-DDTs, but the second most dominant genera in the nappy-treated SENN-DDT was *Yersinia*, while it was *Rahnella* in the phthalic acid- treated SENN-DDT. *Acinetobacter* proportions were similar in the nappy- and phthalic acid- treated SENN-DDT. *Yersinia* was not present in the phthalic acid-treated SENN-DDT (Figure 3B).

Overlapping species

An analysis was performed to find the shared and unique bacterial families, genera and species for each strain after exposure to different plastic treatments. In SENN, there were 26 shared families between all treatments. The most unique genera were present in the nappy treatment (18), followed by phthalic acid (13), BPA (10), untreated (8) and beads (7) (Figure 5A). In terms of bacterial genera, nappies and phthalic acid treatments result in more unique bacterial species (42 and 36 respectively) compared to the 26 genera shared by all treatments. BPA-treated SENN had 21 unique genera, beads-treated had 18 and the untreated had 15 unique genera (Figure 5B). There were 29 shared bacterial species shared across all treatments against SENN. Nappy treatment of SENN resulted in 76 unique species, phthalic acid resulted in 55 unique species, BPA resulted in 43 unique species, while the untreated and beads-treated SENN had 33 unique species respectively.

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In the SENN-DDT, the patterns were different. The untreated SENN-DDT had the most unique bacterial families (28), while there were 23 shared families across all treatments. BPA-treated SENN-DDT had 11 unique families, while phthalic acid and nappy-treated SENN-DDT both had 10 unique families. There were 7 unique families in the beads-treated SENN-DDT (Figure 5D). In terms of bacterial genera, again the untreated-SENN-DDT had the most unique genera (59). There were 24 genera shared between the treatments. BPA-treated SENN-DDT had 25 unique genera, phthalic acid had 22 unique genera, nappy treatment had 21 unique genera, and the beads had 15 unique genera (Figure 5E). There were fewer species shared between all the treatments (28) than the unique species in each treated SENN-DDT. There were 89 unique species in the untreated SENN-DDT group, 50 in the phthalic acid, 47 in the BPA treatment, 34 in the nappy and 33 in the beads (Figure 5F).

Differential abundance

Differential abundance is a pairwise comparison of the difference in abundance of bacterial genera between two groups. The abundance of treated samples was compared to that of untreated samples, and those with a log2 fold change with an alpha value of under 0.01 were plotted. In general, the SENN strain had more differentially abundant genera in the plastic-treated samples. When comparing the beads-treated SENN to the untreated SENNs there were 13 more differentially abundant genera in the bead treatment compared to 12 in the untreated samples (Figure 5A). In the BPA-treated SENN, there were 16 differentially abundant genera compared to 9 in the untreated SENN (Figure 5B). In the phthalic acid-treated SENN, there were 11 genera differentially abundant compared to 8 in the untreated SENN (Figure 5C). In nappy-treated SENNs, there were 27 differentially abundant genera compared to 8 in the untreated SENN (Figure 5D).

This pattern was generally reversed in the SENN-DDT strain. The beads-treated SENN-DDT had 11 differentially abundant genera compared to 18 in the untreated SENN-DDT (Figure 5E). The BPA-treated SENN-DDT samples had 19 differentially abundant genera compared to 17 in untreated SENN-DDT (Figure 5F). The phthalic acid-treated SENN-DDT samples had 10 differently abundant genera compared to 18 in the untreated SENN-DDT samples (Figure 5G). For the nappy-treated SENN-DDT samples, 11 bacterial genera were differentially abundant compared to 22 in the untreated SENN-DDT samples (Figure 5H).

Discussion

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Although the challenge of plastic pollution is well documented (Welden, 2020), the focus tends to be on marine environments. The effects on freshwater organisms, particularly insects are less well described. Of the few descriptions of plastic on insects, the focus was on *Aedes* and *Culex* mosquitoes, and these studies focused on the effects of microplastics (Al-Jaibachi *et al.*, 2018, Al-Jaibachi *et al.*, 2019; Edwards *et al.*, 2023; Griffin *et al.*, 2023; Li *et al.*, 2024). This study focused on the major malaria vector *An. arabiensis* and focused on plastic derived from natural sources (latex beads), plastic additives (BPA, phthalic acid) and a degraded common plastic pollutant (disposable nappies). These plastic sources were chosen to represent a common pollutant in general, as well as the breeding sites of the *An. gambiae* complex in South Africa (Kordecki *et al.*, 2022; Muringaniza *et al.*, 2023; Schenck *et al.*, 2023; Rosser *et al.*, 2024). As such, this study is a generalised study on the effect of plastic pollution on the mosquito microbiome rather than a study of microplastics alone.

Furthermore, the effects of an insecticide-resistant phenotype are considered as this is known to affect the gut microbial composition (Dada *et al.*, 2018; Singh *et al.*, 2022). However, it was found that the insecticide-selected phenotype (SENN-DDT) had a limited effect on the alpha and beta diversity of microbial composition after treatment. In general, the different plastic treatments had a limited effect on the alpha diversity in both strains. In the SENN strain, the only differences in alpha diversity were between untreated and specific treatments rather than between the treatments themselves. In the SENN-DDT strain, the nappy-treated specimens had greater species richness than bead and BPA treatments but not the untreated. In terms of species diversity in SENN-DDT, BPA treatment was notable as having greater diversity than the control and bead treatment. Although there is not a large precedent for comparison, it is worth noting that microplastic treatment increased alpha diversity in both *Ae. albopictus* and *Ae. aegypti* (Edwards *et al.*, 2023). The only notable change in richness was in the nappy treatment in SENN-DDT, and this change may be analogous to the findings in *Aedes*. In both the present study and the *Aedes* study, plastic treatment altered beta diversity. The effect of the different treatments had a significant effect on beta diversity, but this effect was not strain-specific. This suggests that the insecticide-resistant phenotype plays a limited role in affecting the changes in diversity seen after plastic treatment.

When examining the relative abundance of the five most common bacterial genera, the untreated samples were the most distinct in both strains. In terms of the treatments, for both strains, the beads and BPA-treated mosquitoes were more similar to each other than to the nappy and phthalic acid-treated mosquitoes, which were similar to each other. The dominant

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genera in both strains were *Rahnella*, *Elizabethkingia* and *Acinetobacter*. It is notable that microplastic pollution increased the abundance of *Elizabethkingia* in both *Ae. albopictus* and *Ae. aegypti* increased (Edwards *et al*., 2023) as well as *Cx. quinquefasciatus* (Li *et al.*, 2024). This is notable as *Elizabethkingia* has been known to form biofilms on plastic surfaces(Jacobs and Chenia, 2011) suggesting that changes in this genus may be directly due to the interactions with plastic and the bacteria present on the plastic surfaces. When examining the overlapping species, it is notable that there were fewer species shared among treatments than the numbers of species unique to the treatments. This was also observed in *Ae. aegypti* and *Ae. albopictus* (Edwards *et al.*, 2023) . Furthermore, it is when looking at specific genera and species, rather than overall diversity, that the effect of insecticide-resistant phenotype starts coming into effect. In the SENN strain, the nappy-treated samples had the most unique bacterial species, followed by phthalic acid, BPA, untreated and beads. In contrast, for the SENN-DDT strain, the untreated samples had the most unique species, followed by phthalic acid, BPA, nappy treatment and beads treatment.

When looking at differential abundance, there is a clear pattern in SENN where the treatment resulted in more abundant genera, while in SENN-DDT the untreated samples had more differentially abundant genera when compared to the treatments. This was also a pattern that was observed when larvae were reared in metal-treated water (Singh *et al.*, 2024). This pattern may be related to the insecticide-resistant phenotype. SENN-DDT larvae typically survived better in more toxic pollutants than SENN (Oliver and Brooke, 2018b, Oliver and Brooke, 2018a). This suggests that the elevated detoxification enzymes contributed to the improved survival in SENN-DDT, but SENN did not have a protective effect. The increased abundance of bacteria, particularly pesticide and plastic-degrading bacteria, in treated SENN suggests that this may be a response to the pollutant. This is particularly notable as this has been observed before in the same strains. Thus, the finding suggests that in the absence of an established metabolic detoxification system, bacteria could protect the larvae from toxicants as this effect carries over to the adult.

Plastics are associated with enriched microbial growth (Wu *et al.*, 2019; Sheridan *et al.*, 2022) but bacteria are also capable of degrading plastics (Cai *et al.*, 2023). It should therefore not be surprising that there was an increased abundance of bacterial genera and species associated with plastic degradation which were dominant in the plastic-treated samples. The most common plastic-degrading genera found in both strains were *Acinetobacter* (Mohanan *et al.*, 2020), *Corynebacterium* (Helleckes *et al.*, 2023) , *Cutibacterium* (Park *et al.*, 2021),

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Enterococcus (Hou and Majumder, 2021), *Gluconobacter* (Kim *et al.*, 2019) , *Herbapirillum* (Li *et al.*, 2018), *Microbacterium* (Sun *et al.*, 2022) , *Pseudomonas* (Wilkes and Aristilde, 2017), *Sphingomonas* (Pinyakong *et al.*, 2004), *Staphylococcus* (Nowak *et al.*, 2011), *Streptococcus* (Venkatesh *et al.*, 2021), *Veillonella* (Zhu *et al.*, 2023). It is worth noting that nappy treatment in SENN resulted in the most plastic-degrading bacteria while BPA resulted in the most pesticide-degrading bacteria.

However, larval exposure to a pollutant not only results in an enhanced response to the specific xenobiotic but xenobiotics in general (Oliver and Brooke, 2018b, Oliver and Brooke, 2018a). This holds true in the microbial response as well. Larval exposure to metal increased insecticide resistance in the strains, even after a new generation bred in unpolluted water. This was due to a generalised increase in metabolic detoxification enzymes (Jeanrenaud *et al.*, 2020). However, there was also an increase in pesticide-degrading bacterial genera in the strains bred in metal-polluted water. This increase in pesticide-degrading bacteria was also found in F1 *An. arabiensis*, demonstrating that this increased effect was present in wild mosquitoes as well (Singh *et al.*, 2024). The most common genera associated with pesticide-degrading bacteria found in the treatments were *Acinetobacter* (Wang *et al.*, 2021), *Cutibacterium* (Muturi *et al.*, 2021), *Corynebacterium* (Muturi *et al.*, 2021), *Herbaspirillum* (Venkatachalam *et al.*, 2023), *Microbacterium* (Almeida *et al.*, 2017), *Micrococcus* (Dar and Kaushik, 2022), *Pseudomonas* (Dada *et al.*, 2019; Wang *et al.*, 2021; Gómez-Govea *et al.*, 2022) and *Sphingomonas* (Lv *et al.*, 2023) . Like with the plastic-degrading bacteria, in SENN, the nappy treatments resulted in the most bacterial genera and BPA treatment resulted in the most pesticide-degrading genera.

An additional concern is the potential effect of plastic pollution on vector competence. This capacity would be modulated by alteration of the immune system which can occur by two mechanisms. The first mechanism is the alteration of the balance of bacteria associated with protection against *Plasmodium*. This balance was not widely affected by any of the experimental treatments. When looking at differential abundance, there were not many genera found abundant in either the treated or untreated groups. In the SENN strain, although few, most *Plasmodium* protective genera were differentially abundant in the untreated strains. When analysing the beads treatment, there were three *Plasmodium* protective genera in the untreated, one shared and one present in the bead treatment. In phthalic acid, there were three in the untreated and one in the phthalic acid treatment. The nappy treatment had three, one shared with the untreated and two in the untreated. When comparing BPA, there was one *Plasmodium* protective genera in the untreated, one shared and three in the BPA treatment.

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This was similar in SENN-DDT. There were three *Plasmodium* protective genera differentially abundant in the untreated compared to the beads and one genus was shared. There was no *Plasmodium* protective species differentially abundant in the bead treatment. Five *Plasmodium* protective genera were differentially abundant in the untreated, one was shared, and one was differentially abundant in BPA treatment. When comparing phthalic acid, three genera were differentially abundant in the untreated, one was shared and two were differentially abundant in phthalic acid. Three genera were differentially abundant in the untreated, one shared and one differentially abundant in the nappy treatment. Although this is not a clear pattern, there appears to be fewer protective genera in the treated mosquitoes than in the untreated mosquitoes This observation needs to be examined further, particularly in wild specimens. Therefore, it is still unclear as to whether microbial manipulation by plastic exposure will directly alter mosquito vectorial capacity (Jones *et al.*, 2024) .

What is known is that microplastic exposure does alter the mosquito's immune response. Microplastic ingestion has been demonstrated to increase phenoloxidase activity as well as oxidative stress (Silva *et al.*, 2021a; Silva *et al.*, 2021b). Both of these factors would reduce *Plasmodium* infection. Inflammatory-type responses occur in insects as this is an innate response (Chain and Anderson, 1983) . Several bacteria associated with inflammatory responses occur after plastic exposure. The most common of these genera found in the treatments are *Acinetobacter*, *Aeromonas*, *Corynebacterium*, *Cutibacterium*, *Enterococcus*, *Gluconobacter*, *Limnosilactobacilus*, *Pseudomonas*, *Sphingomonas*, *Staphylococcus*, *Streptococcus* and *Veillonella*. If inflammatory responses are more common in plastic-treated mosquitoes, this could potentially reduce the capacity to transmit parasites.

There were two notable findings in this study. The genera *Afpia* was found differentially abundant in beads-treated SENN. The first report of *Afpia* was in *Aedes* in 2022 (da Silva *et al.*, 2022). As such, to the best of our knowledge, this is the first report of *Afpia* in the *Anopheles* mosquitoes. The second finding is the differential abundance of *Wolbachia* and *Asaia* in nappy-treated SENN-DDT. This is notable in two regards. The first is that *Wolbachia* was found in a laboratory strain of *An. arabiensis*, and this genus is not common in *Anopheles* mosquitoes, particularly in laboratory strains where the microbiota is reduced (Chrostek and Gerth, 2019; Wong *et al.*, 2020) . *Asaia* has been known to impede the transmission of *Wolbachia*, so finding both of these differentially abundant in treated SENN-DDT is unusual.

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It must be noted that this study has several limitations. We do not know whether the concentrations of plastic pollutants used in this study are relevant to mosquito breeding sites. This is a general problem with plastic studies, as although there are records of microplastic and additive pollutants in water, the concentrations of these pollutants in the temporary breeding sites are unclear (Jones *et al.*, 2024). Therefore, the concentrations used allowed for the survival of the strains to allow reasonable numbers of mosquitoes. As such, they may not be fully ecologically relevant. Crucially, this study must be replicated with wild *An. arabiensis*. It has been demonstrated that wild mosquitoes have a greater bacterial diversity than their laboratory-reared counterparts (Saab *et al.*, 2020). Therefore, despite the large-scale changes observed, the findings are likely to be an underrepresentation of the changes in wild microbiota. Finally, further studies are crucial to determine the exact effects of plastic on the immune system of *An. arabiensis*.

In conclusion, larval exposure to plastic pollution had a large-scale effect on the microbiota of adult *An. arabiensis*. Although insecticide-selected phenotype was not as important as treatment in altering diversity in general, it did affect the patterns of bacterial abundance in the adults. The insecticide-unselected strain had a greater abundance of bacteria in response to degraded plastic while the insecticide-selected strain had more genera abundant in the untreated group. This suggests that the increased abundance of bacteria in response to plastic treatment may serve a protective function in the absence of a pre-existing resistant phenotype. Larval exposure to plastic products increased the abundance of genera associated with both plastic and insecticide degradation. The abundance of *Plasmodium*-protective genera was not significantly affected by the exposure to plastics, but bacteria associated with inflammation were significantly more abundant. These results suggest that larval exposure to plastic products has the capacity to alter microbial composition in a manner that could have epidemiological consequences.

Figure legends

Figure 1: Alpha diversity in *Anopheles arabiensis* adults reared in plastic-treated water. A: Species richness measured by Chao1 index in SENN. B: Species richness measured by ACE index in SENN. C: Species diversity measured by Shannon index in SENN. D: Species diversity measured by Simpson index in SENN. E: Species richness measured by Chao1 index in SENN-DDT. F: Species richness measured by ACE index in SENN-DDT. G: Species

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diversity measured by Shannon index in SENN-DDT. H: Species diversity measured by Simpson index in SENN-DDT. The *p*-values were determined by the Wilcoxon rank-sum test. Significant *p*-values (<0.05) are shown in larger text.

Figure 2: Beta diversity in *Anopheles arabiensis* adults reared in plastic-treated water. Non-metric multidimensional scaling ordination of diversity in SENN and SENN-DDT adults bred in water treated with plastics. The NMDS plot was based on the Bray-Curtis distance measurements. The stress value was 0.19. A PERMANOVA indicated $p<0.001$ for treatments, $p=0.05$ for strain and $p<0.001$ for treatment: strain. Circles represent the SENN strain and triangles represent SENN-DDT.

Figure 3: Relative abundance of the top 5 most abundant bacteria in *Anopheles arabiensis* adults reared in plastic-treated water. A: Relative abundance of SENN adults reared in water treated with beads, BPA, nappies, phthalic acid or untreated water. B: Relative abundance of SENN-DDT adults reared in water treated by beads, BPA, nappies, phthalic acid or untreated water.

Figure 4: Upset plots displaying overlapping bacteria in *Anopheles arabiensis* adults reared in plastic-treated water. A: Overlapping families in SENN. B: Overlapping genera in SENN. C: Overlapping species in SENN. D: Overlapping families in SENN-DDT. E: Overlapping genera in SENN-DDT. F: Overlapping species in SENN-DDT. Individual dots indicate numbers of individual bacterial families, genera or species. Joined dots indicate the numbers of shared bacterial families, genera or species between the different plastic treatments.

Figure 5: Differentially abundant bacterial genera in *Anopheles arabiensis* adults reared in plastic-treated water. A: Comparison of beads-treated samples (left) vs untreated samples (right) in SENN. B: Comparison of BPA-treated samples (left) vs untreated samples (right) in SENN. C: Comparison of phthalic acid-treated samples (left) vs untreated samples (right) in SENN. D: Comparison of nappy-treated samples (left) vs untreated samples (right) in SENN. E: Comparison of beads-treated samples (left) vs untreated samples (right) in SENN-DDT. F: Comparison of BPA-treated samples (left) vs untreated samples (right) in SENN-DDT. G: Comparison of phthalic acid-treated samples (left) vs untreated samples (right) in SENN-DDT. H: Comparison of nappy-treated samples (left) vs untreated samples (right) in SENN-DDT. Numbers on either side of the 0 represent log2fold change of bacterial genera that are significantly different $\alpha = 0.01$. Each dot represents a single OTU.

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Authors Contributions:

Shristi Misser: investigation, formal analysis, visualisation, data curation, software, writing-review and editing. **Chia-Yu Chen:** supervision, formal analysis, software, writing- review and editing. **Arshad Ismail:** resources. **Shüné V. Oliver:** supervision, conceptualisation, funding acquisition, project administration, resources, writing-original draft preparation. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

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Institutional Review Board Statement

The animal study protocol was provided with an ethics waiver by the Animal Research Ethics Committee of the University of the Witwatersrand as all work was conducted on Dipteran invertebrates (protocol code: S Oliver 29-02-2024-O approved on 29 February 2024).

Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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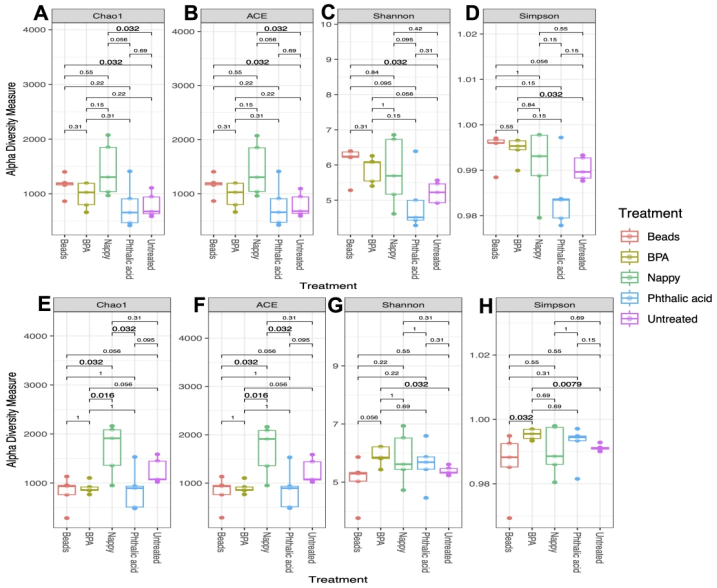


Figure 1: Alpha diversity in Anopheles arabiensis adults reared in plastic-treated water. A: Species richness measured by Chao1 index in SENN. B: Species richness measured by ACE index in SENN. C: Species diversity measured by Shannon index in SENN. D: Species diversity measured by Simpson index in SENN. E: Species richness measured by Chao1 index in SENN-DDT. F: Species richness measured by ACE index in SENN-DDT. G: Species diversity measured by Shannon index in SENN-DDT. H: Species diversity measured by Simpson index in SENN-DDT. The p-values were determined by the Wilcoxon rank-sum test. Significant p-values (<0.05) are shown in larger text.

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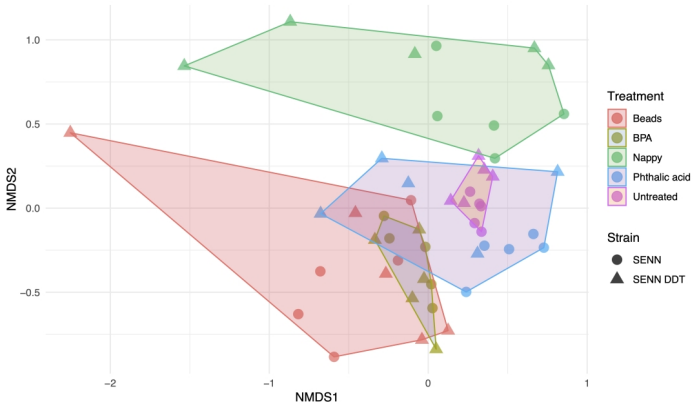


Figure 2: Beta diversity in Anopheles arabiensis adults reared in plastic-treated water. Non-metric multidimensional scaling ordination of diversity in SENN and SENN-DDT adults bred in water treated with plastics. The NMDS plot was based on the Bray-Curtis distance measurements. The stress value was 0.19. A PERMANOVA indicated $p<0.001$ for treatments, $p=0.05$ for strain and $p<0.001$ for treatment: strain. Circles represent the SENN strain and triangles represent SENN-DDT.

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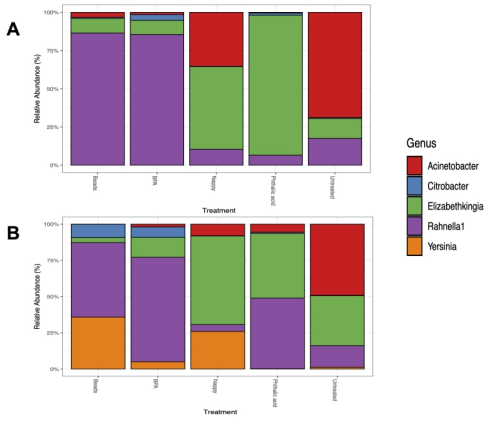


Figure 3: Relative abundance of the top 5 most abundant bacteria in Anopheles arabiensis adults reared in plastic-treated water. A: Relative abundance of SENN adults reared in water treated with beads, BPA, nappies, phthalic acid or untreated water. B: Relative abundance of SENN-DDT adults reared in water treated by beads, BPA, nappies, phthalic acid or untreated water.

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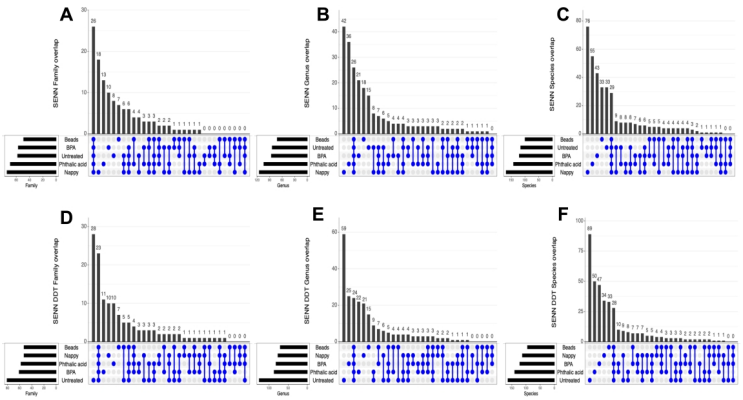


Figure 4: Upset plots displaying overlapping bacteria in Anopheles arabiensis adults reared in plastic-treated water. A: Overlapping families in SENN. B: Overlapping genera in SENN. C: Overlapping species in SENN. D: Overlapping families in SENN-DDT. E: Overlapping genera in SENN-DDT. F: Overlapping species in SENN-DDT. Individual dots indicate numbers of individual bacterial families, genera or species. Joined dots indicate the numbers of shared bacterial families, genera or species between the different plastic treatments.

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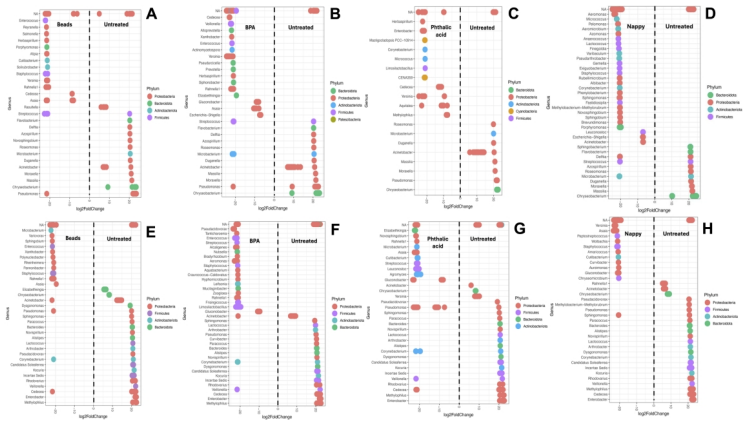


Figure 5: Differentially abundant bacterial genera in Anopheles arabiensis adults reared in plastic-treated water. A: Comparison of beads-treated samples (left) vs untreated samples (right) in SENN. B: Comparison of BPA-treated samples (left) vs untreated samples (right) in SENN. C: Comparison of phthalic acid-treated samples (left) vs untreated samples (right) in SENN. D: Comparison of nappy-treated samples (left) vs untreated samples (right) in SENN. E: Comparison of beads-treated samples (left) vs untreated samples (right) in SENN-DDT. F: Comparison of BPA-treated samples (left) vs untreated samples (right) in SENN-DDT. G: Comparison of phthalic acid-treated samples (left) vs untreated samples (right) in SENN-DDT. H: Comparison of nappy-treated samples (left) vs untreated samples (right) in SENN-DDT. Numbers on either side of the 0 represent log2fold change of bacterial genera that are significantly different α = 0.01. Each dot represents a single OTU.

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Appendix 5: Turnitin report

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