SURVIVAL, BEHAVIOURAL CHANGES AND GEOTAXIS RESPONSE OF *CULEX* SP. LARVAE (CULICIDAE) AFTER EXPOSURE TO CARBARYL AND PYMETROZINE

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Declaration

I, Nonhlahla Radebe, declare that the Master's degree research dissertation or interrelated, publishable manuscripts/ published articles, that I herewith submit at the University of the Free State is my independent work, and that I have not previously submitted it for qualification at another institution of higher education.

Signature:

Radebe

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Table of contents

Abstract

Pesticides play a crucial role in agriculture and are extensively used in developing countries to ensure food security and economic prosperity. However, as the climate is changing (rise in temperature and precipitation), the efficacy of pesticides is reduced leading to more frequent application and extensive use of pesticides causing greater adverse effects on aquatic systems. South Africa is not spared from this pesticide pollution. To mitigate the environmental effects of pesticides in South Africa, (particularly QwaQwa where many farms are situated), the least toxic pesticides such as pymetrozine (a selective "Environmental Protection Agency Reduced Risk pesticide") should be preferably used and recommended for Integrated Pesticides Management (IPM) whereas highly toxic ones such as carbaryl should be phased out to reduce pesticide pollution and unintended death of non-target aquatic organisms. The present comparative study aimed to evaluate the effects of carbaryl and pymetrozine on the behaviour of mosquito larvae (*Culex* sp.). Mortality experiments showed that carbaryl (0, 12.50, 25, 50 and 100 µg/L) caused more mortality than pymetrozine (0, 6.25, 12. 50, 25 and 50 mg/L), and exposure to both insecticides altered the breathing, swimming and resting behaviours of the mosquito larvae. There was mortality in all tested concentrations, except for distilled water (control). The highest mortality was recorded in 100 µg/L of carbaryl. Carbaryl had an LC$_{50}$ of 0.028 mg/L, whereas pymetrozine generated a lethal concentration 50 (LC $_{50)}$ of 181.950 mg/L, this proves the high toxicity of carbaryl as the LC$_{50}$ of carbaryl is more than 100 times smaller than that of pymetrozine. The larvae spent more time breathing in the absence of these insecticides, more time swimming in the presence of carbaryl and more time resting in the presence of pymetrozine. Moreover, these toxicants altered the geotaxis of these organisms thus compromising their fitness. The larvae predominantly displayed negative geotaxis (spent more time breathing) when exposed to both these insecticides. From these findings, it can be noted that pymetrozine was the least toxic of the two pesticides. Hence, pymetrozine can be recommended for IPM. This was supported by a systematic review of 24 years of carbaryl effects on insect behaviour. The overall results revealed that carbaryl should be phased out in favour of less toxic yet effective insecticides like pymetrozine.

Keywords: Behaviour, Carbaryl, Climate change, Culicids, Pollution, Pymetrozine,

List of Figures

Chapter 2

Chapter 3

Chapter 4

List of tables

Chapter 2

Chapter 3

Appendices

List of abbreviations

AChE: Acetylcholinesterase

APVMA: Australian Pesticides Veterinary Medicines Authority

CDC: Centers for Disease Control and Prevention

CI: Confidence Interval

CNS: Central Nervous System

EPA: Environmental Protection Agency

EU: European Union

IPM: Integrated Pest Management

IRAC: Insecticide Resistance Action Committee

LC: Lethal Concentration

NPIC: National Pesticide Information Centre

ns: not significant

PCR: Polymerase Chain Reaction

PPC: Pyridazine Pyrazole Carboxamides

SD: Standard Deviation

sp: species

Std: Standard

TRPV: Transient Receptor Potential Vanilloid

US: United States

USDA: United States Department of Agriculture

USEPA: United States Environmental Protection Agency

WHO: World Health Organization

Chapter 1

General Introduction

1.1 Pesticides use in the environment

Pesticides are chemicals intentionally released into the environment to control pest populations, which include insecticides, fungicides, nematicides, herbicides, rodenticides and molluscicides (Tudi *et al.* 2021). Pesticides can be naturally occurring (for example, pyrethrins and rotenone) or man-made (synthetically produced) (for example, carbamates and organophosphates) (Mahmood *et al*. 2016). Among all the categories of pesticides, the most toxic ones are insecticides (Mahmood *et al*. 2016). Carbamates, neonicotinoids, organophosphates, organochlorines and pyrethroids are examples of classes of pesticides commonly used (Mahmood *et al*. 2016). There are over 3000 different types of pesticides registered for use worldwide, with some being neurotoxic toxicants while others are endocrine-disrupting (Chetty-Mhlanga *et al*. 2021). Pesticides play a critical role in agriculture by protecting crops from pest damage, and in public health by controlling vectors of diseases (disease management) (Mahmood *et al*. 2016). The use of pesticides in agro-ecosystems and public health quickly increased after World War II and the first effective pesticides were introduced in the 20th century (Tudi *et al*. 2021, Riaz *et al*. 2024). These chemicals have contributed significantly to alleviating hunger, promoting an abundant supply of high-quality food, fostering disease control, and boosting economies (Tudi *et al*. 2021). Pesticide exposure in animals and humans causes acute and chronic poisoning, Guillan- Barrè syndrome, autism, endocrine disruption and birth defects in humans (Pabrowski 2014). Moreover, there is evidence that pesticides cause different types of cancers, such as bladder cancer and leukemia (Sabzevari & Hofman 2022).

The advantages of using pesticides to control pests are that the toxicants are quick to act, easy to use, efficient and cost-effective (Aktar *et al*. 2009). However, extensive use of these chemicals causes damage to flora and fauna and stops plants from absorbing important nutrients needed for growth and survival (Sharma *et al.*2019). Pesticides have become huge stressors in aquatic ecosystems (Trekels *et al*. 2013). The residues of these toxicants decimate aquatic larvae of insects and crustaceans, including zooplankton (Sànchez-Bayo 2011). Insecticides have been noted to mostly influence aquatic organisms as opposed to terrestrial organisms because aquatic organisms are evolutionarily old and their primitive isoenzymes of cytochrome P450 are not able to degrade or get rid of toxicants that enter their bodies (Sànchez-Bayo 2011).

Pesticides enter water bodies by leaching through the soil, drift, and direct application into water when controlling or killing mosquito larvae and other pests (Mahmood *et al*. 2016). When pesticides enter the water, they negatively affect aquatic organisms, which may lead to death and loss of biodiversity of both aquatic and terrestrial organisms that feed on aquatic prey (Mahdavi *et al*. 2013, Zhang *et al*. 2018). Pesticides have been noted to decrease dissolved oxygen in water, cause oxidative stress and cause behavioural and physiological changes in exposed populations (for example, fish such as the Chinook salmon) (Scholz *et al*. 2012). These chemicals may also cause changes in the ultrastructure of the exposed organism, for example, malformations of mitochondria (Adamski 2007). These chemicals can completely change the ecological structure of earthworms in soil and result in the death of birds that feed on contaminated earthworms (Sànchez-Bayo 2011). It was estimated that about one percent of the released insecticides reach their target, and the rest ends up as toxicants in the environment, affecting unintended organisms (Tolera 2020).

Pesticide application mostly causes dire effects and behavioural changes in unintended organisms (Erhunmwunse *et al*. 2012). For example, fat soluble pesticides get absorbed by fatty tissues in animals, a process known as bioamplification, resulting in the death of organisms at higher trophic levels (Mahmood *et al*. 2016). Pesticides have been observed to cause a decline in bee populations, causing colony collapse disorder, direct mortality of bees, and increased risks of *Nosema* infection (fungal infection) (Sharma & Abrol 2014, Patrzalek *et al*. 2021). In addition, insecticides also decrease the populations of beneficial insects such as insects' natural enemies and parasitoids, leading to an increased number of pests and secondary pest outbreaks (Wang *et al*. 2013). Additionally, insecticide application causes imbalances between hosts and natural enemies. For example, these chemicals cause changes in the mobility of parasitoids, reducing their ability to successfully catch and control pests (Madhavi *et al*. 2013).

Pesticides are persistent in the environment. The degradation of pesticides in the environment results in the formation of new toxic chemicals, posing a threat to aquatic and terrestrial organisms years after the release of the chemicals (Tudi *et al*. 2021). Pesticide residues have been previously found in food, milk, and water consumed by people, making them sick (Ulfah *et al*. 2022). Furthermore, one study shows that pesticides may have delayed impacts on the exposed organisms and future generations of the exposed organisms, as some chemicals do not immediately cause anatomical malformations and behavioural deficits during the development of the young but may cause these defects as time progresses (Weis 2014).

As the climate changes (with rising temperature and precipitation), the efficacy of pesticides is reduced, leading to more frequent applications and extensive potentially causing greater adverse effects on the environment (Perin *et al*. 2022). Moreover, it has been observed that some organisms can develop resistance to commercially available insecticides, which limits the effectiveness and use of these chemicals (Rezende- Teixeira *et al*. 2022). Because of repetitive application, exposed organisms may end up developing cross-resistance (Jaffar *et al*. 2022). In the long run, the frequent use of pesticides may cause them to lose their effectiveness in the future. To help mitigate or prevent more pesticide pollution, there are regulatory laws in place in more developed countries such as Canada and the United States of America. The EU recently proposed a law to protect and restore soils through a reduction of 50% usage of the most hazardous pesticides by 2030 (da Graça Silva *et al.* 2022).

1.2 Carbaryl

Carbaryl is a broad-spectrum insecticide that is registered for use in agriculture and homes for insect pest control (Mahajan *et al*. 2007). Carbaryl is used to control sucking and chewing insect pests such as beetles and moths that cause drastic damage to crops (Gao *et al*. 2022). It is also used as an ectoparasiticide for the treatment of lice in humans (Mahajan *et al.* 2007). Carbaryl inhibits the AChE enzymes in the nervous system resulting in uncontrolled movement and death of the organism (Margus *et al.* 2021). It is also classified as an Endocrine-disrupting chemical, and such chemicals tend to be highly toxic and highly persistent in the environment because they accumulate in body tissues (Mnif *et al.* 2011). Because this insecticide accumulates in body tissues, it causes reproductive abnormalities, reduce immunity and increase susceptibility to diseases (Clotfelter *et al*. 2004). Carbaryl mostly affects non-target organisms and causes adverse effects in populations of aquatic organisms, disrupting their population dynamics (Clotfelter *et al.* 2004). For example, when rainbow trout (*Oncorhynchus mykiss*) individuals were exposed to carbaryl, the fish became more vulnerable to predation by largemouth bass, increasing mortality of the trout (Little *et al.* 1990). In addition, when tadpoles were exposed to the same chemical, feeding in the presence of predators increased, heightening the chances of death of these juvenile gray treefrog (*Hyla versicolor*) (Bridges 1999). Carbaryl is also proven to be highly toxic to beneficial insects such as bees (Bond *et al*. 2016). Unfortunately, bees are rapidly decreasing currently (Zattara & Aizen 2021), so the availability and use of carbaryl should be reconsidered.

Many highly toxic insecticides, such as carbaryl, have been banned in Western nations but are still readily available on the world market (Ecobichon 2001). Yet this pesticide is extremely toxic and often poorly controlled and managed due to a lack of education on handling and use, as witnessed by known cases of carbaryl poisoning and deliberate use in suicide attempts (Eyhorn *et al*. 2015, Poudel *et al.* 2020). 99 % of deaths that occur due to pesticide poisoning worldwide, occurs in developing countries (Kesavachandran *et al.* 2009). Part of the reason carbaryl is banned and its use is restricted in other parts of the world is due to its ability to be detected in the environment (water and soil) years after application (Chow *et al*. 2023). For example, carbaryl was detected in soil 1 to 2 years after its initial application (WHO 1994).

1.3 Pymetrozine

Pymetrozine {4, 5-dihydro-6-methyl-4-[(3-pyridylmethylene)-amino] − 1, 2, 4-triazine-3(2H)-one} is a novel insecticide that was relatively recently registered for use in the 1990s (Ausborn *et al.* 2005). It possesses a remarkable selectivity for plant-sucking insects, including aphids, whiteflies and plant hoopers (Ausborn *et al.* 2005). Pymetrozine is also known by the trade names Fulfill, Endeavor, and Chess 250 WP (Bextine *et al*. 2004). In addition, pymetrozine is classified as a selective “EPA Reduced Risk pesticide” (Smitley *et al.* 2019). This insecticide selectively targets the feeding behaviour of exposed organisms, causing the death of the organisms due to hunger, though the mode of action of this insecticide is not completely understood (EPA 2000). This insecticide is proven to be less toxic to non-target organisms such as birds and beneficial insects such as *Apis mellifera* (honeybees) and *Coccinella undecimpunctata* (predators of aphids), and other insects such as predatory true bugs and lacewings (Chang & Snyder 2008; Cabral *et al*. 2011, Badaway *et al*.2015), making it ideal for Integrated Pest Management - IPM (Cabral *et al*. 2011).

In China, pymetrozine has been widely applied on rice and other crops as a substitute for organophosphorus pesticides because of its low toxicity and effectiveness (Yu *et al*. 2018). This insecticide has been classified as a harmless pesticide according to the International Organization for Biological and Integrated Control Scale (Yu *et al*. 2018). Pymetrozine has low toxicity in the environment, is not persistent and poses little to no threat to human health and the environment (Jansen *et al.* 2011). It has been documented that this pesticide has short half-lives in water and soil, and degrades relatively quickly in the environment, becoming undetectable within 42 days. (Tudi *et al*. 2023).

1.4 Mosquitoes

Mosquitoes are divided into three main genera *Aedes, Culex and Anopheles,* known to disseminate human disease such as Zika virus, malaria, yellow fever and dengue (Dale & Knight 2008. They play a critical role in public health because there is no efficient pharmacological treatment to control these parasitosis (Dale & Knight 2008, Cuervo-Parra *et al*. 2016, Rezende- Teixeris *et al*. 2022). Furthermore, *Culex* mosquitoes can transmit yeast infection *Candida parapsilosis* (Bozic *et al*. 2017). Because of their transmission of these deadly diseases, it is important to control mosquitoes as they negatively affect human health and the economic growth of countries. For instance, *Culex* sp. transmits Japanese encephalitis virus, which kills approximately 30% of the infected patients annually (Dahmana & Mediannikov 2020), hindering people from working, hampering the economic development of the affected areas (Tomori 1999, Bloom *et al*. 2018). An outbreak of the virus can overwhelm health facilities, thus causing the need for more money to be invested in the health system for vaccinating and treating infected people (Bloom *et al*. 2018, Kehnort 2021). According to Campbell- Lendrum *et al*. 2015, yearly, there are more than one million deaths caused by vector-borne and neglected tropical diseases such as malaria. Despite female mosquitoes being vectors of illness, male mosquitoes are pollinators and play an important role in food production.

Larval mosquitoes are aquatic and do not transmit diseases (Day 2016). Larvae are important in the food chain because they are a food source for a wide range of animals including dragonflies, damselflies, larvae of caddisflies, frogs and fish. *Toxorhynchites* (a genus of mosquitoes) are also predators of larval mosquitoes (Vinogradov *et al*. 2022). Due to climate change and increase in temperature, the populations of mosquitoes are also expected to increase, leading to the possibility of an increase in epidemic outbreaks (Perrin *et al*. 2022). To mitigate epidemic outbreaks, larval stages of mosquitoes must be controlled, and increased pesticide application might be necessary. The proven most effective way to decrease infection by mosquitoes is to kill the vectors themselves through pesticide application (Baz *et al*. 2022), but unfortunately mosquitoes have developed resistance to most pesticides (Liu 2015). Pesticide application to control mosquitoes is linked to disruption of wetlands (Dale & Knight 2008). This is bad for the ecosystem because wetlands are habitats to a diverse organism such as insects and birds (Hu *et al*. 2017). These habitats help refill groundwater and purify water from other aquatic systems (Hu *et al*. 2017). Thus, to reduce wetlands degradation other ways to control mosquitoes such as biological control of mosquitoes and the use of essential oils from plants such as garlic (*Allim sativum*), sweet violet (*Viola odorata*) *and* green tea (*Camellia sinensis)* have been considered (Baz *et al*. 2022).

1.5 Behavioural changes and geotaxis responses

In behavioural ecotoxicology, it is vital to assess all the factors that may contribute to changes in the behaviour of organisms when exposed to toxicants (Motz & Alberts 2005). Seldom studies have investigated non-ubiquitous behaviour factors that may be significantly altered by chemical toxicity, such as local enhancement and geotaxis (Stander *et al*. 2019). Geotaxis is the directional movement of organisms in response to gravity (Known *et al*. 2016). Geotaxis responses can be positive or negative. An organism displays positive geotaxis by moving downwards whereas negative geotaxis is displayed when an organism orients itself and moves upwards against gravitational forces (Shirley & Shirley 1988, Hader & Hemmersbach 2017). Geotaxis is an innate response in all living organisms that has remained constant in relation to changing environmental parameters such as climate and vegetation (Balaban *et al*. 2011, Bae *et al.* 2016).

The direction of gravity in living organisms does not change during evolution, but climate and vegetation have changed (Balaban *et al*. 2011). This makes geotaxis a valid and valuable parameter to consider when studying the behaviour of organisms (Motz & Alberts 2005). Moreover, geotaxis has been studied for more than 100 years in unicellular organisms (Pettersson & Ekelund 2006). Geotaxis is an important factor in both aquatic and terrestrial organisms. In aquatic organisms, it helps the organism stay or move in water to optimal conditions for growth, survival and reproduction (Sineshchekov *et al.* 2000). In terrestrial organisms, gravity helps flying organisms to fly successfully (Bae *et al.* 2016), insects to find food oviposit in proper environments (Dethier 1952), and other animals to choose correct habitats with resources that will help them survive (Popsuj & Stolfi 2020).

Other behavioural assays such as breathing, swimming and resting of aquatic organisms such as mosquito larvae are as important in ecotoxicology. Breathing occurs when the larvae is suspended upside-down in the water column getting oxygen via its siphon. Swimming refers to the exploration of water by the larvae, and resting is when the larva is at the bottom of the water column and not moving (Mellanby 1958). Breathing is important for the proper development of the mosquito. The late-stage instar larvae spend most of their time on the water surface breathing so that they become fully grown functional adults (Alverez-Costa 2024). Resting assists in hiding the larvae when there is a danger present. The larvae are observed swimming down in water and staying there when they sense vibrations or shadows, hence this behaviour is important in predation evasion (Mellanby 1958). Many activities that are performed by mosquito larvae strictly depend on swimming. These activities include breathing, foraging, navigation to find suitable habitats and escaping predators (Tomè *et al.*

2014). Since swimming plays such a crucial role in the survival of the mosquito larvae, it is important to study the changes induced by insecticides application to the mosquito population (Tomè *et al.* 2014).

The presence of toxicants such as insecticides increases the chances of larval mortality and alterations in behaviour that might not be favourable for the larvae. After exposure to xenobiotics or toxicants, the swimming speed of mosquito larvae is likely to decrease and this might cause them to be at high risk of dying due to predation (Reynaldi *et al.* 2011). This behavioural alteration might also affect the foraging success of the organism because larvae tend to decrease swimming speed after they have encountered a food-rich area (Lutz *et al.* 2020). However, the most important parameter is breathing because without oxygen, they could suffocate and die. Additionally, without sufficient oxygen, the larvae would not successfully pupate because the mosquitoes need to obtain air to separate the cuticle of the fourth instar larvae from the pupa's cuticle (Alvarez- Costa *et al*. 2024).

1.7 Significance of study

Pesticides play a critical role in our lives and are extensively used for food security and to control vectors of Neglected Tropical Diseases such as malaria, especially in developing countries (European Parliament 2021, Rezende- Teixeria *et al.* 2022). Because of this, they are expected to remain widely used in the foreseeable future (European Parliament 2021). However, extensive use of these chemicals is a problem and causes pesticide pollution in water bodies and soil, threatening the lives of non-target organisms (Sharma *et al.* 2019). The extensive use of pesticides has resulted in their recurrent detection in water resources, increasing the likelihood of the exposure of aquatic organisms to these chemicals (Kibuthu *et al.* 2016). Because of climate change, the performance of pesticides might be compromised, leading to more frequent pesticide application and causing adverse effects in aquatic and terrestrial environments (Duchenne- Moutien 2021, European Environment Agency 2023). South Africa has been reported to be at risk for high pesticide pollution (Tang *et al*. 2021), and this country is the number one pesticide user in Sub-Saharan Africa (Motsoeneng & Dalvie 2015). This study was carried out to find ways to mitigate environmental pollution and to lessen the effects of pesticide pollution, less toxic insecticides (Eyhorn *et al*. 2015) such as those that have pymetrozine as the active ingredient could be regularly used and considered for IPM and those that are highly toxic such as those that have carbaryl as their active ingredient could be phased-out because they cause adverse effects in the environment (Gupta & Sexena 2003, Donley 2019). There is a gap in the literature regarding how insecticides affect essential behaviours like breathing, swimming and resting in mosquito larvae. Most studies focus on how exposure to different insecticides results in insecticide resistance and avoidance (Wang *et al.* 2020, Andreazza *et al.* 2021, Dhiman *et al.* 2021, Koto Yérima Gounou Boukari *et al*. 2024). Whereas little to no studies focus on the breathing, swimming and resting behaviours of mosquito larvae (Tsotesti *et al.* 2022). The results of this study can provide baseline information on altered behaviour in mosquito larvae caused by carbaryl and pymetrozine and help raise awareness among farmers and household owners about the toxicity of commonly used insecticides.

1.8 General aim and objectives

1.8.1 Aim

Assess the sub-lethal effects of carbaryl and pymetrozine on the survival and behaviour of *Culex* sp. larvae.

1.8.2 Specific objectives

- Assess the effects of carbaryl and pymetrozine on the survival of *Culex* sp. fourth instar larvae under controlled conditions after acute exposure.
- Assess the effects of carbaryl and pymetrozine on the breathing, swimming and resting behaviours of *Culex* sp. fourth instar larvae under controlled conditions after acute exposure.
- Assess the effects of carbaryl and pymetrozine on the geotaxis responses of *Culex* sp. fourth instar larvae after acute exposure.
- Compare the effects of carbaryl and pymetrozine on the breathing, swimming and resting behaviours and geotaxis responses of *Culex* sp. fourth instar larvae under controlled conditions.
- Conduct a systematic review of studies conducted between 2000 and 2024 on behavioural changes of insects after exposure to carbaryl

Chapter 2

Behavioural changes of insects after exposure to carbaryl insecticide: A systematic review

2.1 Introduction

Insects are known to play a critical role in maintaining ecological balance as they contribute significantly to soil fertility and as decomposers, pollinators, bio-indicators, control agents to other insect pests, and as a food source for insectivores (Losey & Vaughan 2006, Weisser & Seimann 2008, van Huis 2013). However, the widespread use of insecticides, particularly ones such as carbaryl in insect pest management, has raised concerns regarding their impact beyond the targeted pest species. Many non-target organisms are exposed to sublethal insecticide concentrations, which can affect various life traits such as development, physiology, and behaviour, ultimately compromising the health and ecological function of the exposed insects (Müller 2018). Thus, understanding the behavioural changes caused by carbaryl exposure is crucial, especially given the ecological importance of insects. Changes in their behaviour may reduce their efficiency in fulfilling these ecological roles, and such insights can guide improvements in Integrated Pest Management (Hoy 2019).

Behaviour, defined as any observable action an organism performs in response to stimuli or its environment (Hoy 2019), is broadly classified into innate and learned categories. Innate behaviours, for example, flight in butterflies, are genetically encoded, whereas learned behaviours, such as opening puzzle boxes and getting food rewards, are not genetically encoded but can be acquired (Beny & Kimchi 2014). The concept of insect behaviour dates back to Aristotle's Historia Animallium (Sheehan & Miller 2021). Insects display a wide range of complex behaviours allowing them to adapt and thrive in diverse ecological niches (Ramesha *et al.* 2024). These behaviours are generally controlled by the physiological processes of the nervous, muscular, and endocrine systems (Chu *et al.* 2024).

Carbaryl is one of the most used carbamate insecticides in the world to control insect pests on agricultural land (Iyer 2001). Under exposure to this insecticide, insects are poisoned by ingestion and absorption through the cuticle (van der Werf 1996). It acts by inhibiting acetylcholinesterase (AChE), a critical enzyme in the nervous system responsible for hydrolysing acetylcholine in cholinergic synapses. This disruption leads to uncoordinated muscular activity, paralysis, and ultimately death of the affected insects (Koshlukova & Reed 2014). Unfortunately, carbaryl is highly hazardous to a wide range of untargeted organisms including insects, birds, fish, and anurans (US EPA 2003). While it is less to moderately persistent in the environment than organochlorine, its degradation rates vary from slow to rapid with half-lives of 231 days in aquatic conditions (sea water) and 72 to 78 days in anaerobic soils (Xu 2000, Derbalah *et al.* 2020). The degradation rate is relatively quicker in aerobic soils with half-life of 4 to 27 days (Lima *et al.* 2015). Because of the relatively high carbaryl's degradation rate in the environment and its low mammalian toxicity, it has been permitted to substitute some organochlorine pesticides (Sunaryani & Rosmalina 2021). However, despite its low mammalian toxicity, the use of this insecticide has been restricted in some countries such as the United Kingdom, due to its high toxicity to non-target organisms causing biodiversity loss, and its potential carcinogenicity in humans (Elston 2012, Scanlan & Koshlukova 2024).

Pesticide exposure can alter various insect behaviours, including reproductive behaviour, locomotion (including flight), social behaviour (including calling behaviour, for example, mating calls produced by male field crickets), and defensive mechanisms (Harrison *et al.* 2013, Bartling *et al.* 2024). Exposure to carbaryl can significantly affect the mating behaviour of insects, simultaneously affecting reproductive success, which can result in population declines over time. For example, the exposure to carbaryl reduced attraction to pheromones and lessened calling patterns in moths, leading to lower mating rates (Wei *et al.* 2004). Despite the adverse effects caused by insecticides on reproduction, other insecticides may cause hormesis, known as positive responses at reproduction (França *et al*. 2017). In social insects such as ants, exposure to this insecticide can impair digging behaviour and trail-following patterns, which may reduce foraging success (Chen 2006).

The neurotoxic effects of carbaryl also impair insect locomotion, causing paralysis and reducing coordination in many insect species (Lee *et al.* 2015). For instance, brown marmorated stink bugs and Western corn rootworms exposed to carbaryl showed reduced mobility and impaired standing ability (Behle 2001, Lee *et al*. 2013). Such impairments can affect the ability to escape predators, navigate their environment, or reduce the foraging success of these insects. Moreover, carbaryl has been documented to cause paralytic flight patterns in lime butterflies (*Papilio demoleus*), compromising survival (Gosh Shreya *et al*. 2015). Unfortunately, exposure to carbaryl weakens these responses, making insects more vulnerable to predators and disrupting predator-prey dynamics. For instance, insects exposed to carbaryl are more susceptible to predation by birds because of impaired escape responses. Moreover, insect death due to exposure to an insecticide can disturb the food webs due to a cascading effect on predator-prey dynamics (Burn 2000).

Another behaviour susceptible to disruption due to exposure to insecticide is feeding. For example, mosquito vectors (of diseases such as malaria) exposed to carbaryl show reduced feeding efficiency, lowering pathogen transmission potential and their fitness (Andreazza *et al.* 2021). Carbaryl can also affect insect oviposition with various outcomes depending on the insect groups. For example, it has been observed that in *Culex* mosquitoes, there is a tendency for laying eggs in carbaryl-contaminated pools, according to a study by Vonesh & Kraus (2009), as it is a natural behaviour for this mosquito species to lay eggs in contaminated water. In contrast, in certain cases, carbaryl can have no significant impact on insect oviposition, as seen in green lacewings *Chrysoperla externa* (Moura *et al.* 2001).

Avoidance behaviour is another crucial survival strategy in insects. In fact, some carbaryl-resistant insects tend to avoid areas that are contaminated with the insecticide (Kang & Jung 2017). These insects tend to stop feeding when they come across insecticide-contaminated food sources (Nansen *et al.* 2016). Yet, some species like honeybees continue feeding on carbaryl-contaminated flowers, increasing their risk of poisoning (Kang & Jung 2017).

The emergence success of insects after metamorphosis is another behaviour or parameter hugely affected by exposure to carbaryl. Insects exposed to this insecticide generally show reduced rates of successful emergence to the adult stage. For instance, Galvan *et al*. (2005) found that about 40% of Asian lady beetles emerging from carbaryl-treated pupae died soon after. Similarly, the emergence rate of minute wasps *Habrobracon hebetor* was negatively affected by exposure to carbaryl, reducing their fecundity, fertility, longevity and generation time (Mahdavi *et al.* 2011). Additionally, Beuter and colleagues (2019) demonstrated a marked decline in the emergence rates of both the mayfly *Ephemera danica* and members of the caddisfly family Limnephilidae after exposure to carbaryl. Surprisingly, in Redcoat damselflies, carbaryl was shown to stimulate the development of larvae and had only a minor effect on the emergence success, with a consistent decrease in the time to peak emergence as carbaryl concentration increased (Hardersen *et al.* 2000).

Carbaryl exposure does not only alter the behaviour of insects, this chemical also alters the behaviour of other animal species such as earthworms, chickens, rats, and fish (Farage-Elawar 1989, Takahashi *et al.* 1991, Gupta & Saxena 2003, Chaudhari & Saxena 2021). In earthworms, carbaryl exposure disrupted locomotion and reproduction, by impairing cocoon production (Gupta & Saxena 2003). In the fish species *Channa punctatus,* erratic movements were observed after exposure to carbaryl (Chaudhari & Saxena 2021). In chickens, carbaryl exposure altered locomotion, increasing stride width while decreasing stride length (Farage-Elawar 1989). In rats, acute exposure reduced motor activity, and chronic exposure resulted in both reduced and impaired motor activity (Ruppert *et al.* 1983, Takahashi *et al.* 1991).

Most studies investigating insect mortality and behavioural changes after exposure to carbaryl have been conducted in developing countries, including China, Brazil, India, Mexico, and Saudi Arabia (Zhao *et al.* 2012, Bacci *et al.* 2018). Developed nations such as the United States, Australia, and Germany have also contributed to this research (Suwanchaichinda & Brattsten 2002, Liang *et al.* 2010, Beuter *et al.* 2019). These studies have been mainly carried out in laboratory settings, with adult insects being the frequently tested life stage, honeybees have commonly served as model species in these investigations (Wilde *et al.* 2001, Gao *et al.* 2018, Nogrado *et al.* 2019).

For the efficient management of insects and insecticide resistance, it is important to understand how insecticides act on insects, including how these chemicals affect their behaviour (Hierlmeier *et al*. 2022). In recent decades, several studies have helped to understand how carbaryl affects insect behavioural life traits. However, for a better understanding of trends and to capitalise on the knowledge generated by these recent research developments, a comprehensive review of these findings is critically needed.

For this review, the focus was on carbaryl and not pymetrozine because, of the two insecticides data are available for carbaryl, whereas for pymetrozine the available data remains sparse. Nevertheless, a few studies document behavioural changes in insects after exposure to pymetrozine. For example, insects exhibit behavioural changes such as changes in feeding activity, movement patterns, mating and reproduction success after exposure to pymetrozine. Wang *et al.* (2020) showed that exposure to this insecticide impairs mating behaviours and reduces female fecundity in brown planthoppers and fruit flies. In Potato Psyllid and aphids, pymetrozine is known to reduce feeding time, leading to starvation and ultimately death (Butler *et al.* 2011; Gorman *et al.* 2001). Additionally, aphids exhibit decreased movement which hinders their ability to find mates, food, and escape predators (Gorman *et al.* 2001).

2.2 Aim and objectives

2.2.1 Aim

The current review aims to critically appraise literature evidence on the behavioural changes of insects after exposure to carbaryl from studies conducted between 2000 and 2024. Not enough data is currently available to produce a systematic review on behavioural changes of insects after exposure to pymetrozine.

2.2.2 Objective

The objective was to gather all the existing research on the effects of carbaryl on the behaviour of insects after exposure in ecotoxicological studies. This review also aims to identify trends and point to gaps in the literature regarding the choice of insect species investigated in these studies.

Primary question

How do insects behave when they are exposed to carbaryl?

Secondary question

Does acute and chronic exposure to carbaryl cause death/ mortality in insects?

2.3 Methodology

2.3.1 Article search

The search aimed to retrieve and quantify as much information and investigations as possible on the mortality and behavioural changes induced by the carbaryl insecticide in insects (Hexapoda class) worldwide. The search focused on published articles. Literature was selected from PubMed, Web of Science, Scopus, United States Environmental Protection Agency, Wiley Online Library and SETAC Press using keywords such as 'carbaryl exposure,' 'behavioural changes,' and 'insects.' The key inclusion criterion was studies published between 2000 and 2024 in peer-reviewed journals. Other review articles, and academic dissertations/theses were excluded. Review articles, dissertations, and theses were excluded as they did not align with the study’s focus on original research papers.

2.3.2 Search conditions

English was used as the language for our search. The search was also restricted to a select time frame comprising all the peer-reviewed published papers ranging from the year 2000 to the current year 2024.

The search terms were conducted according to the Mnkandla *et al.* (2021) method where the necessary literature was sorted into categories. For the search, those categories were combined using Boolean operators “AND” and/ or “OR”. Those four categories were test organism, contaminant, mortality and behavioural changes.

2.3.3 Criterion used to extract relevant articles.

For a study to be included in the current review, it had to have been published between the year 2000 and 2024 and be written in English. The search produced more than 10 000 results. Of these, 85 research articles were deemed relevant to the title/ topic and included in the analysis. The articles were considered relevant if they assessed mortality or behavioural changes in insects after exposure to carbaryl, the study had to be conducted either in the laboratory or in the field, the life stage had to be from the eggs to the adults, and the study could be conducted in any country in the world. The included studies, thus met the following eligible criteria:

Eligible population: Test organism (aquatic/ terrestrial) and group (insects).

Eligible intervention: Carbaryl insecticide

Eligible comparator: Control versus various chemical concentrations/ dosages.

Eligible study type: Both field and laboratory studies that included carbaryl and an insect test organism.

2.3.4 Keywords

Test organism terms

- Ground-dwelling insects
- Aquatic insects
- Flying insects
- Terrestrial insects
- Swimming insects
- Soil insects

Contaminant terms

- Carbaryl

Parameters and behaviour terms

- Mortality: death
- Calling: vocalizations often used by group-living organisms, for example, calls to attract females during the mating period.
- Mobility: the ability to move around
- Avoidance: behaviour whereby individuals avoid situations or stimuli that cause discomfort.
- Digging: behaviour where insects like ants use their mandibles to scrape or move materials.
- Flight: the ability to fly or manoeuvre in the air using wings.
- Emergence: the process of insects transforming from immature stages to adults, this typically happens at the end of metamorphosis.

2.3.5 Search string

https://scholar.google.com/scholar?q=behavioural+changes+of+insects+after+exposure+to+carbaryl+&hl=en&as_sdt=0%2C5&as_ylo=2000&as_yhi=2024

(“behaviour* change”) AND (“ground-dwelling insect* OR aquatic insect* OR flying insect* OR terrestrial insect* OR swimming insect* OR soil insect*) AND (“expos*) AND (toxic* OR “carbaryl” OR “insecticide exposure” OR “pesticide exposure”)

The meaning of the star symbol at the end of search terms indicates that the word can be in various forms. For example, behaviour* includes “behavioural” and expos* includes “expose”, “exposed” or “exposure”, etc...

2.3.6 Academic database

- Scopus
- Web of Science
- Science direct
- Pub Med
- United States Environmental Protection Agency

- SETAC Press
- Wiley Online Library

Google Scholar was mainly used for our internet search. In addition, ResearchGate (https://www.researchgate.net/) was also used to access articles that could not be accessed through Google scholar.

Table 1: Data coding

Code	**Variable**	**Description**
Bibliography	Author Publication year Title Publication type	Only the surname of the first author, and used *et al,* for the other contributed authors. The year the paper was published. Full title of the study Type of publication i.e., journal article, report, etc
Location of the study	Country	The country where the study was conducted
Study site	Field/Laboratory	Was the study conducted on the field or at the laboratory?
Study habitat	Aquatic/Terrestrial	Did the study use aquatic species or a terrestrial species?
Contaminant	Chemical/Insecticide	Insecticide carbaryl used as a contaminant for the experiment?
Comparison	Control vs treated/test	Compared the control and different concentrations or dosages
Study organism(s)	Insects	Were the study test organism(s) insects?
Organism's stage	Eggs, larvae, pupae, adult	Which life stage of the organism was tested?
Behaviour / endpoint	Mortality, calling behaviour, digging behaviour, mobility, avoidance, behaviour, emergence behaviour	Which behaviour was assessed during the exposure.

2.4 Results

2.4.1 Countries that reported the use of carbaryl on insects

In our analysis, we found that the United States (35%), particularly the states of Florida (6%), Kansas (2%), Mississippi (1%), Maryland (1%), Tennessee (1%), West Virginia (1%), and California (1%), followed by China (24%), accounted for the highest number of studies investigating the effects of carbaryl on insects (Figure 1). In contrast, other regions of the world contributed no more than 3% of the total studies retrieved. Countries with the lowest proportions of carbaryl research, each representing less than 3% of the studies, were dispersed across various continents including Egypt (Africa), Germany and Poland (Europe), several countries in Asia, and regions of South America. Similarly, countries like New Zealand were also represented by less than 1% of the research output (Appendix 1, Figure 1).

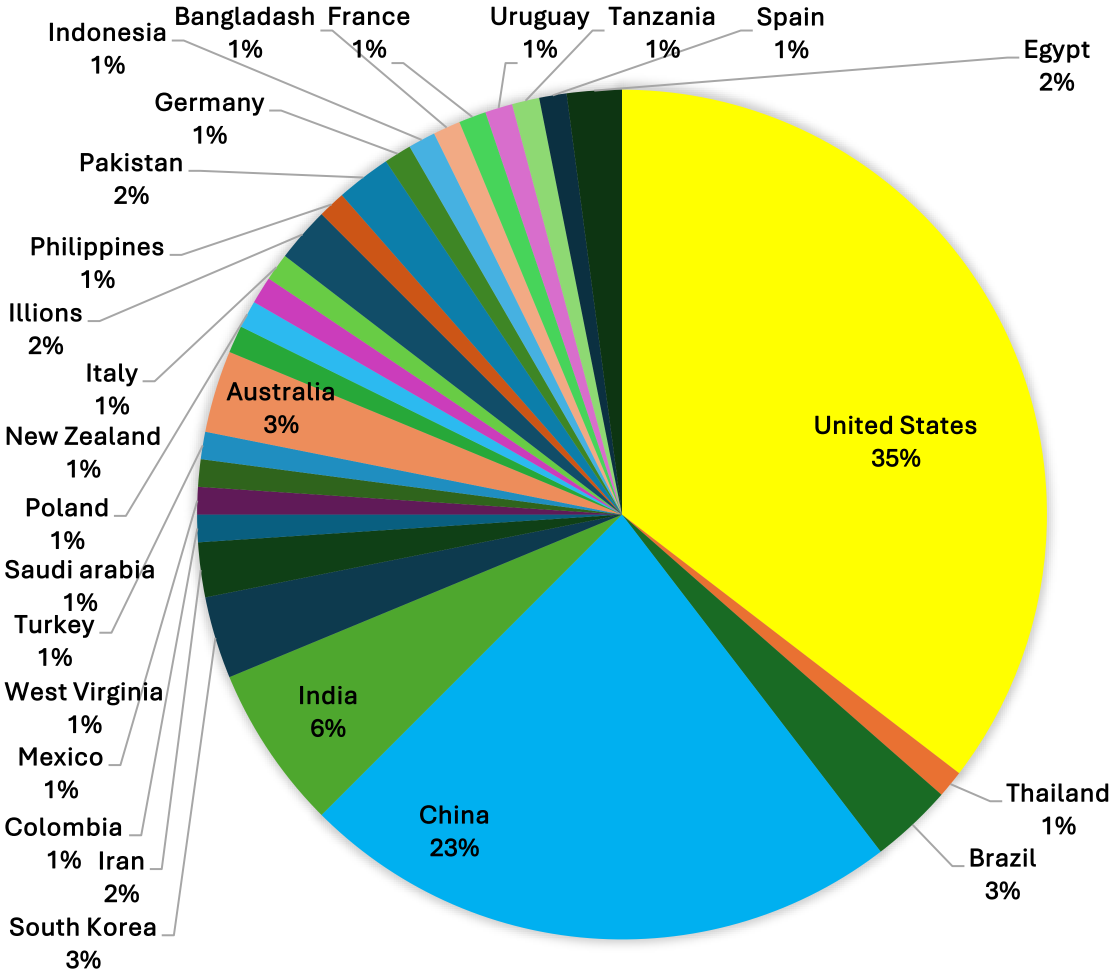

Figure 1: Worldwide distribution, in percentage, of published articles mentioning the use of carbaryl on insects.

2.4.2 Species used to assess mortality and behavioural changes induced by exposure to carbaryl

A wide variety of insects were used to assess the effects of carbaryl, ranging from flying, walking, to swimming insects. As shown in Figure 2 (Appendix 1), honeybees were the most commonly used test organism (11 %), followed by diamondback moths (4%), fall armyworms (4 %), and the western corn rootworm (4 %). Less frequently used organisms included the Asian tiger mosquito (0.9 %), the lime butterfly (0.9 %), bumble bees (0.9 %), sharpshooters (0.9 %) and red imported fire ants (0.9 %).

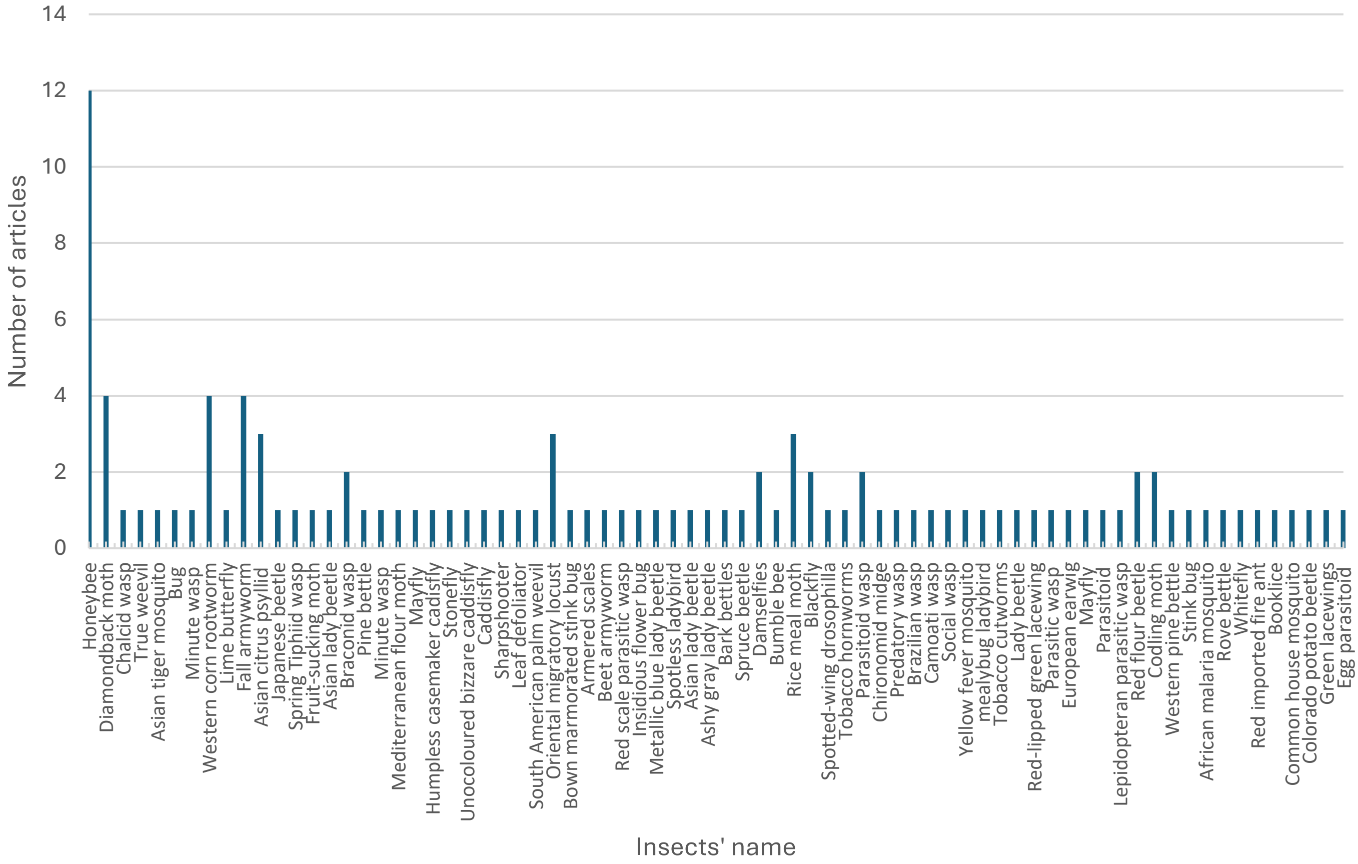

Figure 2: Diversity and frequency of use of the insects used to assess the effects of carbaryl on insect mortality and behaviour.

2.4.3 Study design

Laboratory settings were indicated as the most frequently used or preferred environment for conducting research, with 74% of the reviewed studies taking place in a controlled laboratory context. A further 19% of the research was carried out in both the laboratory and field setting, while only a small proportion (7%) of studies were exclusively conducted in the field (Appendix 1, Figure 3).

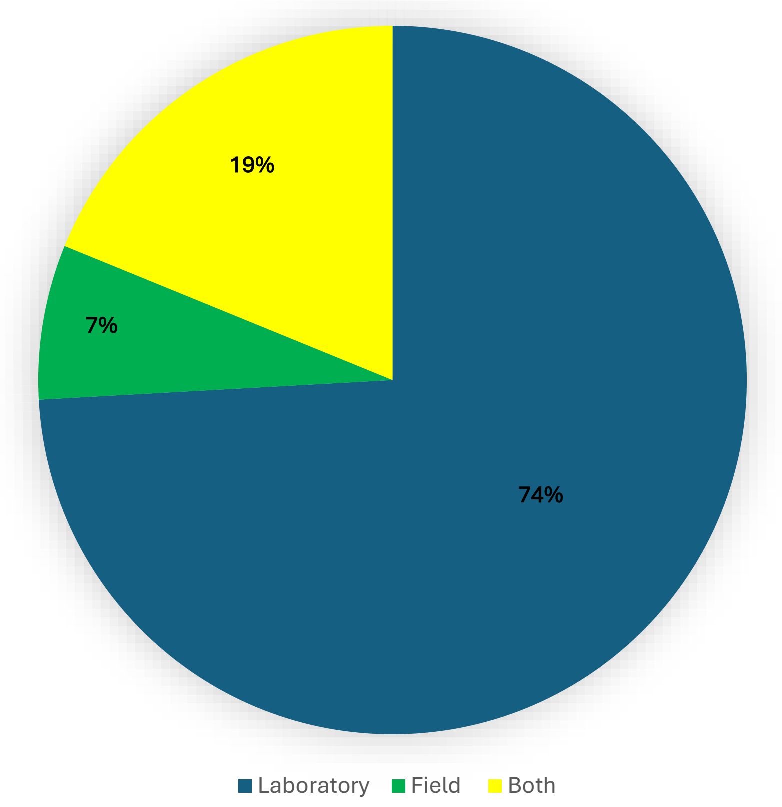

Figure 3: Distribution, in percentage, of published articles based on studies carried out in the laboratory, field or both settings.

2.4.4 Life cycle stages of insects used

It was noted that adults were the most frequently used life stage in the tests (40 %) of the studies), compared to eggs (3 %), larvae (31 %), and nymphs (3%). Some studies did not specify the life stage used (6%), while other indicated that the age of the organisms was unknown (1 %) (Appendix 1, Figure 4).

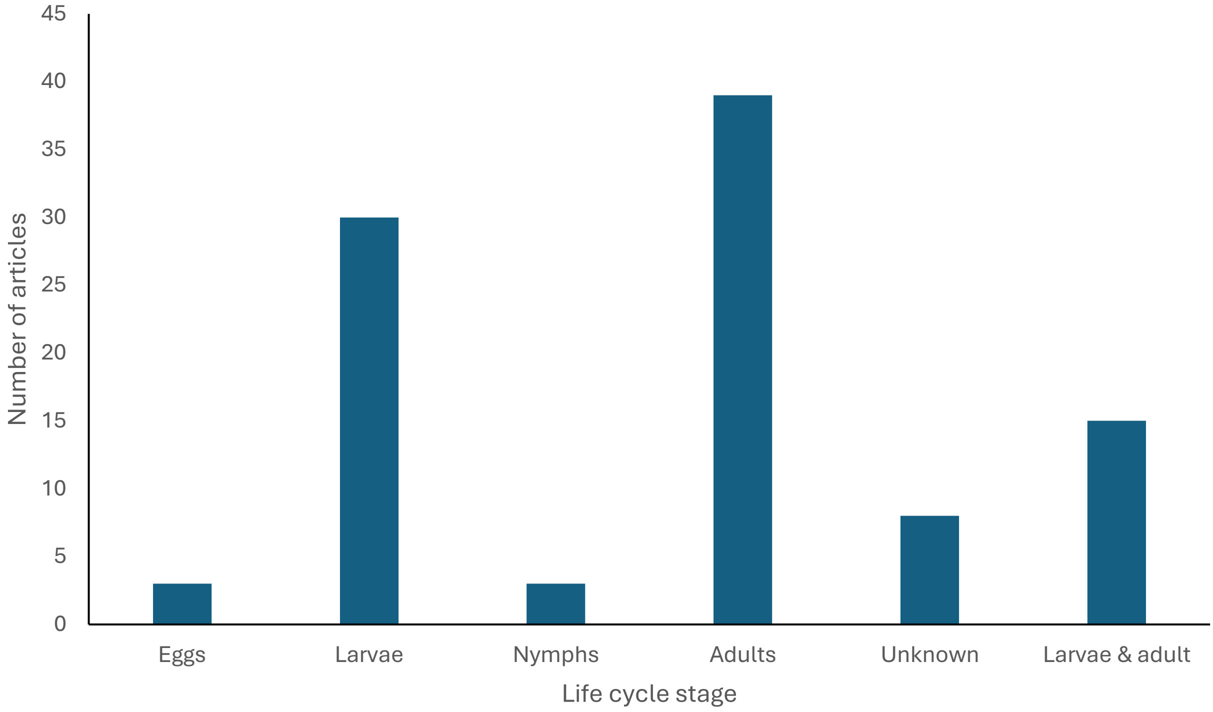

Figure 4: The number of published articles mentioning the life cycle stage of the insects tested during the experiments.

2.4.5 Behaviours or parameters assessed

Survival or mortality were the most commonly investigated experiment endpoints, followed by toxicity and susceptibility. Behavioural endpoints such as calling, digging, copulation, flight, and detachment were less assessed in the reviewed literature (Appendix 1, Figure 5).

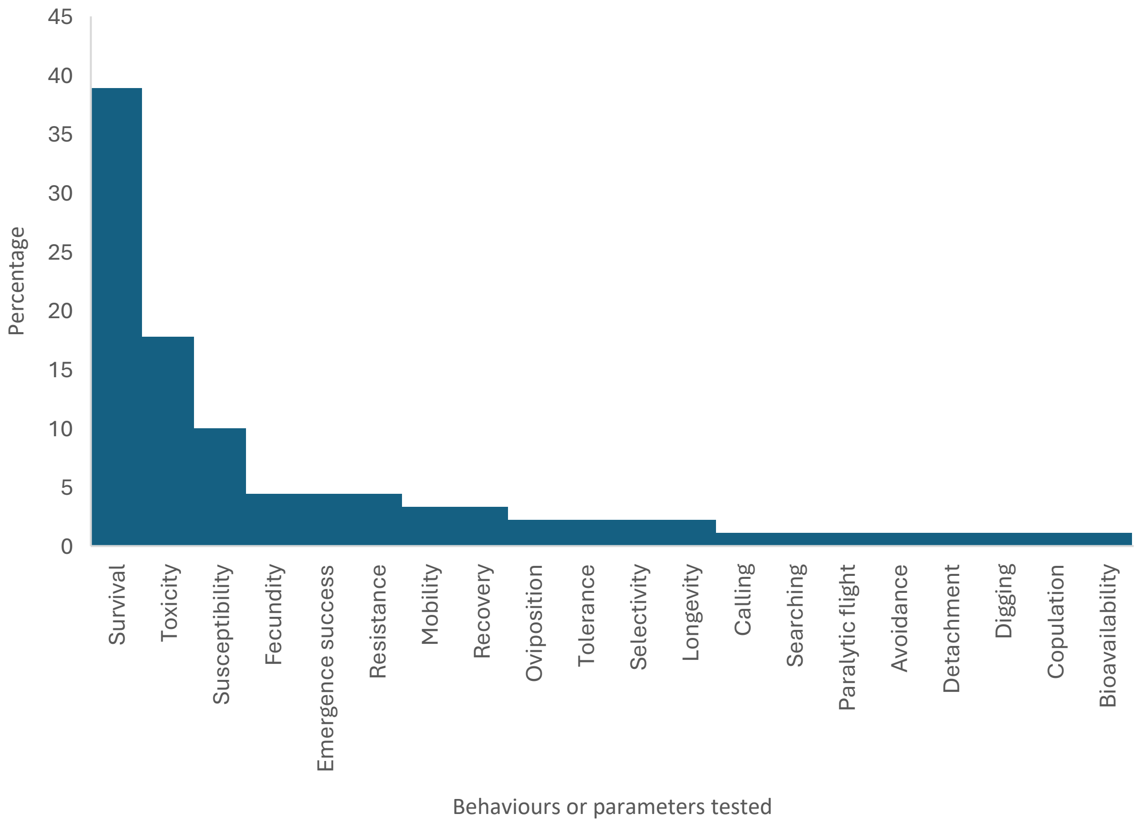

Figure 5: Behaviour endpoints and other parameters assessed in published articles reporting the effects of carbaryl on insects.

2.4.6 Carbaryl and other chemicals used

Of the 85 reviewed articles, 16 (18.8%) specifically tested the effects of carbaryl alone on insects. The remaining articles 69 (81.2%) examined carbaryl in combination with other chemicals, such as permethrin (12 %), chlorpyrifos (32 %), imidacloprid (32 %), and deltamethrin (19 0%), among other (Appendix 1, Figure 6).

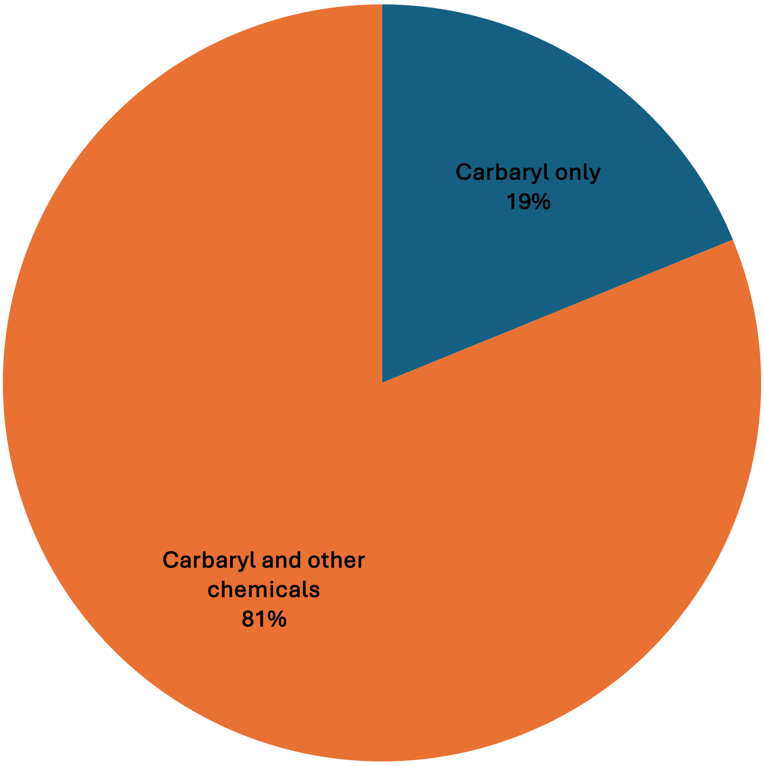

Figure 6: Distribution, in percentage, of articles mentioning carbaryl and other chemicals in the retrieved literature.

2.5 Discussion

2.5.1 Countries that reported the use of carbaryl on insects

In this work, the results highlighted in Figure 1 show that the U.S. has the highest proportion of studies followed by China. In the U.S., the three most commonly used pesticides are organophosphates, pyrethroids and carbamates (USDA 2001). The country's large agricultural sector, one of the largest in the world, likely contributes to the significant use of carbaryl to control insects (Donley 2019). Carbaryl is particularly effective against bark beetle attacks, and the literature reviewed frequently used beetles, such as pine beetles and Asian lady beetles, as test organisms. This focus may explain the high number of studies conducted in the United States (Hastings et al. 2001). Moreover, carbaryl is the second most detected insecticide in water surfaces in the US (Tiwari *et al.* 2019), reinforcing its widespread application. In fact, carbaryl has historically been one of the most heavily used insecticides in the U. S. (Mora-Gutiérrez *et al*. 2021).

The high number of studies on carbaryl in China may be attributed to the fact that China has become the world's largest pesticide consumer accounting for approximately 43% of the total pesticide used (Zhang *et al*. 2022). Additionally, China's large population, combined with having only 9% of the world's arable land (Wang *et al.* 2022), increases the pressure to maximize crop yields, which may explain the extensive use of carbaryl in the country's agricultural practices.

Most countries that use carbaryl to control insect pests are developing nations, among them are Brazil, India, Italy and South Korea (Marletto *et al*. 2003, Abdullah *et al*. 2006, Moura *et al*. 2012). Surprisingly, despite Brazil being one of the four largest agricultural producers in the world along with the United States, Europe, and China (Panis 2021), the use of carbaryl there appears to be less prevalent. This lower use could be due to national regulations or alternative pest control methods (Erickson 2022).

Even though the use of carbaryl is mostly reported in developing countries, the literature often includes only one study per country, which may be a result of this insecticide being banned in certain places, such as India (Choubey 2023) or a lack of research focusing on the use and impact of such chemicals in these countries. Despite these bans, many insecticides, including those restricted by the European Union, are still exported to developing countries, leading to increased usage in these regions (European Parliament 2021). There is an increased demand for food production due to population growth, and many farmers in developing countries rely on these chemicals as the best option to protect their crops from pests (Islam & Karim 2019), as pesticide use is currently viewed as a critical means of ensuring food security (European Parliament 2021). As an illustration, some developing countries export or sell fruits and vegetables to other countries, and this could increase the use of pesticides because more production is needed to meet the market demand (Galt 2008, Tudi *et al*. 2021).

In the African context, only one study from Egypt reported the effects of carbaryl on insects, highlighting a significant gap in the literature. This underscores the need for more research in Africa, as carbaryl is used in both households and agricultural settings across the continent.

2.5.2 Species used to assess mortality and behavioural changes induced by exposure to carbaryl

From the retrieved articles, honeybees (*Apis mellifera*) were the most commonly used test organisms, likely because of their role as bioindicators of environmental pollution and high sensitivity to contaminants (Cunningham *et al*. 2022). Honeybees are particularly valuable for continuous biomonitoring, as they are regularly exposed to pollutants such as pesticides and heavy metals during foraging, making them effective samplers of environmental conditions (Papa *et al*. 2024).

Other terrestrial insects, including the diamondback moth (*Plutella xylistella*)*,* the Western corn rootworm (*Diabrotica virgifera virgifera*), the fall armyworm (*Spodoptera frugiptera*), the rice moth (*Corcyra cephalonica*) and the Asian citrus psyllid (*Diaphorina citri*) were also frequently used as test species (Behle 2001, Yu *et al*. 2003, Feng & Zhang 2017, Kishk *et al.* 2017, Pilotos *et al.* 2020). These insects are agricultural pests known to cause crop damage in agriculture, resulting in crop losses (Philips *et al.* 2014, Matova *et al.* 2020, Gassmann 2021, Vincent *et al*. 2021, Leong *et al*. 2022).

For example, the Western corn rootworm is estimated to be responsible for loss of at least $2 billion dollars yearly in crop losses and control costs in the U.S (Sappington & Spencer 2023). The diamondback moth, a global pest of cruciferous crops, has developed resistance to various major insecticide classes, further complicating management efforts (Tonnang *et al.* 2009). Therefore, it is not surprising that several of the retrieved studies explored the development of insecticide resistance, particularly investigating the effectiveness, selectivity and tolerance levels of carbaryl on the diamondback moth (Bacci *et al.* 2009, Bacci *et al.* 2018, Rahardjo *et al.* 2021).

The fall armyworm is another major threat to global food security, responsible for estimated crop losses of approximately 70 billion US dollars, enough to feed millions of people (Gouda *et al.* 2024). Additionally, the rice moth is a serious pest of stored grains (Vincent *et al*. 2021), while the Asian citrus psyllid causes Huanglongbing (citrus greening), a disease that devastates citrus crops worldwide (Agarwal *et al.* 2023).

Aquatic insects are among the most commonly used macroinvertebrates for water quality assessment and monitoring worldwide because of their sensitivity to changes in their environment (Azmi *et al.* 2018). In the reviewed articles, caddisflies (*Brachycentrus americanus, Lepidostoma unicolor, Psychoglypha* sp.) were the primary aquatic insects used as test organisms (Peterson *et al.* 2001). Caddisfly larvae are widely used in aquatic pollution monitoring due to their sensitivity to contaminants (Cunningham et al. 2022). Their role as bioindicators stems from their ability to respond to water quality perturbations, making them ideal for assessing pollution levels (Bowles *et al.* 2020, Thamsenanupap *et al.* 2021). Typically, aquatic insects such as caddisflies, mayflies and stoneflies serve as indicators of good water quality, while midges as indicate poorer conditions (Let *et al.* 2022, Prat & Castro- Lòpez 2023). However, in the current review, only caddisflies were predominantly used to assess the effects of carbaryl, whereas mayflies, stoneflies and midges were rarely utilised.

Some insects that cause plant diseases, such sharpshooter (*Homalodisca coagulate*), which transmits Pierce's disease, and the brown plant hopper (*Nilaparvata lugens*), responsible for yellowing, browning and drying of crops, were among the least commonly used test organisms (Jing *et al.* 2017, Krugner *et al.* 2019). In addition, the European earwig (*Forfificula Auricularia*) and red imported fire ants (*Solenopsis invicta*) were also less frequently studied (Chen 2006, Shaw & Wallis 2010). Moreover, medically important vectors of zoonotic diseases such as mosquitoes of the species *Aedes albopictus*, *Aedes aegypti*, *Anopheles arabiensis* and *Culex pipiens* were similarly underrepresented in the studies reviewed (Suwanchaichinda & Brattsten 2002, Tong & Bloomsquit 2013, Merdan & Ghareeb 2022, Urio *et al*. 2022). Given the medical importance of these species, there is a need for more research into the effects of carbaryl on these vectors (Flores & O'Neill 2018).

Overall, this literature review has revealed that the effects of carbaryl were more extensively tested on terrestrial insects than on aquatic species. As this pollutant is likely to spread in environmental waters, there is a need for tests on aquatic insects as a key approach to extensively investigating the effect of carbaryl on aquatic ecosystems.

2.5.3 Study design (conducted in laboratory or field)

As we could have expected, the majority of studies were conducted in laboratory settings. Out of the 86 articles reviewed, 63 were exclusively laboratory-based. Laboratory studies are favoured over field studies for several reasons: they allow for controlled, optimal testing conditions, where environmental factors such as temperature, light, and humidity can be carefully regulated. Additionally, laboratory exposures are short-term, replicable, and generally less costly (Spurgeon 2020). Researchers in these studies often assessed outcomes such as mortality and behavioural changes, including avoidance behaviour and alterations in gene expression induced by exposure to carbaryl, using laboratory protocols like Polymerase Chain Reaction (PCR) tests (Guo *et al.* 2015, Yang *et al*. 2016).

Most laboratory studies assessed acute and chronic exposures ranging from 24 hours to several days. Laboratory results are invaluable for hazard assessments, providing critical insights into the toxicity of chemicals such as pesticides, pharmaceuticals, and metals in the environment (Ezerins *et al*. 2022). However, field-based studies are also necessary to assess carbaryl's toxicity under real environmental conditions, where factors like variability in temperature and exposure duration may increase toxicity levels (Spurgeon 2020).

2.5.4 Life stages of insects exposed to carbaryl

The life stages assessed in the reviewed articles included eggs, larvae, nymphs, pupae and adults. Notably, six studies did not specify which life stages were exposed during their experiments. Among the studies that did specify, adults were the most commonly used, followed by larvae, with eggs, nymphs and pupae being utilsed to a lesser extent. In some studies, both adults and larvae were used concurrently. Indeed, adults are often selected to assess whether their sensitivity aligns with that of larvae or other juvenile stages of the same species (Bruus *et al.* 2020). This is particularly relevant for terrestrial adults of freshwater insects, which have shown increased sensitivity to pesticides, especially insecticides (Bruus *et al.* 2019, Bruus *et al.* 2020). However, it was expected that larvae would be the most studied life stage, as they are generally more sensitive to the effects of environmental contaminants (Theodorakis 2005). This is especially true for insects like moths and beetles, which tend to be more destructive as larvae (Ofuya *et al.* 2023). In bees, for example, the larval stage is often considered the most important for evaluating pesticide impacts on population dynamics (Eeraerts *et al.* 2020). Furthermore, studying larvae is advantageous for assessing overall susceptibility and tolerance to the tested toxicants (Rand *et al.* 2014). Finally, insecticides are used on eggs in agriculture to reduce the number of hatchlings from insect pests (Santos *et al.* 2017).

2.5.5 Behaviour(s) or parameter(s) assessed after exposure of insects to carbaryl

Carbaryl has been shown to significantly affect insect behaviour. For example, the Lime butterflies (*Papilio demoleus*), exposed to carbaryl exhibited slow larval movement and paralytic flight in adults (Gosh Shreya *et al.* 2015). The sluggish movement in larvae makes them more vulnerable to predation, while the paralytic flight in adults equally compromises their ability to forage and escape predators. Speed and manoeuvrability are essential for avoiding capture, so when flight abilities are impaired, the likelihood of predation and death increases (Srygel & Dudley 2008, Altshuler & Srinivasan 2016). Moreover, carbaryl affects various other behaviours in insects, such as searching efficiency, calling behaviour, and avoidance responses (Mahdavi *et al*. 2013, Wei *et al*. 2004, Kang & Jung 2017). Chen (2006) observed that exposure to carbaryl reduced the digging effort of worker ants (*Solenopsis Invicta)* in sand. Ants rely on their underground nests for protection from predators and environmental hazards. Reduced digging ability not only increases the risk of predation but also impacts the ants' role as "soil engineers," as they play a critical role in soil aeration and nutrient enhancement (Cammeraat & Risch 2008; Bruce *et al.* 2019).

Calling behaviour is crucial for mating success in many insects species, as it facilitates communication between potential mates (Wei *et al.* 2004). In a study on moths (*Spodoptera litura*), females exposed to carbaryl during their peak calling period (8-9 hours after initiation of the scotophase which is the dark phase in a cycle of light and darkness) showed a significant reduction in calling behaviour. Only 53% of the females exhibited calling behaviour after exposure (Wei *et al.* 2004). This decrease can negatively affect the mating success, resulting in fewer offspring. Over time, such an effect could result in population declines, potentially causing a loss of these species in localised areas or entire regions a few generations down line.

Additionally, insect exposure to carbaryl also led to detachment behaviour and impaired movement in several insect species (Behle 2001, Overmyer & Noblet 2003). For instance, blackfly larvae (*Simulium* spp.) exposed to carbaryl detached from the surface of flask, sinking to the to the bottom, a behaviour typically associated with unfavourable conditions (Overmyer & Noblet 2003). Similarly, the Western corn rootworm (*Diabrocita virgifera virgifera*) stopped feeding and lost the ability to stand shortly (a few minutes) after being exposed to carbaryl, behaviours that would likely result in starvation and death if the insects could not access new food sources (Behle 2001). Inevitably, insects must also be able to avoid contaminated environments to minimize exposure to harmful chemicals (Skelhorn & Ruxton 2008). However, honeybees showed less avoidance of carbaryl, leading to higher rates of poisoning and death in a field study (Kang & Jung 2017). This finding is concerning given the already declining honeybee populations, suggesting that the use of carbaryl should be minimized to prevent further harm to these critical pollinators (Zattara & Aizen 2021).

Furthermore, carbaryl affects the emergence rate and can cause developmental malformations in insects. For example, exposure to this insecticide may cause malformations of certain organs or body parts in many insects (Gosh Shreya *et al.* 2015). It may also cause wrinkled skin in butterfly larvae and wrinkled wings in adults (Gosh Shreya *et al.* 2015). In another case, exposure of the beet armyworm *Spodoptera exigua*, has been shown to cause stunted development, with only the head fully formed after pupation (Adamski *et al.* 2007).

2.5.6 Carbaryl and other chemicals used in the studies

Carbaryl was the primary chemical of interest for this review. However, only 15 articles focused solely on testing the effects of carbaryl on insects. All the other articles tested carbaryl in combination with other chemicals, such as imidacloprid and deltamethrin (Bacci *et al*. 2009, Sharma & Abrol 2014). It was noted that imidacloprid and deltamethrin were mostly studied due to their high toxicity, which raises concerns about environmental impact (Lu *et al*. 2019, Sarker *et al.* 2024). These insecticides are known to kill a wide range of non-target organisms, contributing to biodiversity loss. As a result, many countries have banned or restricted their use (Groh *et al*. 2022, Sigmund *et al*. 2023).

2.6 Conclusion

The present review covered published articles from different parts of the world focusing on the effects of carbaryl on insects' behaviours, covering a period of 24 years. It highlighted whether experiments were mostly done in the laboratory or field. This information is critical because it reveals in what settings future studies should be carried out to produce optimum results and to generate new data. The limitation of this review is that only published articles were considered, other academic writing like dissertations and theses were not included. Future reviews should include other academic writing and consider looking at how carbaryl affects other more complex aquatic species such as frogs and fish, including zebrafish because there is plenty research done on how insecticides affect such species. To conclude, carbaryl should be phased out because of its negative effects on the behaviour of unintended target organisms.

Chapter 3

Survival and behavioral changes of mosquito larvae (*Culex sp.*) after exposure to carbaryl and pymetrozine

3.1 Introduction

Pesticides are chemicals used to control pest populations and regulate plant growth in agricultural and household settings (Rother & Ricado 2008; Quinn *et al.* 2011; Usman *et al.* 2024). They help increase a country's economy by controlling insect pests, leading to more production of quality food and sales, especially in developing countries (Carvalho 2017; European Parliament 2021). However, the extensive use of these chemicals results in them running off to aquatic ecosystems through leaching, causing pesticide pollution and causing abnormalities to non-target organisms or killing them (Vonesh & Kraus 2009). South Africa is not spared from pesticide pollution and has been added to the top 30 countries at risk of pesticide pollution (Tang *et al.* 2021). Because South Africa is a water-scarce country (Quinn *et al.* 2011), water resources should be protected from chemical contamination/ pesticide pollution.

A well-known toxic pesticide is Carbaryl, a whitish or greyish, odourless carbamate pesticide with the chemical formula $C_{12}H_{11}NO_2$ (Caro *et al.* 1974). Carbaryl was first registered for use in the 1950s, is highly toxic and can be fatal to non-target organisms such as beneficial insects, amphibians, birds, mammals and other marine organisms (USDA 2019). Carbaryl kills insects, invertebrates and other animals by disrupting their nervous system, which leads to convulsions and ultimately death. This pesticide is proven to be hazardous to invertebrates such as earthworms (*Eisenia fetida*), insects (honeybees, stone flies), shrimps and water fleas (EPA 2003; APVMA 2006) and vertebrates such as birds, fish, and rodents (Alavanja & Bonner 2012; Sahana & Agarwal 2018). It has also been documented to be hazardous to both humans and other higher mammals when inhaled or absorbed through the skin ultimately causing skin cancer (melanoma) (Dennis *et al.* 2010). Exposure to this insecticide can also cause infertility, pregnancy loss and stillbirths in humans (Sahana & Agarwal 2018). Although there are currently no reported cases of human deaths caused by carbaryl, this insecticide is extremely toxic and potentially detrimental to both human and environmental health (Bond *et al.* 2016). In addition, carbaryl is less to moderately persistent in the environment, with half-lives (the time it takes for a substance such as a pesticide to decrease to half of its original dose) of 4 days in water and 16 days on soil surfaces (Bond *et al.* 2016).

Moreover, a pesticide product of carbaryl, Blue Death, containing carbufuran and camphechlor (although camphechlor was banned in South Africa since 1970) showed a positive correlation with birth defects of the male reproductive structures of babies born from mothers from the Eastern Cape, South Africa (Heeren, 2003; Quinn *et al.* 2011). Even though the toxicity of this pesticide is highlighted in non-target organisms such as humans, it is still used in South Africa to this day. In South Africa, this chemical is readily available in retail stores and can be overused if not correctly managed or administrated. According to Pesticide Management Policy for South Africa (2010), pesticides that have endocrine disrupting properties, and that are carcinogenic and have immunotoxic potential should be considered for phasing-out, severe restrictions and bans. Carbaryl disrupts the endocrine system of insects and is carcinogenic to rodents and mice, hence this pesticide should be considered for phasing-out or severe restrictions (National Pesticide Information Centre 2003; U.S. Environmental Protection Agency 2004; Fattahi *et al.* 2012; Sabarwal *et al.* 2018).

Based on literature evidence, pymetrozine could be comparatively less toxic than carbaryl (Chang & Snyder 2008; Yu *et al.* 2018; El-Bouhy 2024). Pymetrozine is a pyridine azomethine insecticide which was not previously used as an insecticide until the 1990s (EPA 2002; Calvo-Agudo *et al.* 2020). This insecticide is a water-dispersible granule and works by inhibiting the feeding behaviour and other behaviours of the insect through disruption of the chordotonal organ Transient Receptor Potential Vanilloid (TRPV) channel complex (Calvo-Agudo *et al.* 2020; US EPA 2021; IRAC 2022). Pymetrozine only targets sucking insect pests such as aphids (Sechser *et al.* 2002) that cause damage to plants and lead to a decrease in crop production, thus possibly affecting the economy of a country. Due to this insecticide's novel mode of action, it is not easy for it to target unintended organisms or lead to the development of resistant strains (Sechser *et al.* 2002). This insecticide is comparatively less toxic to terrestrial and aquatic vertebrates and is determined to be less toxic to birds, humans, other mammals and insects including honeybees (Ishaaya *et al.* 2007; Chang & Snyder 2008; Patrzalek *et al.* 2021). It has also been found to be relatively harmless to beneficial insect species such as lady bird beetle (Sechser *et al.* 2002).

Pymetrozine does not pose a risk of contaminating groundwater and is less persistent in soil (Joseph *et al.* 2011) with a half-life of approximately 4 days, both in water and soil. Some have advocated that pymetrozine should be used regularly by farmers in Integrated Pest Management and private households, because it has minimal effect on the environment and human health, and has low leaching potential (Jansen *et al.* 2011, Sun *et al.* 2019, Calvo-Agudo *et al.* 2020). However, long exposure (78 weeks) to pymetrozine may result in reproductive toxicity (damaging fertility or the unborn child) and may be carcinogenic (USEPA 2010); Dewhurst & Zarn 2014). Trivia 500 WDG, a pymetrozine-based pesticide sold in South Africa, has been documented to have adverse or chronic effects on aquatic organisms such as Rainbow trout, Sheepshead minnows & Common carp after 96 hours exposure, and may be very toxic to these organism (LC50 >100 mg/l) (Trivia 500 WDG 2022).

In behavioural ecotoxicology, it is vital to assess all the factors that may contribute to changes in the behaviour of organisms when exposed to toxicants (Ford *et al.* 2021; Gerhardt, 2007; Hellou 2010; Peeters *et al.* 2009). For example, swimming in aquatic organisms is of importance because the survival of an organism is dependent on it. This behaviour allows organisms such as mosquito larvae to breath, forage and run away from predators, allowing the organisms to grow and survive (Tomè *et al.* 2014). Studying behaviours such as swimming, breathing, and resting of the organism in presence of contaminants such as pesticides is also important when assessing water quality, because changes to these behaviours can be used as early warning signs of toxic effects (Mnkandla & Otomo 2022). Organisms behave differently from how they normally would when exposed to contaminants, they would either spend more time and energy in one activity, such as swimming, as opposed other activities, and this might negatively affect their growth and survival.

Exposure to contaminants such as carbaryl hinders swimming behaviour in aquatic organisms and, this behavior can be changed at low concentrations of contaminants, as little as approximately 5% of the lethal concentration that kills 50% of the exposed population can cause changes in swimming of some organisms (fish) (Little & Finger 1990). Hence it is vital to look at them when assessing the toxicity of chemicals. To further prove that swimming is an important behaviour in ecotoxicology studies, it is used as a biomarker in toxicity assessments in *Daphnia* (Bownik 2017), which is a recommended test organism in ecotoxicology studies. Breathing is equally important, because without oxygen, the organism would suffocate and die (de Jager & Kruger 2019). The larvae require to spend more time breathing before turning into an adult and disturbances to the larvae's breathing time may slow their development resulting in smaller and less competitive adults (Roberts *et al*. 2014). Finally, resting in mosquito larvae is also important because after the larvae swims; while avoiding predation or disturbed, the larvae would need to rest to regain this energy (Roberts *et al*. 2014).

Mosquito larvae, which are considered model systems in testing behavioral ecology were used as test organisms in this study (Juliano 2010). The larvae were used in this study because they are easy to handle, restricted only to water and can easily be controlled during the experiments (Lee *et al.* 2017). These organisms also complete their life cycle in a short period of time, meaning that they can be used in abundance in a short space of time (Crans 2004, Centers for Disease Control and Prevention 2024). Additionally, the larvae do not transmit any diseases, since most adult mosquitoes are known to be vectors of many deadly diseases such as Zika virus, Yellow fever, malaria and West Nile fever (Cuervo-Parra *et al.* 2016), thus using the larvae to carry the experiments is considerably safe.

3.2 Aim and objectives

The aim of this chapter is to determine survival and assess behavioral changes of *Culex* sp. after exposure to carbaryl and pymetrozine.

The objectives are to:

- Determine the survival rate of mosquito larvae, *Culex* sp. after exposure to insecticides carbaryl and pymetrozine.
- To assess the breathing, swimming, and resting behaviors of mosquito larvae after exposure to carbaryl and pymetrozine.
- To compare the toxicity of carbaryl and pymetrozine in *Culex* sp. mosquito larvae after exposure to carbaryl and pymetrozine.

3.3 Methodology

3.3.1 Test organisms

Culex sp. larvae were collected from a private garden in Harrismith (28° 17′ 0′′ S, 29° 08′ 8′′ E), Free State, South Africa, and transported to the Ecotoxicology Research Laboratory at the University of the Free State (QwaQwa campus), Free State, South Africa. Behavioural changes (swimming, breathing and resting) and mortality of the larvae were assessed. The larvae were identified and confirmed by the help of Dr Judicaël Obame (Entomologist). Only 4th instar larvae (Figure 1) were used for these tests. This was done because this is the last larval stage before pupation. At this stage, the larvae have developed enough and can be easily seen and recorded during behavioural testing.

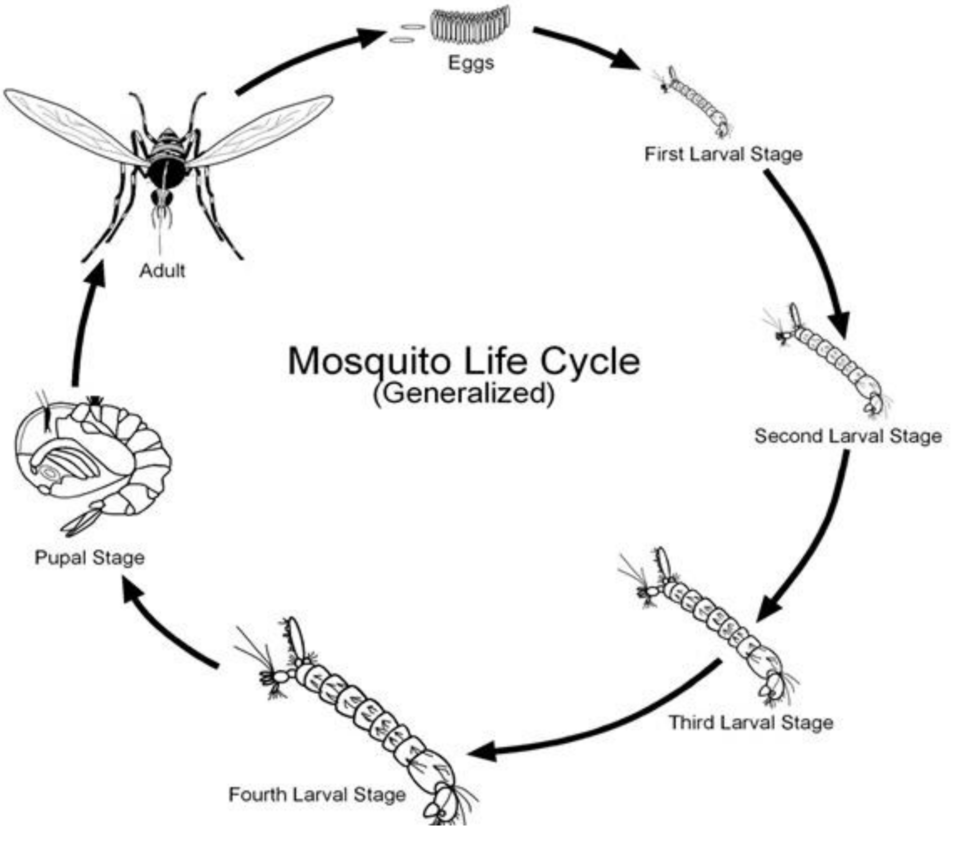

Figure 1: Illustration of general life cycle of mosquitoes. Obtained from www.mosquitoes.org/LifeCycle.html

3.3.2 Test pesticides

Doom Blue Death- Multi Insect powder (carbaryl) was purchased from a PEP store, Phuthaditjhaba (28° 32′ 00′′ S, 28° 49′ 00′′ E), Free State, South Africa. Doom Blue Death-Multi Insect powder contains 15g of carbaryl (carbamate) per one kg of this powder (15g/kg) as an active ingredient, and 3g of permethrin (pyrethroid) per one kg (3g/kg) (information from packaging). Trivia 500 WWDG 500g/kg pymetrozine was purchased from Villa Crop, Kempton Park (26° 06′ 00′′ S, 28° 15′ 00′′ E), Gauteng, South Africa. Trivia 500 WWDG contains 500g of pymetrozine (pyridine azomethine) per one kg (500g/kg) as an active ingredient (information from packaging).

3.3 Endpoints

3.3.1 Survival

Exposures were carried in 250ml measuring cylinders, the cylinders were filled up to 100ml mark with the solution. Using 5ml plastic Pasteur pipettes individual larvae were transferred to the exposure solutions. Cohorts of five randomly selected larvae were put in different concentrations of each active ingredient for 24 hours and this exposure was repeated four times, assessing mortality rates. The larvae (200 individuals) were exposed to both carbaryl and pymetrozine using four different concentrations for each active ingredient (12,5; 25; 50; and 100 µg/L for carbaryl and 6,25; 12.50; 25; and 50 mg/L for pymetrozine), including the control (distilled water). Range-finding tests were carried out to determine suitable carbaryl concentrations. In the first trial, concentrations of 0; 125; 250; 500; and 1000 µg/L were used to test for mortality and all organisms in the exposures died (except for the control). On the second trial, concentrations of 0; 2,5; 5; 10; and 15 µg/L were used, and all the organisms survived. In the last trial, concentrations of 0; 12,5; 25; 50; and 100 µg/L were used. For pymetrozine, concentrations of 0; 12,5; 25; 50 and 100 µg/L were used but caused very low (~10% , and we were aiming for at least 30% morality) mortality. Consequently, concentrations of 0; 6,25; 12.50; 25; and 50 mg/L were used. No food was provided for the larvae during the exposure period because the exposure only lasted 24 hours. These exposures were carried at 20°C. After 24 hours, when the exposure period ended, the number of immobilized larvae, even after being pricked with a pipette, were declared dead and counted.

3.3.2 Breathing, swimming, and resting behaviours

Individual larvae were exposed to either the control or one of the chemical treatments of carbaryl and pymetrozine and filmed for ten minutes. Exposures were carried in 250ml measuring cylinders, the cylinders were filled up to 210ml mark with the test solution (210 ml mark was chosen to allow the test organisms to have sufficient solutions to explore). Using 5ml plastic Pasteur pipettes individual larvae were gently picked or transferred to the exposure solutions. The behaviours (breathing, swimming, and resting) were recorded for 10 minutes using an iPhone 11 camera (1080p HD video recording at 60 fps), and this exposure was repeated for 15 different larvae for each active ingredient and each exposure concentration, including the negative control (distilled water). These experiments were carried at 20°C because this is the recommended temperature for chemical testing in ecotoxicology. According to Mnkandla & Otomo (2022), breathing was defined as the behaviour that occurs when the larvae are positioned upside-down with their breathing tubes (tail ends) protruding beyond the surface of the water column. Swimming was defined as the free exploration of the water column by the larvae and the time spent by the larvae when positioned neither at the bottom nor at the top of the water column. And resting was defined as the behavior typified by the larvae lying motionless at the bottom of the water column.

3.3.3 Statistical analysis

To test for the normality of the data, Shapiro- Wilk normality test was used. The mortality and survival data was found to be not normally distributed. Kruskal Wallis test was used to analyse data, followed by Dunn's test of comparison as a post-hoc test for further analysis of the data. In addition, the behavioural data was also not normally distributed, to analyse this data, Kruskal Wallis test, followed by Dunn's test of comparison were used. The software used for analysing behavioural responses was Graph Pad Prism (GraphPad Prism version 6.00 for Windows, GraphPad software, San Diego, CA, USA, www.graphpad.com). Finney's probit analysis spreadsheet calculator (Mekapogu 2021) was used to calculate the 24 hours lethal concentrations (LC_1- LC_{99}) of carbaryl and pymetrozine in *Culex* sp. larvae.

3.4 Results

Figure 2 represents the survival of mosquito larvae after 24 hours of exposure to carbaryl in four replicates. There was a significant difference ($p < 0.05$) in the survival rates of *Culex* sp. larvae in the control (0 µg/L) and the highest concentration (100 µg/L) after exposure to carbaryl for 24 hours. This indicates that exposure to carbaryl affected the survival of the mosquito larvae (there was 100% survival in the control, 75% in 12.5 µg/L, 60% in 25 µg/L, 30% in 50 µg/L and 10% in 100 µg/L). However, there was no significant difference ($p > 0.05$) in the other concentrations (12.5, 25, and 50 µg/L) when compared to the control and the highest concentration. Exposure to carbaryl in these concentrations slightly affected the survival of mosquito because as the concentration range increased, the number of surviving larvae decreased. The calculated LC_{50} was 0.028 mg/L.

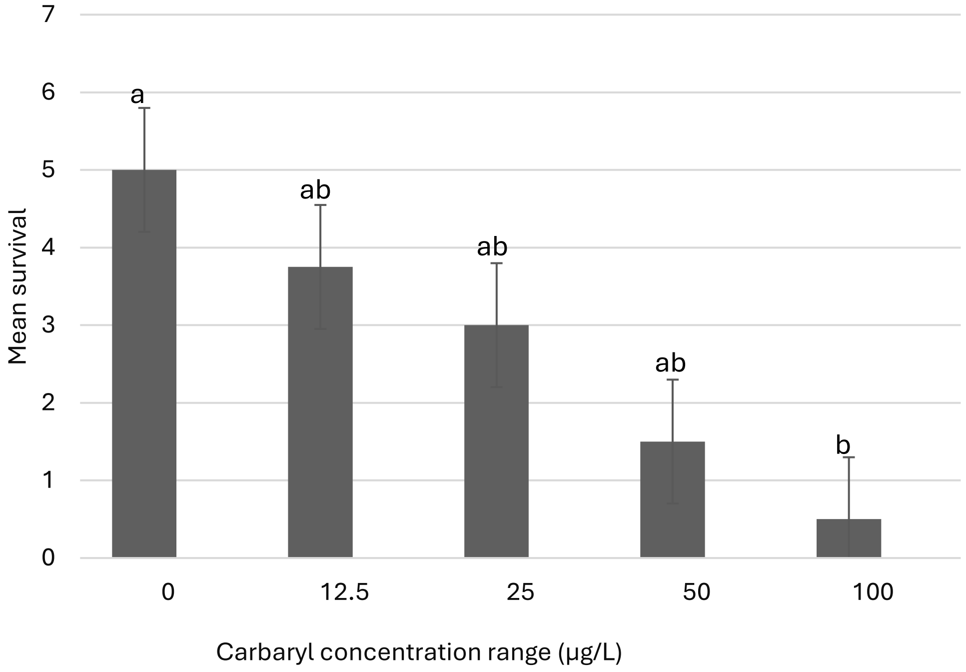

Figure 2: Average of surviving number of mosquito larvae after exposure to different concentrations of carbaryl for 24 hours. Values are presented as ±SD and letters represent statistically significant differences ($p<0.05$). n=100 individuals, 20 per concentration/ treatment.

Figure 3 illustrates the survival of mosquito larvae after 24 hours of exposure to pymetrozine, in four replicates. There was 100% survival in the control, 100% in 6.25 mg/L, 100% in 12.5 mg/L, 85% in 25 mg/L and 75% in 50g/L) Overall, there was no significant difference ($p > 0.05$) in the survival rates of *Culex* sp. larvae after exposure to pymetrozine for 24 hours. This indicates that exposure to pymetrozine did not affect the survival of the mosquito larvae. However, highest mortality was observed in the highest concentration of the insecticide (50 mg/L). The calculated LC_{50} is 181.95 mg/L.

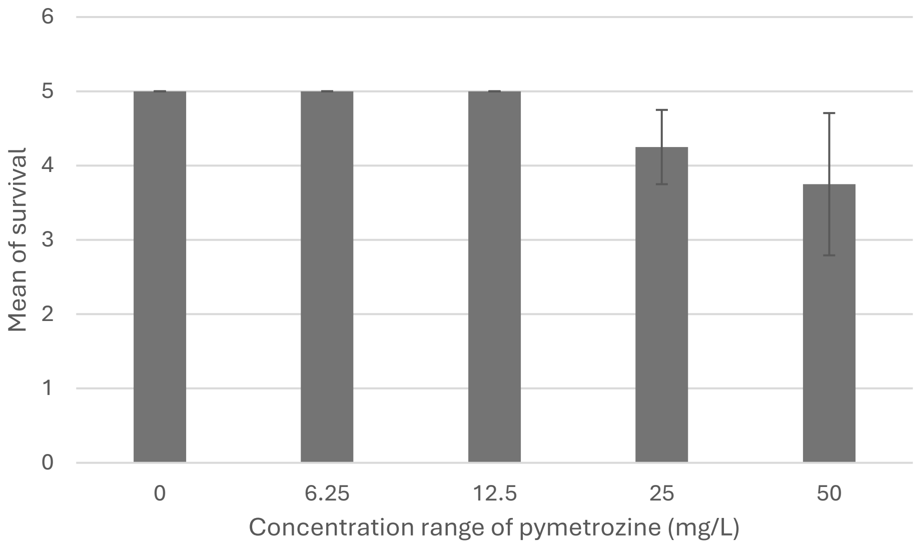

Figure 3: Average of surviving number of mosquito larvae after exposure to different concentrations of pymetrozine for 24 hours. Values are presented as ±SD. n= 100 individuals, 20 per concentration/ treatment.

Table 2: Lethal concentrations 30, 50 and 90 (generated in Finney's probit analysis spreadsheet calculator) of carbaryl and pymetrozine (in mg/ L) of *Culex* sp. larvae after 24 hours exposure.

	Carbaryl	Pymetrozine
LC_{30}	0.016 (0.010; 0.025)	66.650 (24.941; 178.111)
LC_{50}	0.028 (0.018; 0.044)	181.950 (68.086; 486.224)
LC_{90}	0.107 (0.069; 0.198)	2117.471 (792.368; 5658.589)

Figure 4 represents the effects of carbaryl on the breathing behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae in total) after 10 minutes of exposure. In the control, the larvae spent most of the time (average of 476 seconds, which is 78% of the time) breathing. When compared to other treatments, the breathing time decreased to 53% in 12.5 µg/L, then increased to 68% in 25 µg/L, further decreased to 40% in 50 µg/L and finally increased to 41% (1% more from 50 µg/L) in 100 µg/L. There was significant difference ($p < 0.05$) in the control and 50 µg/L, and in the control and 100 µg/L. The was no statistical difference ($p > 0.05$) between the control and 12.5 µg/L, and 50 µg/L. Moreover, there was no significant difference ($p > 0.05$) between 12.5 µg/L, 25 µg/L, 50 µg/L and 100 µg/L.

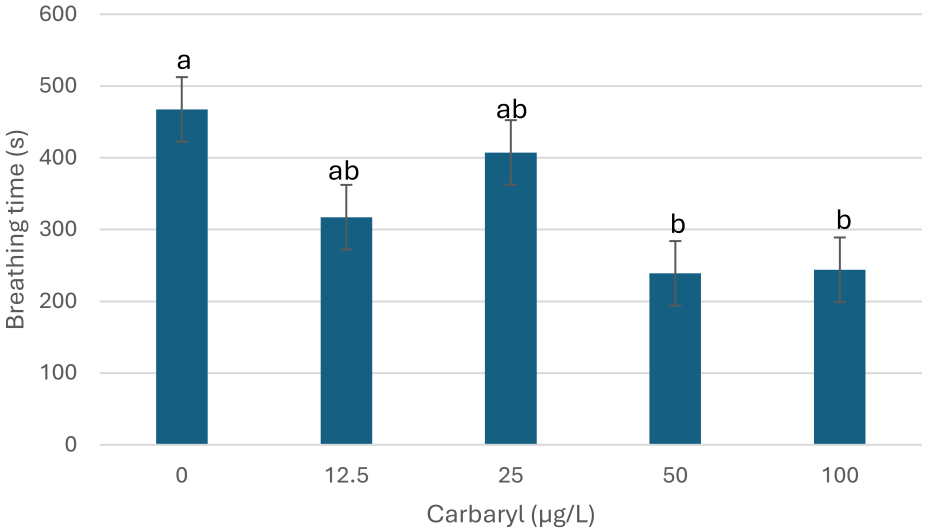

Figure 4: The effects of carbaryl on the breathing time of *Culex* sp. mosquito larvae. Error bars represent standard error, and letters represents significant differences. n= 15 larvae per treatment.

Figure 5 reflects the effects of carbaryl on the swimming behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae) after 10 minutes of exposure. The larvae spent most of the time (average of 269 seconds, which is 45% of the time) swimming, in the highest concentration (100 µg/L). When compared to other treatments, swimming time decreased to 31% in 50 µg/L, further decreased to 12% in 25 µg/L, then increased to 24% in 12.5 µg/L and finally decreased to 15% in the control. There was significant difference in swimming ($p < 0.05$) when comparing the control and 100 µg/L, as well as when comparing 25 µg/L and 100 µg/L. The was no statistical difference ($p > 0.05$) between the control and 12.5 µg/L, and 50 µg/L. Moreover, there was no statistical difference ($p > 0.05$) between the control and 25 µg/L.

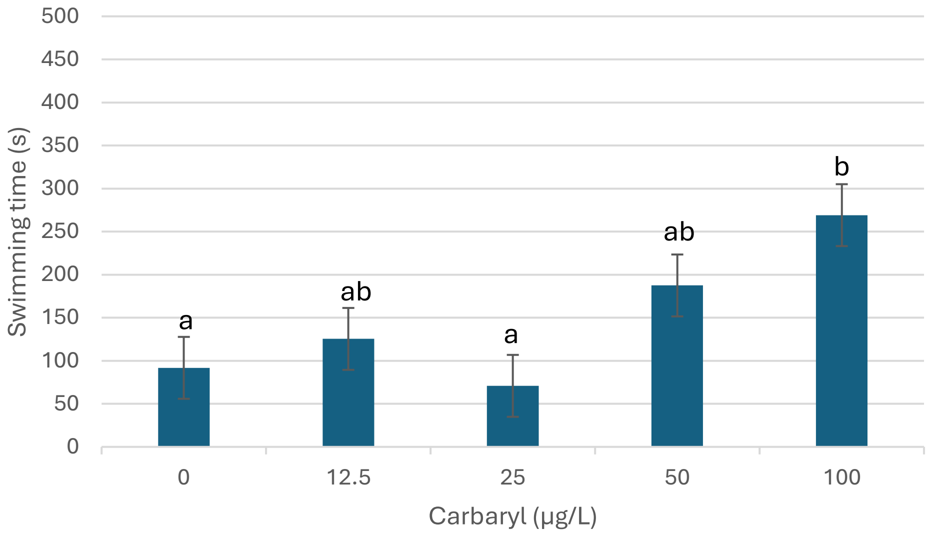

Figure 5: The effects of carbaryl on the swimming time of *Culex* sp. mosquito larvae. Error bars represent standard error, and letters represents significant differences. n= 15 larvae per treatment.

Figure 6 represents the effects of carbaryl on the resting behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae) after 10 minutes of exposure. The larvae spent most of the time (average of 175 seconds, which is 29% of the time) resting, in 50 µg/L. The larvae hardly rested in the control, the resting rate was 7%, while the resting rate increased to 26% in 12.5 µg/L, then decreased to 20% in 25 µg/L. In the highest concentration, the resting rate was 15%. Statistically, significant difference ($p < 0.05$) was only observed when comparing the larvae in the control and 50 µg/L. The was no statistical difference ($p > 0.05$) between the control and 12.5 µg/L, 25 µg/L and 100 µg/L.

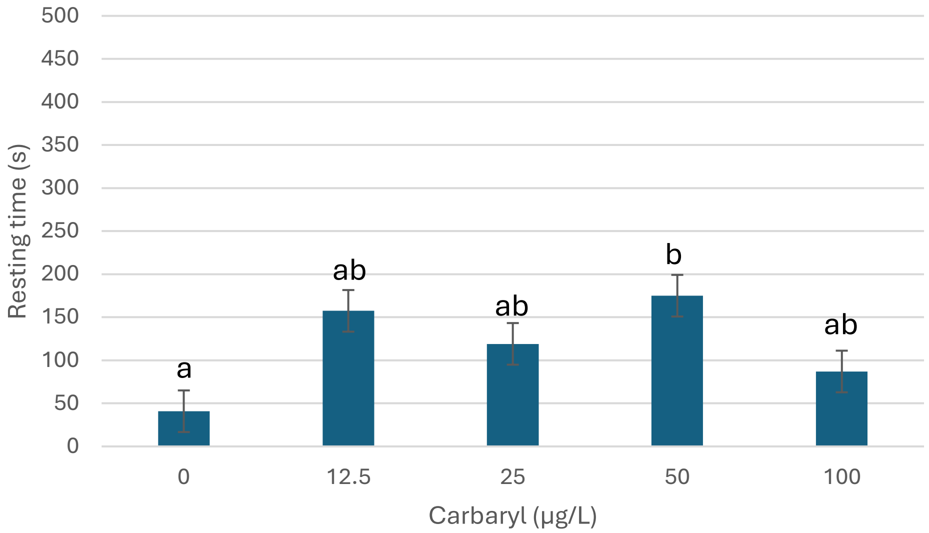

Figure 6: The effects of carbaryl on the resting rate of *Culex* sp. mosquito larvae. Error bars represent standard error, and letters represents significant differences. n= 15 larvae per treatment.

Figure 7 displays the effects of pymetrozine on the breathing behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae) after 10 minutes of exposure. In the control, the larvae spent most of the time (average of 495 seconds, which is 83 % of the time) breathing. When compared to other treatments, the breathing time decreased to 56.50% in 6.25 mg/L, then slightly increased to 57.33% in 12.50 mg/L. Furthermore, the breathing time increased to 69% in 25 mg/L and then decreased to 41% in 50 mg/L. There was significant difference ($p < 0.05$) in the control and 50 mg/L. The was no statistical difference ($p > 0.05$) in breathing in the larvae exposed in 6.25 mg/L, 12.50 mg/L and 25 mg/L. There were also no statistical differences in breathing time of larvae exposed to 12.50 mg/L and 25 mg/L. Moreover, there was no significant difference ($p > 0.05$) in the breathing time between the control, 6.25,12.50, and 25 mg/L.

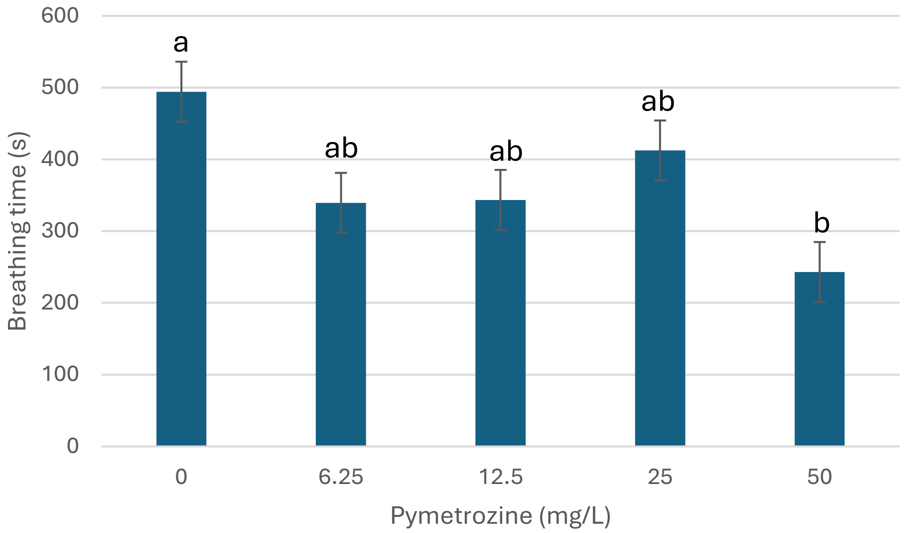

Figure 7: The effects of pymetrozine on the breathing rate of *Culex* sp. mosquito larvae. Error bars represent standard error, and letters represents significant differences. n= 15 larvae per treatment.

Figure 8 exhibited the effects of pymetrozine on the swimming behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae) after 10 minutes of exposure. The organisms spent most of the time (approximately 167 seconds, 28% of the time) swimming in the 50 mg/L solution. And the least amount of time in the control (56 seconds, 9%). In other treatments, the swimming time was 19%, 21% and 22% in the 25 mg/L, 6.25mg/L and 12.50 mg/L solutions, respectively. There was significant difference ($p < 0.05$) in the control and 12.50 mg/L, and the control and 50 mg/L. The was no statistical difference ($p > 0.05$) between the 6.25 mg/L and 25 mg/L. Moreover, there was no significant difference ($p > 0.05$) between the control, 6.25, and 25 mg/L.

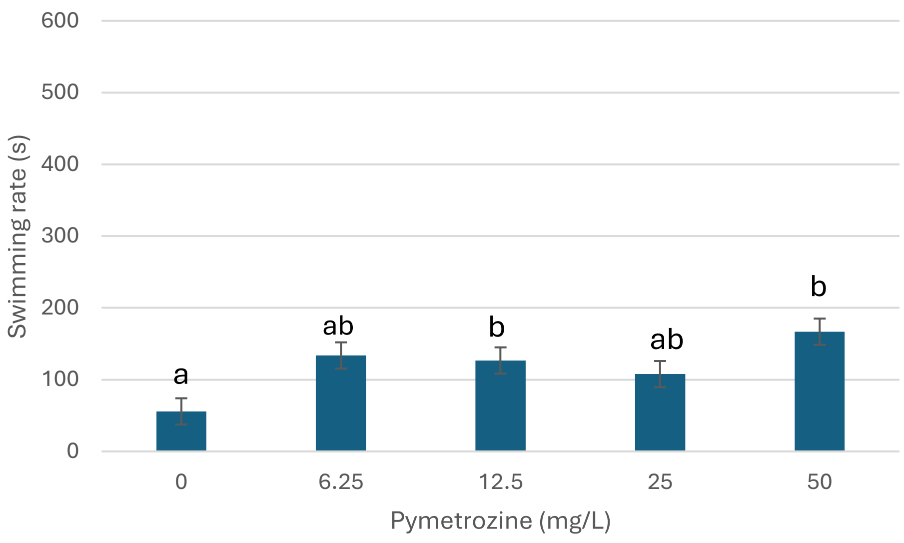

Figure 8: The effects of pymetrozine on the swimming rate of *Culex* sp. mosquito larvae. Error bars represent standard error, and letters represents significant differences. n= 15 larvae per treatment.

Figure 9 represents the effects of pymetrozine on the resting behaviour of the mosquito larvae for 15 different larvae per treatment (75 larvae) after 10 minutes of exposure. The larvae spent most of the time (average of 177 seconds, which is 29.5% of the time) resting, in the 50 mg/L solution. The larvae spent least time resting in the control, the resting rate is 8%, and in 25 mg/L (13% of the time). The resting rate was 21% in 6.25 mg/L, then increased to 22% in 12.50 mg/L. The was no statistical difference ($p > 0.05$) between the control and all the other concentrations (6.25 mg/L, 12.50 mg/L, 25 mg/L and 50 mg/L) observed.

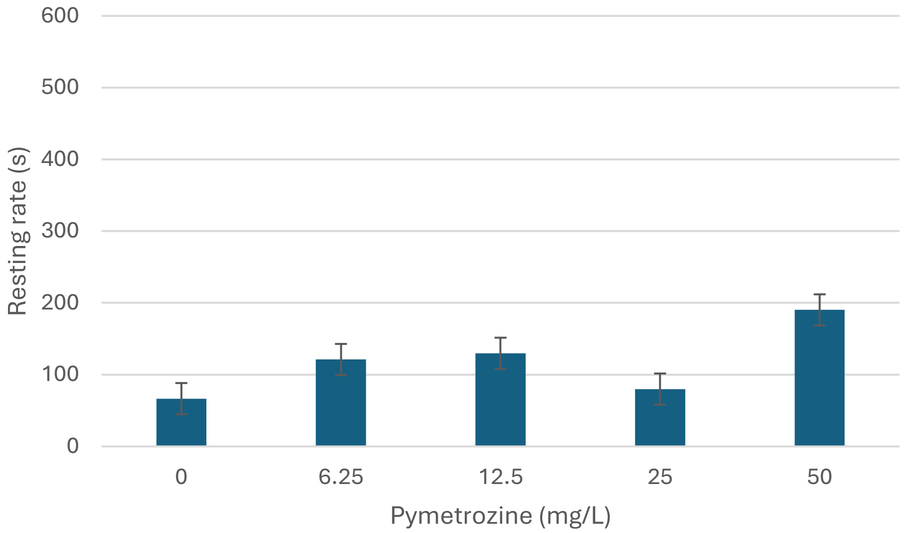

Figure 9: The effects of pymetrozine on the resting rate of *Culex* sp. mosquito larvae. Error bars represent standard error. n= 15 larvae per treatment.

Figure 10 represents the effects of distilled water (control) of different behaviours (breathing, swimming, and resting) on the mosquito larvae. The larvae spent most of the time (average of 481 seconds, which is 81% of the time) breathing, followed by swimming (approximately 74 seconds, 12%) and spent least amount of time resting (approximately 45 seconds, approximately 8%) in the absence of insecticides (either carbaryl or pymetrozine).

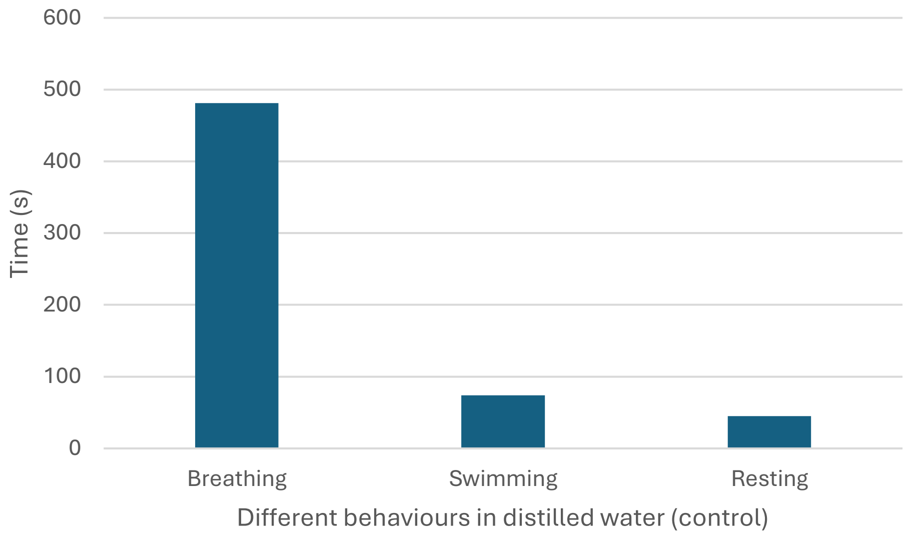

Figure 10: The effects of distilled water on different behaviours of *Culex* sp. mosquito larvae. n= 30 larvae per treatment.

3.5 Discussion

Comparing the mortality results of both carbaryl and pymetrozine, carbaryl was the most toxic insecticide because as little as 100 µg/L caused significant mortality of the larvae (Figure 2). This was also confirmed by the fact that LC_{50s} of 0.0281 mg/L (0.0179; 0.0439) and 181.95 mg/L (68.086; 486,224) were generated for carbaryl and pymetrozine, respectively (Table 2). Carbaryl is a fast-acting and relatively toxic insecticide because acute exposure (24 hours) to it caused significant mortality to mosquito larvae (Figure 2). The results of the present study differ from those of Rettich (1977) who generated an LC_{50} of 333 µg /L for *Culex pipiens* larvae and a LC_{50} of 376.6 µg/L for *Aedes contans* when larvae of these mosquito species were exposed to carbaryl for 24 hours. These differences were attributed to the different concentration ranges used in our study and that of Rettich (1977). It is worth noting that our study was one of the few studies to report LC_{50}'s of carbaryl and pymetrozine on mosquito larvae. Furthermore, Jansen *et al*. (2011) and Calvo-Agudo *et al*. (2020) also highlighted that pymetrozine is a less toxic insecticide and can be used to minimize negative environmental effects and human health hazards.

The high mortality in carbaryl could be attributed to the fact that this chemical is a cholinesterase-inhibiting pesticide. This means that exposure to it affects the nervous system of the organism resulting in paralysis, convulsions, and ultimately death (Caro *et al*. 1974, IRAC 2012). The low mortality in pymetrozine could be because this insecticide affects the feeding behaviour of the organism (Ausborn *et al.* 2005, Raj Boina *et al*. 2009), and produces the best efficacy through uptake by feeding and much less by direct topical contact (Wyss & Bolsinger 1997). In our experiment, the larvae were not fed pymetrozine but were exposed to it through the swimming medium during the 24-hour exposure. Perhaps due to the concentration range (0-50 mg/L) or the duration of exposure (24 hours), the results largely indicated limited toxicity. In addition to pymetrozine affecting the feeding behaviour of the organisms, pymetrozine is also identified to have neurotoxic effects on insects by IRAC (Könemann 2022). This insecticide can alter the normal functioning of chordotonal mechanoreceptors, resulting in changes in insects' coordination and sense of gravity (Peng *et al.* 2023).

Yang *et al*. (2016) also proves that pymetrozine is not highly toxic or lethal to mosquito larvae (*Culex pipiens pallens*). According to their study, at least 10 mg/kg is needed to cause 90% mortality and 100 mg/kg to cause 100% mortality. However, in our study, we recorded no mortality in the 12.5 mg/L treatment (Figure 3). The difference in our results and Yang *et al.* (2016)'s results can be due to the different temperatures used during exposure, our exposures were done at 20°C and theirs at 25±1 °C. In addition, when tested in lady beetles (*Hippodamia veriegata*), pymetrozine was proved to be more harmless to 4th instar larvae and adults at 2500 ppm (parts per million) in tap water after 24 hours (Almasi *et al*. 2013), further proving that pymetrozine is a less toxic insecticide.

Tsotesti *et al.* (2022) conducted a similar study where they looked at the breathing, swimming, and resting behaviours of culicid larvae after exposure to imidacloprid. Tsotetsi and colleagues found that in the control, the larvae spent nearly 75% of the time breathing and less time in imidacloprid solutions. In the present study, the larvae spent approximately 81% of the time breathing (Figure 10). Breathing is an essential behaviour in mosquito larvae because for the larvae to reach adulthood (via metamorphosis), they must spend most of their time close to the surface of the water to breathe (Hawkes & Hopkins 2022, Alverez-Costa 2024). Any change to the time spent breathing by the larvae might compromise the growth of the larvae, and potentially affect the metamorphosis process and adult emergence rate. Moreover, most mosquito larvae spend most of their time being inactive and feeding on the surface of the water (Roberts *et al*. 2019), which could possibly also explain why the larvae spent most of the time breathing in the absence of the insecticides as this is their natural behaviour. In the presence of carbaryl, the breathing time was drastically reduced because carbaryl affects acetylcholine esterase functions, which causes the affected nerves to be continuously stimulated, leading to the larvae's inability to swim up to the water surface to breathe (Bond *et al.* 2016). Nevertheless, the behaviour that dominated was the breathing behaviour.

Tomè *et al*. (2014), carried out a study similar to the present one. However, in that study, the authors assessed the survival and swimming behaviour of *Aedes aegypti* larvae and pupa after exposure to imidacloprid, spinosad, deltamethrin, and azadirachtin. It was found that larvae that were exposed to imidacloprid and spinosad decreased the distance swum and increased resting time. Unlike Tomè *et al.* (2014,) the present study did not look at the distance swum but looked at the resting time of the larvae after exposure to carbaryl and pymetrozine. It is expected that neurotoxic insecticides cause impairment to swimming, surprisingly, the larvae spent more time swimming in the 100 µg/ L carbaryl solution (Figure 5). However, it is worth noting that "swimming", as defined in our experiments, was not necessarily active swimming but time spent by the mosquito larvae when positioned neither at the top nor the bottom of the water column.

Carbaryl has been known to reduce the swimming velocity of Natricine snakes (Hopkins *et al.* 2005), impair the motility of Zebra fish embryos (Schock *et al.* 2012), and affect the swimming behaviour of Daphnia (Dodson & Hanazato 1995). This asserts that carbaryl does indeed affect the swimming behaviour of not only mosquito larvae but also of other non-target organisms. Exposure to insecticides like carbaryl also affects the swimming speed of aquatic organisms such as tadpoles. Upon exposure to this chemical, the organisms' swimming activity is decreased, and this behaviour may make them more accessible to predators. On the other hand, the decrease in swimming activity may result in lower predation rates through reduced detection by visually oriented predators (Bridges 1997, Denoël *et al.* 2013).

It is expected that imidacloprid, Spinosad and carbaryl affect the behaviour of the mosquito larvae because they have more or less the same mode of action. According to Tomè *et al.* (2014), when 4th instar *Aedes aegypti* larvae were exposed to imidacloprid and spinosad, increase in concentrations of these pesticides resulted in an increase in resting time. However, when the larvae were exposed to increasing concentrations, the chemical did not have any effect on the resting time. According to our results, carbaryl did not affect the larvae, in the same manner. When we exposed 4th instar larvae of *Culex sp*. to carbaryl, there was no correlation between the resting time and the concentration range i.e. an increase in concentration range did not necessarily result in an increase in the resting time. The highest concentration used was 100 µg/ L carbaryl. Yet, the highest and statistically significant resting duration was observed at 50 µg/ L carbaryl. The difference between the present study and that of Tomè *et al.* (2014), could be because different species were used, and the studies largely used different methodologies. Moreover, all these chemicals are not of the same chemical groups and should be expected to exert different levels of toxicity.

The larvae did not spend much time resting in carbaryl solutions, but they spend more time swimming and exploring their environment (Figure 6). The resting time for this study was the highest at 50 µg/L and not 100 µg/L which was the highest concentration (Figure 6). It was expected that the larvae would spend more time resting in the highest concentration since exposure to carbaryl causes insect paralysis (Schock *et al.* 2012). Maybe increased resting would be observed if the larvae were exposed to the chemical for a longer period. These results are contrary to a study by Tomè and colleagues in (2014) where the resting time of the larvae increased when the concentrations of imidacloprid and spinosad were increased. However, Tsotesti *et al.* (2022) found that the increase in resting time was not directly proportional to an increase in concentration range, i.e. the highest concentration used was 2 mg/L of imidacloprid and the larvae spend most of the time resting in 1 mg/L imidacloprid solution, and what they found is the same as what this current study found.

Carbaryl is not as selective as pymetrozine and affects most untargeted essential organisms such as bees (USEPA 2003), birds (George *et al.* 1992), and fish (Tilak *et al.* 1981). Earthworms are known for their important role in soil ecosystems, these organisms enhance soil structure and fertility (Saxena *et al.* 2014), and carbaryl is proven to be extremely toxic to earthworms (Cathey 1982, Gupta & Sexena 2003, Sithole *et al.* 2023). The high toxicity of carbaryl was also reported freshwater fish *Colisa fasciatus* (Singh *et al.* 2008). Our results show that carbaryl is toxic to *Culex* sp. mosquito larvae because there was significant difference between the control and 100 µg/L carbaryl solution which is equivalent to 0.1 mg/ L and the USEPA Acute Response Dose (0.1 mg/kg/ d), in addition, the field recommended application rate (25 mg/kg) (Mostert *et al.* 2002, Sithole *et al*. 2024).

Research is very limited on how pymetrozine affects the swimming, resting, and breathing behaviour of mosquito larvae. In the present study, mosquito larvae spend most of their time breathing in the distilled water than in pymetrozine solutions. Even though Pymetrozine is documented to be less toxic, it lowered the breathing rate of *Culex* sp. in the highest concentration (50 mg/L) tested ($p < 0.001$) (Figure 7). In the absence of pymetrozine, breathing rates were as high as the rates observed by Tsotesti *et al.* (2022). This further proves that larvae tend to spend more time breathing in the absence of any chemical (insecticide), and this behaviour is essential for the normal development and growth of the mosquito.

In the current study, it was observed that mosquito larvae exposed to pymetrozine swam and rested the most in the highest concentration (50 mg/L). However, Tomè *et al.* (2014), noted that deltamethrin (a pesticide that affects insects through ingestion and direct contact), did not really affect the resting behaviour of the *A. aegypti* mosquito larvae upon exposure to different concentrations (0.001, 0.01, 0.05, 0.1, 0.5, 1.0 and 10.0 ppm), this could be most likely due to the fact that the tested concentrations were different between our study and that of Tomè *et al.* (2014). Pymetrozine changes the insect's coordination due to changes in the normal functioning of chordotonal mechanoreceptors and the observed increase in the resting behaviour in 50 mg/L may be because the larvae could no longer swim as much as in the control because of the changes in the chordotonal mechanoreceptors (Könemann 2022).

Additionally, pymetrozine did not cause concentration-dependant reduction in locomotion on zebrafish (Könemann 2022). These results are similar to ours, as we observed that the increase in resting time was not precisely dependent on the concentration (e.g., in 12.5 mg/L, the resting rate is much higher than in the 25 mg/L). The swimming behaviour of mosquito larvae upon exposure to lambda-cyhalothrin, a pyrethroid insecticide, was studied in prey-predator interactions and insecticide resistance (Valbon 2019). Valbon (2019) observed that in *A. aegypti* larvae, insecticide resistance increased the swimming duration and distance of the larvae.

Yu *et al.* (2018) demonstrated that pymetrozine can be highly lethal to the juveniles of *Procambarus clarkii*, the red swamp crayfish which is known as a key species because of its use in pollution determination and water quality. Exposure to 0.1,0.3,0.5,0.7,0.9 and 1.1 mg/L of pymetrozine resulted in locomotory behavioural changes, i.e., the crayfish initially showed fast movement, then lost equilibrium and ultimately sank to the bottom and this concentration range was lower than the one used in the current study (6.25, 12.5, 25, 50 mg/L). Nevertheless, in our study the mosquito larvae spent more time swimming in 50 mg/L, and we did not look at swimming speed, however it is important to note that pymetrozine does have an effect on the swimming rate of mosquito larvae. Similar behavioural changes (erratic swimming and loss of equilibrium) were also noted in the common carp after exposure to flonicamid (Ghelichpour *et al.* 2019). Flonicamid is a pyridine carboxamide insecticide that kills insects by stopping their feeding behaviour, which results in starvation (Ghelichpour *et al.* 2019). This mechanism or mode of action is like that of pymetrozine. Yu et al. (2018) study on the red swamp crayfish and Ghelichpour *et al.* (2019) study on the common carp further prove that the aforementioned insecticides (pymetrozine and flonicamid) do affect the swimming behaviour of aquatic species.

The desired outcome of insecticides would be to affect only the organisms they were designed to target and kill and cause less harm to non-target organism and the environment. As such, pymetrozine can be classified as a better pesticide because of its unique mode of action and tendency to affect targeted organisms. Jansen *et al.* (2011), noted that pymetrozine is a highly selective pesticide because pymetrozine did not affect or cause mortality of insects that play essential roles in the environment. These insects include rove beetle (*Aleochara bilineata*), parasitic wasp (*Aphidius rhopalosiphi*), ladybird (*Adalia bipunctata*), hoverfly (*Episyrphus balteatus*), and carabid beetle (*Bembidion lampros*), which are bioindicators, pollinators and aphid predators. This insecticide was proven to be practically less toxic to fish Rainbow trout (*Oncorhynchus mykiss*), Sheepshead minnow (*Cyprinodon variegatus*) and Bluegill sunfish (*Lepomis macrochirus*) (El- Bouhy *et al.* 2024). However, the safety data sheet of trivia 500 WDG (2022), shows that pymetrozine could be highly toxic to fish, rainbow trout, sheepshead minnows and common carp with LC_{50s} (96h) of less than 100 mg/L. Moreover, pymetrozine can be highly lethal to the juveniles of red swamp crayfish (Yu *et al.* 2018). Overall, nevertheless, pymetrozine is proven to be less toxic than carbaryl.

In conclusion, the findings of this study reveal that carbaryl is more toxic than pymetrozine. This is proven by the LC's and by carbaryl causing more mortality at 100 µg/L when compared to pymetrozine solution at 50 mg/L. Exposure both carbaryl and pymetrozine does affect the breathing, swimming and resting behaviours of the larvae. Due to the altered behaviours because of chemical exposures, the survival and fitness of the larvae may be negatively affected.

Chapter 4

Geotaxis responses of *Culex* sp. larvae after exposure to carbaryl and pymetrozine.

4.1 Introduction

Geotaxis also known as gravitaxis is defined as organisms' response to gravity, it is a binary response that can be either positive or negative (Suklin 1984). Positive geotaxis is defined as downward movement of an organism towards the centre of Earth whereas negative geotaxis is the upward movement of an organism away from the centre of Earth (Kanda 1915, Shirley and Shirley 1988). Geotaxis could be a valuable tool in ecotoxicological study because gravity plays an important role in directing or orienting organisms such as vertebrates and invertebrates to proper environments which are suitable for their survival (Bownik & Wlodkowic 2021). An advantage of studying this endpoint is that geotaxis is easy to observe and can be easily analysed, however, geotaxis can be confused with other behavioural responses such as reaction to light (phototaxis) (Ruhela *et al.* 2019, Bownik & Wlodkowic 2021).

To sense gravity, different organisms use different organs. For example, aquatic larvae of *Limnobiidae* utilizes statocysts (Schwartzkopff 1974), crustaceans (Bender & Frye 2009) and terrestrial mollusks (snails and slugs) also use a pair of statocysts (organ located in the brain), and *Drosophila* uses Johnston's organ (sensory cells located in the pedicel of antennae) (Sun *et al.* 2009). Geotaxis studies are mostly done in invertebrates including oysters (Wheeler *et al.* 2017), decapods (Almeid *et al.* 2021), tea mosquito bugs (Das 2022), zebra fish (Sabadin *et al.* 2022) and *Drosophila* flies (Rhodenizer *et al.* 2008, Peng *et al.* 2023), and these organisms usually show negative geotaxis. Negative geotaxis is extensively studied in the fruit fly *Drosophila* because this fly is a sensitive model organism that reproduces within a short time, allowing more generations to be tested in a short period of time. Nevertheless, this endpoint is also tested in other organisms such as crabs (Shirley & Shirley 1988), fish (Thoré *et al.* 2023), trematode cercariae and in vertebrates such as rats, negative geotaxis is used to study different central nervous system disorders (CNS) (Ruhela *et al.* 2019). Geotaxis plays an essential role in helping parasites locate their hosts, increasing their chances of survival, helping flies escape their predators and other organisms in the aquatic system (Bauer *et al.* 2005, Chen *et al.* 2021). Geotaxis also plays an important role in the alarm reaction of mosquito larvae (Mellanby 1958).

Not all organisms display negative geotaxis, some organisms generally display positive geotaxis, especially terrestrial vertebrates. Young rodents and rats have the tendency to move downhill (displaying positive geotaxis) when placed at different angles (Alberts *et al.* 2004, Ben-Shaul *et al.* 2022). This tendency is usually because these animals live in burrows (Torres *et al.* 2003). Other animals that usually exhibit positive geotaxis are snails (Lervi & Clark 2015, Lervi *et al.* 2017). Some organisms display a geotaxis switch in the presence of external stimuli and others in the absence of external stimuli. A geotaxis switch is a theoretical point where the geotaxis behaviour of an organism changes from negative to positive or vice versa. The walleye pollock, a fish, is an example where young juveniles exhibit positive geotaxis, and relatively older ones display negative geotaxis in the absence of chemicals (Davis & Olla

1994). Another example is in *Euglena* (single-cell flagellate eukaryotes) juveniles where a change in geotaxis has occurred in the presence of copper and mercury (Stallwitz & Häder 1994).

Chemical exposure can alter the geotaxis orientation of many organisms and lead to death (Al-Baggou 2004, Qiao *et al*. 2022). The extensive use of pesticides to control pest populations has resulted in the alteration of natural behaviours of exposed organisms resulting in decreased chances of survival in these organisms. Once an animal gets exposed to pesticides through dermal contact or oral administration, that animal's fitness is reduced. Carbaryl (1-naphthyl methylcarbamate), is a neurotoxic systematic carbamate insecticide that reversibly inhibits the acetylcholinesterase activity in animals. The inhibition of acetylcholinesterase by carbaryl is reversible because carbaryl poisoning tends to be of shorter duration (Fishel 2005). Even though poisoning happens in shorter duration, the buildup of acetylcholine in nerve synapses occurs leading to the continuous firing of nerve pulses in the nervous system (U.S.EPA 2024), resulting in paralysis, uncontrolled movement and death (Mora-Gutiérrez *et al*. 2021).

This carbamate is classified as a class II toxicant that can cause effects via ingestion, inhalation and skin contact (Koshlukova & Reed 2014). Carbaryl is a highly toxic, widely used insecticide that is used to control insects (NPIC 2003). This insecticide affects the natural behaviour of many exposed organisms (Gupta & Saxena 2003). For instance, carbaryl negatively affected bosminids' natural defence mechanism against predators increasing the mortality rate of such organisms and potentially altering population dynamics (Sakamoto 2009). Insects such as ants and bees rely on geotaxis responses to navigate and forage efficiently. Exposure to carbaryl can negatively affect the navigation and foraging success of these insects. In ants for example, exposure to carbaryl alters their ability to successfully navigate in their surrounding and forage effectively, decreasing their chances of survival (Mora-Gutiérrez *et al.*2021). In addition, carbaryl affects the burrowing behaviour of earthworms, and this disadvantageously affects the organisms because this behaviour is crucial in earthworms for escape from chemical exposure and predators (Gupta & Saxena 2003). Moreover, burrowing is also required for reproduction and feeding (Gupta & Sundararaman 1991), and short exposure to carbaryl results in mortality of *Eisena fetida* (Sithole *et al*. 2023). The effects of carbaryl on geotaxis has also been observed in aquatic crustaceans (freshwater decapods) that were exposed to carbaryl-contaminated water and became unable to maintain proper orientation, affecting the survival of these organisms (Naddy & Rodrigues 2015).

Many studies suggest that acute and chronic exposure of aquatic organisms to neurotoxic pollutants such as carbaryl can have long-term effects and pleiotropic effects on these species (Bownik & Wlodkowic 2021). For example, in American toads (*Bufo americanus*), carbaryl decreased the survival of toads to metamorphosis and increased time to metamorphosis in these organisms, negatively affecting their fitness (Distel *et al*. 2009). Hence, it is important to study the impacts of insecticides on life parameters and the behaviour of organisms in the aquatic environment to better understand how certain chemicals affect aquatic life, because changes induced by insecticides on the nervous system in aquatic ecosystems are understudied (Bownik & Wlodkowic 2021).

Another insecticide that causes neurotoxic effects on insects is pymetrozine, however the way in which this insecticide affects the nervous system is poorly understood (EPA 2000, Ausborn *et al.* 2005). Pymetrozine (group 9B insecticide) is highly effective against sucking insects such as aphids, leafhoppers and whiteflies, with low toxicity in bees (Fuog *et al.* 1998). This insecticide is highly selective, mostly affecting sucking and piercing insect pests by blocking the feeding tube (stylet), therefore, stopping the feeding behaviour of these pests (Somar *et al.* 2019). After the stylet has been blocked, the insect remains immobile in the plant and dies shortly (Ring 2019). In beetles, planthoppers and locusts (*Locusta migratoria*), this insecticide acts by affecting the chordotonal sensillae on the tibia of the leg (Ausborn *et al.* 2005), blocking the nerve impulses to the brain, resulting in constant stretching of legs, unbalanced movement and high risk to predation since the organisms become unable to move or jump properly (Slater *et al.* 2017).

Research on the effects of pymetrozine on geotaxis is relatively limited. After exposure to pymetrozine, there were changes in the movement patterns of the melon aphid (Gorman *et al.* 2001). Pymetrozine impaired the normal climbing behaviour of *Drosophila* flies, upon exposure, the flies could not climb up normally because this insecticide had disrupted the function of chordotonal mechanoreceptors (Peng *et al.* 2023). Moreover, pymetrozine affects the normal upward climbing of flies, showing geotaxis defects (Peng *et al.* 2023). Furthermore, pymetrozine is proven to make *Drosophila* flies inactive (Nesterov *et al.* 2015).

4.2 Aim and objectives

The aim of this chapter, therefore, was to assess the geotaxis responses on *Culex* sp. fourth instar larvae after exposure to carbaryl and pymetrozine.

The objectives are to:

- assess the effects of carbaryl and pymetrozine on the geotaxis of the larvae and
- determine whether there is a switch point in the geotaxis of the larvae after exposure to these insecticides.

4.2 Methodology

4.2.1 Test organisms and pesticides

Culex sp. larvae collected in a private garden in Harrismith were used for these experimental exposures. Doom Blue Death- Multi Insect powder (carbaryl) was purchased from a PEP store, Phuthaditjhaba, Free State, South Africa. Trivia 500 WWDG 500g/kg pymetrozine was purchased from Villa Crop, Kempton Park, Gauteng, South Africa.

4.2.2 Experimental setup

The larvae used for geotaxis were not exposed to the insecticides prior to the experiment. A larva was exposed to the control or treatments of the active ingredient (carbaryl and pymetrozine) of the chosen insecticides and filmed for ten minutes. The larvae were exposed to four different concentrations of each insecticide, for pymetrozine the concentrations were 6.25, 12.50, 25 and 50 mg/L, and for carbaryl, the concentrations were 12.50, 25, 50 and 100 µg/L. The concentration ranges of the insecticides were different because both chemicals have different toxicities, and the study intended to measure behaviour and cause less than 30% mortality. Using 5ml plastic Pasteur pipettes individual larva were gently picked up from their living substrate and transferred to the exposure solutions. Exposures were carried in 250 ml measuring cylinders; the cylinders were filled up to 210 ml mark with the exposure solutions. Geotaxis was measured as a function of time. Negative geotaxis was recorded as the time the organism spent at the surface of the water column (breathing) or moving upwards within the water column. Positive geotaxis was recorded as the time the organism lied at the bottom of the water column or moved downwards within the water column. The geotaxis responses were recorded using an iPhone 11 camera (1080p HD video recording at 60 fps). The exposure was repeated 15 times for each treatment (active ingredient and the control), i.e. exposures were carried out in 15 replicates. Absolute geotaxis was calculated by adding cumulative positive geotaxis duration and negative geotaxis.

4.2.3 Statistical analysis

The recordings were viewed using Films & TV in Acer Aspire laptop. In Microsoft Excel, the cumulative time spent by the larvae in negative and positive geotaxis were recorded. Shapiro-Wilk normality test was utilized to test for the distribution of data. For statistical analysis, Kruskal Wallis was used followed by Dunn's test of comparison as a post-hoc test. Graph Pad Prism (GraphPad Prism version 6.00 for Windows, GraphPad software, San Diego, CA, USA, www.graphpad.com) was used for statistical analysis.

4.3 Results

The larvae predominantly exhibited negative geotactic behaviour. The larvae spent approximately 74% of the time displaying negative geotaxis in the control, 35% in the 6.25 mg/L pymetrozine solution, 36% in the 12.5 mg/L pymetrozine solution, 55% in 25 mg/L pymetrozine solution and 9% in the 50 mg/L pymetrozine solution (Figure 1). There was significant difference only in the control and the highest concentration (50 mg/L). When comparing the control and the highest concentration (50 mg/L), there was a decrease in the negative geotaxis (Figure 1).

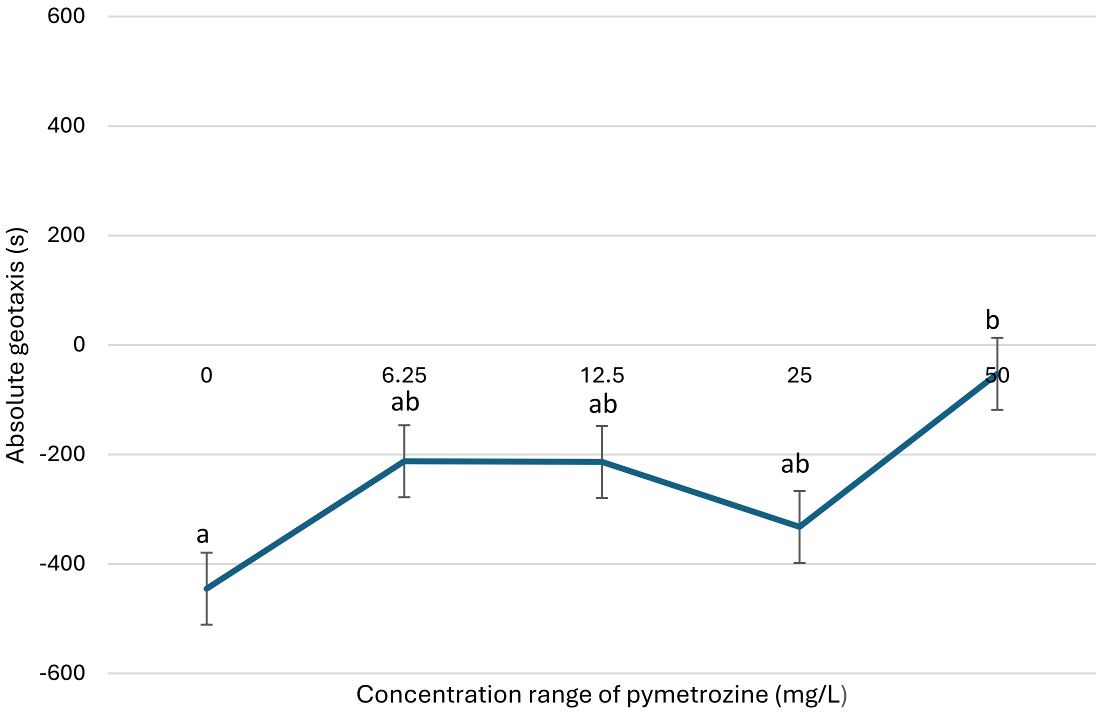

Figure 1: Geotactic behaviour of *Culex* sp. after exposure to pymetrozine. Different letters above error bars represent statistical differences and error bars represent standard error. n = 15 larvae per treatment.

Geotaxis was observed for both insecticides (carbaryl and pymetrozine). Overall, in carbaryl solutions, the mosquito larvae spent most of the time exhibiting negative geotaxis (in the control). The larvae spent about 71% of the time displaying negative geotaxis, 27% in 12.5 µg/L, 48% in 25 µg/L, 11% in 50 µg/L and 26% in 100µg/L respectively (Figure 2). Significant difference was only observed in the control and in the 50 µg/L solution. When comparing the control and to the 50 µg/L solution, there was a decrease in the negative geotaxis (Figure 2).

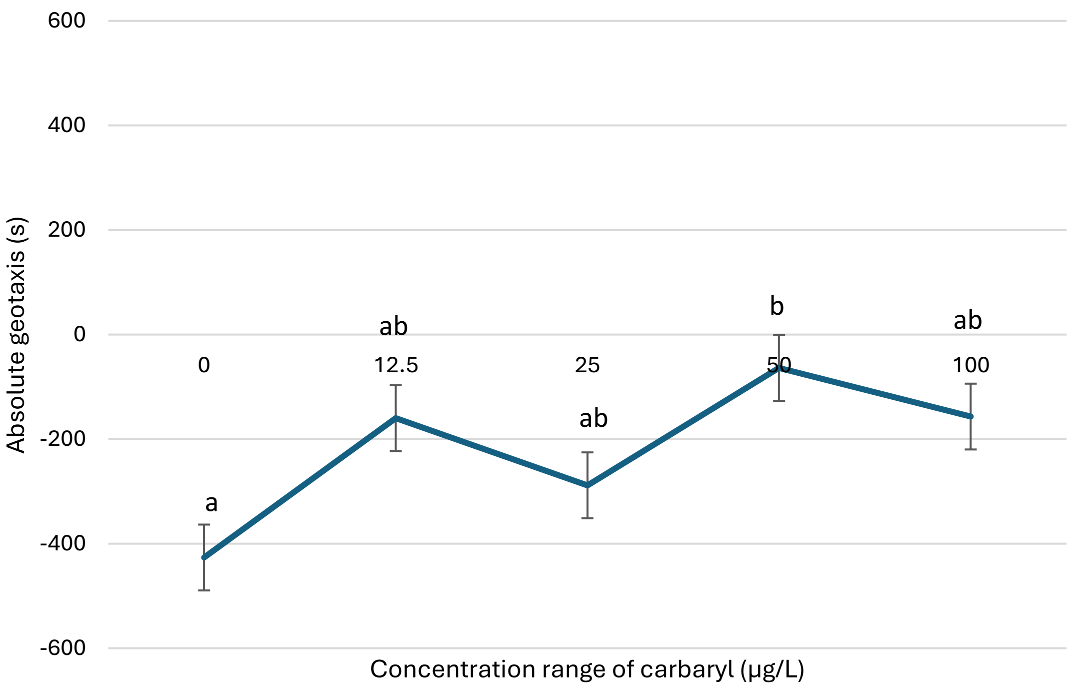

Figure 2: Geotactic behaviour of *Culex* sp. after exposure to carbaryl. Different letters above error bars represent statistical differences and error bars represent standard error. n = 15 larvae per treatment.

4.4 Discussion

This chapter assessed the geotaxis responses of *Culex* sp. larvae in distilled water and after exposure to carbaryl and pymetrozine in distilled water. The culicid larvae spent most of the time (~71 to 74%) displaying negative geotaxis in the control (distilled water). These results are in conformity with that of Tsotesti *et al*. 2022, who reported that the larvae mosquito species spent more time breathing (displaying negative geotaxis) in the absence of pesticides. After exposure to pymetrozine, the negative geotaxis then decreased to about 35% in 6.25 mg/L and 36% in12.50 mg/L, and increased to 55% in 25 mg/L, and finally decreased to 9% in 50 mg/L. Meaning that the larvae spent less time breathing (negative geotaxis) in the presence of this insecticide when compared to the control. A significant effect on the geotaxis behaviour was observed only between the control and 50 mg/L of pymetrozine ($p < 0.05$). Pymetrozine is proven to affect or disrupt the normal geotaxis behaviour of fruit flies *Drosophila melanogaster* by disrupting the normal functioning of the chordotonal mechanoreceptors, affecting the fly's sense of gravity (Peng *et al.* 2023). In the study by Peng *et al*. (2023), *Drosophila melanogaster* was exposed to pymetrozine (for 20 seconds, the lowest concentration was 3.7 mg/L, and the highest concentration was 2700 mg/L), where the negative geotactic movement was affected, and the flies could not normally climb up the testing tubes. In addition, Nesterov *et al*. (2015) further proved the effects of this insecticide in the same species and confirmed that pymetrozine does indeed affect the movement and coordination of this species. The flies in the latter study were inactive after exposure to pymetrozine (at 200µM for 2 hours). Furthermore, Spalthoff *et al.* (2023), documented that *Drosophila* flies exposed to insecticide Pyridazine Pyrazole Carboxamides (PPCs) showed 0 % negative geotaxis after 24 hours exposure after being fed 300 pm of PPCs. PPCs are a new class of insecticides that controls piercing and sucking pests.

Most aquatic organisms tend to display negative geotaxis in the absence of toxicants. For example, zebrafish have been reported to spend more than 80% of their time at the water surface (Sabadin *et al.* 2022). Snow crabs and red snow crabs display strong negative geotaxis at the early stages of their lives (Yamamoto *et al.* 2022). Diatoms, the alga *Clamydomonas reinhardtii,* and larval stages of sea urchins are also known to display negative geotaxis (Kam *et al.* 1999, Sineshchekov *et al.* 2000, Kage *et al.* 2020; Yaguchi *et al.* 2022). This is consistent with the observation made in mosquito larvae in the present study.

Mosquito larvae display mostly negative geotaxis in the distilled water because the larvae need to breath to develop into adults (Spielman & D'Antonio 2001), and this trend was also observed in the present study. Additionally, geotaxis causes many organisms to swim upwards in still water (Dedley & Kessler 1992), possibly a reason as to why the mosquito larvae spent more time swimming upwards in the control. However, this is different for other aquatic organisms, such as adult fish (*Nothobranchius furzeri*), which spend less time at the surface of the water to avoid bird predators (Thorè *et al*. 2023).

After exposure to flonicamid, an insecticide with a similar mode of action as pymetrozine, flonicamid, at concentrations ranging from 200 µM to 2 mM for 30 seconds, impaired the climbing behaviour of *Drosophila* flies (Qiao *et al.* 2022). As the dose was increased (from 200 µM to 2 mM), the impairment of flies was also increased in the lowest dose tested, causing only 38% -44% to reach the top half of the testing tube (Qiao *et al.* 2022). These results clearly show the toxicity of this chemical because even the lowest concentration tested showed impairment to negative geotaxis of flies, affecting the natural behaviour of flies since these flies naturally show negative geotaxis (Liao *et al.* 2012). Even though our study did not look at the impairment of negative geotaxis, there was a decrease in negative geotaxis, possibly showing impairment of negative geotaxis.

In the presence of carbaryl, the culicid larvae displayed only negative geotaxis. Carbaryl affected the geotaxis of the mosquito larvae, the negative geotaxis mainly decreased as the concentration of carbaryl increased. In 12.5 µg/L carbaryl- contaminated solution, the negative geotaxis was 27%, 48% in 25 µg/L, 11% in 50 µg/L and 26% in 100 µg/L of the carbaryl solution. A significant change in geotaxis behaviour was only seen between the control and 50 µg/L of carbaryl ($p < 0.05$). Gupta & Saxena (2003) tested the effects of carbaryl on the geotaxis of earthworms (*Metaphire posthuma*) and showed that when the concentration of carbaryl increased (from 0.125 ppm to 2 ppm for a 40-minute exposure time), the burrowing time also increased. Burrowing is an essential activity in earthworms because this inherent behaviour helps the organism to protect itself from predators and escape exposure to toxicants. Increasing burrowing time could, thus, translate into failure to escape predation and undesirable environmental conditions. Moreover, Gupta & Sundararaman (1991) conducted a similar study using different species of earthworms (*Pheretime posthuman*) and found that carbaryl reduced the burrowing ability of worms as the concentration was increased. As the concentration was increased, the worms could no longer burrow, without this ability, the organisms might die as they will have difficulties feeding and will be exposed to predators and can no longer defend themselves. Carbaryl affected the burrowing ability of worms even at the lowest concentration (1 ppm), this shows that carbaryl is highly toxic and can affect the geotaxis of *Pheretime posthuman* even at lowest concentration (Gupta & Sundararaman 1991). In comparison to this study, carbaryl did affect the geotaxis of culicid larvae. The larvae spent less time breathing (negative geotaxis) when compared to the control. However, no mortality was recorded in carbaryl-contaminated solutions.

Carbaryl administrated to mice orally (150 mg/kg) to mice decreased neuromotor coordination and the general locomotor activity in these vertebrates, delaying negative geotaxis (Al-Baggou 2004). The results clearly shows that carbaryl affected the mice' ability to climb up the experimental apparatus on time, exposure to carbaryl can put the mice at risk when running away from predators in steep areas. Additionally, when rats were exposed to sulfur, a chemical that has a similar mode of action as carbaryl (sulfur poisoning induces muscle fasciculation, convulsions and death), the same effects as that of (Al-Baggou 2004) were observed, the rats required more time to complete negative geotaxis tests and the swimming test scores slightly decreased at doses 4 g/kg and 8 g/kg (Hassan & Al-Baggou 2022). These studies prove that carbaryl have negative effects on the geotaxis of certain organisms.

After the mosquito larvae was exposed to carbaryl, the negative geotaxis decreased. The decrease in negative geotaxis may have negative effects in mosquito larvae because the larvae is required to mostly stay at the surface of the water column before transforming to pupae to get sufficient oxygen. Similarly, Pettersson & Ekelond 2006, showed that exposure of *Euglena* *gracilis* to Avans (herbicide), had inhibitory effects on the negative geotaxis of swimming cells and decreased the percentage of upward swimming (negative geotaxis) with increasing concentration. In contrast, Ziegler *et al.* 2021 exposed larvae of brown trout (*Salmo trutta*) to venlafaxine, 0 to 100 µg/L. Larvae exposed to these concentrations (100 µg/L and more) spent more time at the surface of water as opposed to unexposed fish. In our study, mosquito larvae exposed to 100 µg/L of carbaryl spent less time in the surface of water, this could be because venlafaxine and carbaryl do not affect the organisms the same, and because fish larvae were exposed to the chemical for a long period of time (exposed for more than 24 hours).

The upwards swimming in the fourth instar of mosquito larvae is expected to be dominant not only because the larvae must spend more time at the surface of water breathing for well development, but also because after completion of metamorphosis, the adult is expected to fly upwards (negative geotaxis). In flying insects, positive geotaxis is defined as the downward flying of the insect. The dominantly negative geotaxis in the present study may be attributed to the fact that most flying insects usually fly upwards dominantly display negative geotaxis. Negative geotaxis is observed in other flying insects, such as fruit flies, butterflies and honeybees, to correctly orient their bodies during flight. Monarch butterfly (*Danaus Plexippus*) displays negative geotaxis only (Kendzel *et al*. 2023), *Drosophila* flies showed negative geotaxis when geotactic responses were tested (Rhodenizer *et al*. 2008) and honeybees also show upward migration when there is light (El Hassani *et al*. 2005). In contrast to this behaviour most terrestrial organism such as terrestrial snails (*Potamopyrgus antipodarum*) and slug (*Limax valentianus*) (Kunichika & Matsuo 2022, Levi *et al*. 2017), rats (Ben-Shaul *et al*. 2022), and young rodents (Albets *et al*. 2004) exhibit positive geotaxis.

No geotaxis switch point, or equilibrium was observed in this study, meaning that there were no significant changes in the negative and positive geotaxis of the larvae. The geotactic response of the larvae did not change to positive geotaxis, and there was not a point whereby the time spent by the larvae displaying negative and positive geotaxis was equal. In conformity to our study that the geotaxis might sometimes not switch, in an experiment where Pea aphids that were placed at the bottom of the test vial, remained down throughout the experiment and most of the aphids that were placed at the top stayed there (Zhang *et al.* 2016). In contrast to our study, geotaxis switch point has been documented in other studies, whereby test organisms change from displaying negative geotaxis to displaying positive geotaxis or vice versa. Stallwitz & Häder (1994) found that young cultures of *Euglena gracilis* switched geotaxis after exposure to copper and mercury, these metals reversed the direction of positive geotaxis (downward swimming) to negative geotaxis (upward swimming). *Chlamydomonas reinhardtii* usually show negative geotaxis, but in the presence of a chemical stimuli or light, the direction of geotaxis was observed to be positive (Sineshchekov *et al.* 2000). Dice 1914 demonstrated that *Dapnia pulex* changes geotaxis direction in different temperature. *Daphnia* also showed a strong tendency of showing positive geotaxis at a high temperature (25°C), and negative geotaxis at lower temperature (15°C). From Dice's 1914 study, it is evident that an increase in temperature causes animals such as *Daphnia* to move upwards, so the tendency of displaying positive geotaxis at high temperature cannot be due to temperature but gravity.

Häder *et al*. 2005 proved that *Euglena* cells show both positive and negative geotaxis. Young cells showed positive geotaxis shortly after inoculation, but the application of heavy metal ions resulted in negative geotaxis. Older cells showed negative geotaxis, the negative geotaxis was reverted or reversed by increasing salinity in the exposure medium. Davis & Olla 1994 documented that free embryo of *Theragra chalcogramma* swam downwards, exhibiting positive geotaxis one day after hatching but, seven days after hatching, the organisms started showing negative geotaxis. The positive geotaxis of lower stages of the embryos is because embryos need to stay deep in water to avoid lightened layers of the sea and avoid predators.

Overall, our study did not show geotaxis switch point maybe because the exposure time was rather short (10 minutes) and maybe because only one larval stage (fourth instar) was used for the experiments. In both the chemicals tested (carbaryl and pymetrozine), the larvae showed only negative geotaxis, even though there were concentrations whereby the absolute geotaxis nearly switched to positive geotaxis (at 50 mg/L of pymetrozine). The dominantly negative geotaxis means that the larvae mostly swam up the water column which could mean that carbaryl and pymetrozine did not really affect the natural behaviour of the organisms within the ten minutes exposure time. It is of essence to know the geotactic equilibrium point or switch point because that point might indicate the increase in death rate of the larvae, because when the larvae exhibit positive geotaxis, it means that the larvae cannot reach the surface of the water to breath resulting in death (Burdick 1921). These results may suggest that when the organisms are exposed to different chemicals for a long period of time and in a high concentration range, the geotaxis switch point may be obtained. It is worthy to note that there is limited literature on the effects of pymetrozine and especially carbaryl on the geotaxis responses of aquatic organisms such as culicid larvae, hence, more studies that test effects of insecticides on the geotaxis behaviour of mosquito larvae should be done in future.

CHAPTER 5

General Discussion

In this study, we specifically focused on assessing the mortality of *Culex* sp. mosquitoes and changes in behaviours, namely, their breathing, swimming, and resting behaviours, as well as geotactic responses after acute exposure to carbaryl and pymetrozine. Additionally, a systematic review was written to document how carbaryl exposure induces behavioural changes in exposed insects. After a general introduction, Chapter 2 reviewed mortality rates and behavioural changes in insects after exposure to carbaryl. Chapter 3 assessed the mortality and the above-mentioned behaviour of *Culex* sp. larvae after exposure to carbaryl and pymetrozine whereas Chapter 4 assessed the geotactic responses of the larvae after exposure to these insecticides. All objectives of the different chapters were achieved.

Chemical exposure can induce various behavioural changes by interfering with one or more of the following systems namely, hormonal, sensory, neurological, and metabolic systems, affecting the organism's fitness (Schuijt *et al*. 2021). As previously stated in the 2nd chapter, more studies focus on the mortality of insects after exposure to insecticides, while there is limited published literature regarding behavioural changes in insects after exposure to insecticides. It is vital that future research delve more into changes in insect behaviour in the presence of insecticides because such information can help in understanding how insecticide exposure affects the distribution of insects. Additional studies on the effects of insecticides on behaviours such as changes in mobility, predator scavenging, escape, feeding and reproductive behaviour exposure must be studied to better understand how chemical exposure affects various insect behaviours. The data obtained from such experiments can be used for more informed environmental risk assessment and management.

In the 3rd chapter, we observed that as the concentration of the chemical solution increased, the number of dead larvae also increased, showing a positive correlation between mortality and increasing chemical toxicity. This trend was observed in both the insecticides evaluated and it was proven that carbaryl is more toxic that pymetrozine because carbaryl caused more mortality of the larvae at concentration range 1000 times less than that of pymetrozine. This was further, supported by the LC_{50} (lethal concentration that kills 50 % of the population exposed to the chemical) values, which indicated a significantly lower value for carbaryl. The findings of this chapter highlight the high toxicity of carbaryl on insects and this data can be used as baseline data for future studies looking at the chemical toxicity of carbaryl and pymetrozine on aquatic invertebrates, additionally, this data can be used in setting recommendations for Integrated Pest Management (IPM) and encourage the use of pymetrozine as a less toxic alternative in agriculture.

We recommend that future studies expose more larvae to the carbaryl and pymetrozine solutions i.e. use more than 10 larvae per solution and increase the number of replications to enhance statistical analysis. In addition, future studies can also focus on using biomarkers to find out which physiological pathways might lead to death in the exposed organisms. Moreover, more research can be done to assess the larvicidal effects of carbaryl and pymetrozine at different larval stages to further evaluate the effectiveness of these insecticides for pest control purposes because most mosquitoes are vectors of various diseases, certainly including the *Culex* sp. used in our study.

Quantifying the effects of temperature on the toxicity of insecticides against target pests is also. important to see if insecticides exhibit a temperature- dependent toxicity correlation and make informed selection of appropriate insecticides to use even in realistic environmental conditions for pest control (Raj Boina *et al.* 2009), especially in light of climate change. For instance, some insecticides are more effective in elevated temperatures as opposed to low temperatures. In Asian citrus psyllid *D. citri*, when temperatures were low (10°C to 25° C) pesticides such as pyrethroids were effective in controlling these organisms but at elevated temperatures (25° C to 38° C), pesticides such as carbaryl and chlorpyrifos were most effective (Raj Boina *et al.* 2009). It is, therefore, important to consider different temperatures when assessing mortality and behavioural changes of insects to evaluate the effectiveness at different seasons and correctly choose which pesticides are best to use in specific seasons.

Additionally, simultaneously doing laboratory and field studies is essential to better make recommendations and informed decisions for IPM because insecticide use in these different settings commonly yields different results. Abbate and colleagues (2022) showed that laboratory experiments tend to overestimate toxicity effects of insecticides sprayed in field, thus, future studies should consider assessing the effects carbaryl and pymetrozine in the field to further assess if the insecticides effects in the laboratory and field are going to be the same.

In our study, during behavioural analysis, we observed that exposure to carbaryl and pymetrozine induced jerky and quick movement patterns in the mosquito larvae, whereas, in the control (distilled water), the movement patterns were smooth. Future research can investigate if changes in movement patterns are a result of the mode of action of these insecticides or there are other underlying factors that led to changes in movement patterns. In addition, more studies on the influence of these insecticides on the swimming speed of the exposed mosquito larvae can be conducted by researchers to test whether exposure to these insecticides affects the swimming speed of the larvae. Chanu and colleagues (2017) exposed water bugs *Anisops sardeus* to cadmium (Cd) and noted a decrease in the swimming speed of Cd-exposed organisms, such decrease negatively affected the efficacy of the water bugs to catch its prey, and this may also be the case in mosquito larvae.

In Chapter 4, we observed that in the absence of carbaryl and pymetrozine, the larvae spent most of their time displaying negative geotaxis, however, upon exposure to these insecticides, there is a decrease in time spent displaying negative geotaxis. These results showed that chemical toxicity affect the geotaxis responses of larvae and can be further used in ecotoxicology studies that examine the geotactic behaviour of insects when exposed to insecticides. Future studies should consider doing experiments in different light conditions to see phototaxis responses because most organisms are sensitive to light and light can affect the directional movement of organisms (Harris *et al.* 2009). Future research on geotaxis should delve into understanding the sensory mechanisms responsible for geotaxis responses and how environmental cues like light affect these. Our findings emphasise that carbaryl and pymetrozine do cause changes in geotaxis responses of aquatic organisms such as mosquito larvae after acute exposure. However, more studies on chronic exposure of mosquito larvae to these insecticides should be conducted to observe whether geotaxis responses can switch from negative to positive after long-term exposure. This will help improve the reliability and depth of the findings we are presenting, here for instance (Ford *et al.* 2021). Exploring the influence of insecticides on geotactic behaviour of mosquito larvae could provide insights into their survival strategies when exposed to external stressors.

In conclusion, the findings of our study proved that carbaryl is more toxic than pymetrozine and that both these insecticides induce behavioural changes in the breathing, swimming and resting behaviours of the experimental organisms. Moreover, in the presence of carbaryl and pymetrozine mosquito larvae display negative geotaxis. There is a lack of published research focusing on the assessment of the behavioural changes in mosquito larvae after exposure to insecticides like carbaryl and pymetrozine. This indicates a gap in literature. Overall, therefore, although this research has provided novel insights into the way chemicals might affect the behaviour of aquatic larvae, more research is needed to generate much needed data on the effects of chemical toxicity on insect behaviour in the aquatic and terrestrial environment.

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Appendices

Appendix 1 (Raw data of systematic review)

Table 3: Mortality and behavioural changes of insects after exposure to carbaryl published studies retrieved from the internet used for systematic review.

Reference	**Location (Country)**	**Chemical used**	**Behaviour/ Endpoints**	**Insect's name**
Lee *et al.*2016	Thailand	Carbaryl, thiamethoxam, imidacloprid, and clothianidin	Susceptibility, mortality, LD_{50}	Honeybees *(Apis florea, A. cerana, A. mellifera, and A. dorsata)*
Bacci *et al.* 2018	Brazil	Carbaryl, methamidophos, parathion methyl and permethrin	Selectivity, mortality, LC_{90}	Diamondback moth *Plutella xylostella*, True weevil *P. scutellaris and* bugs *Lasiochilus sp.*
Suwanchaichinda & Brattsten 2002	United States	Carbaryl, Benzothiazole (BZT), rotenone, and temephos	Insect tolerance, insecticide toxicity and activities, & expression of microsomal cytochrome P450s (P450s)	Asian tiger mosquitoes *Aedes albopictus*
Zhao *et al.*2012	China	Carbaryl, chlorpyrifos, fenitrothion, phoxim, profenofos, and triazophos, carbsulfan, isoprocarb, metolcarb, and promecarb	Toxicity (LC_{50})	Minute wasps *Trichogramma japonicum*
Zhu *et al.* 2001	United States	Carbaryl	Mortality, resistance	Western corn rootworm, *Diabrotica virgifera*
Gosh Shreya *et al.* 2015	India	Carbaryl	Larval malformation rates, movement	Lime butterfly *Papilio demoleus*
Wang *et al.* 2022	China	Carbaryl, chlorantraniliprole	Mortality, development time, longevity, and fecundity	Fall armyworm (*Spodoptera frugiperda*)

Nogrado *et al.* 2019	Korea	Carbaryl	Toxicity	Honeybees
Tiwari *et al.*2011	United States	Carbaryl, Chlorpyrifos, Fenpropathrin, Imidacloprid, Spinetoram	Susceptibility, LC_{50}	Asian citrus psyllid (*Diaphorina citri)*
Oliver *et al.* 2005	United States	Carbaryl, imidacloprid, thiamethoxam, halofenozide, chlorpyrifos	Mortality, parasitism rates	Japanese beetle (*Popillia japonica)*
Sharma & Abrol 2014	India	Carbaryl, betacylfluthrin, betacyfluthrin + imidacloprid	Mortality	Honeybees *Apis mellifera*
Deshmukh *et al.* 2009	India	Carbaryl and gamma benzene hexachloride (γ-BHC)	Midgut histopathology and midgut enzyme activities, sublethal effects of chemicals	Fruit-sucking moth *Othreis materna*
Galvan *et al.* 2005	United States	Carbaryl, bifenthrin, thiamethoxam, imidacloprid, malathion	Efficacy of insecticides, mortality	Asian lady beetle *Harmonia axyridis*
Mahdavi *et al.* 2013	Iran	Carbaryl, chlorpyrifos, abamectin and spinosad	LD_{30}, LD_{50}, toxicity	Braconid wasp *Habrobracon hebetor*, Mediterranean flour moth *Anagasta kuehniella*
Fettig *et al.* 2018	United States	Carbaryl	Efficacy of insecticides	Pine beetles
Udayagiri e*t al.* 2000	United States	Carbaryl, fenpropathrin, bifenthrin, methomyl, malathion, propargite, abamectin, benomyl, captan, myclobutanil, thiram, iprodione, sulfur	LT_{50} (Lethal Time for 50% mortality), survivorship	Minute wasp *Anaphes iole,*
Peterson *et al.* 2001	United States	Carbaryl, triclopyr	LC_{1}, LC_{50}, susceptibility	Mayfly, Humpless casemaker caddisfly, stonefly, Unicoloured bizarre caddisfly

Peterson *et al.* 2001	United States	Carbaryl	LC$_{50}$	Stonefly, Mayfly
Walters *et al.* 2003	United States	Carbaryl	Detection and Susceptibility to insecticides	Sharpshooter *Homalodisca coagulata*
Sangha 2011	India	Carbaryl, profenophos, quinalphos	Mortality	Leaf defoliators
Martínez *et al.* 2019	Colombia	Carbaryl, abamectin, deltamethrin, fipronil, imidacloprid and spinosad	LC$_{25}$, LC$_{50}$, effects of insecticides on reproduction of insects	The South American palm weevil
Mullin *et al.* 2010	United States	Carbaryl, aldicarb, chlorpyrifos, imidacloprid, boscalid, captan and myclobutanil, and pendimethalin	Detection and toxicity of insecticides	Honeybees
Guo *et al.* 2015	China	Carbaryl, deltametrin, malathion, DDT	Dentification and functional analysis of a cytochrome P450 gene	Oriental migratory locust *Locusta migratoria*
Jiang *et al.* 2015	China	Carbaryl, diazoxon, paraoxon, fenobucarb, fipronil and ethofenprox	Selectivity to insecticides, mortality	Brown planthopper *Nilaparvata lugens*, Mirid bug *Cyrtorhinus lividipennis*
Guo *et al.* 2012	China	Carbaryl, malathion and deltamethrin	Detoxification of insecticides	Oriental migratory locust *Locusta migratoria*
Armenta *et al.* 2003	Mexico	Carbaryl, chlorpyrifos, methamidopho, cypermethrin)	Effects of insecticides	Fall armyworm *Spodoptera frugiperda*

Lee *et al*.2013	United States	Carbaryl and 26 other insecticides	Locomotory behaviour and mobility	Brown marmorated stink bug *Halyomorpha halys*
Sansar 2021	Turkey	Carbaryl, thiamethoxam, chlorpyrifos	Mortality	Honeybee (*Apis mellifera*)
Liang *et al.* 2010	Australia	Carbaryl, methidathion, *n*C24 horticultural mineral oil (HMO)	Disruptive effects of an *n*C24 horticultural mineral oil (HMO) and two other insecticides (carbaryl and methidathion)	Red and purple armored scales
Al- Rajhy *et al.* 2005	Saudi Arabia	Carbaryl & piperonylbutoxine	LC_{50}	Red Palm Weevil
Adamski 2007	Poland	Carbaryl	Toxicity, disruptive effects in metabolism	Beet armyworm *Spodoptera exigua*
Michaud & Grant 2003	United States	Carbaryl, carbofuran, methomyl, methidathion, esfenvalerate and phosmet, bifenthrin, fenpropathrin, zeta-cypermethrin, cyfluthrin and permethrin, oxadiazine indoxacarb	Toxicity on survival and reproduction	Red scale parasitic wasp, Insidious flower bug, metallic blue ladybeetle, spotless ladybird, Asian lady beetle, Ashy gray ladybeetle
Fettig *et al.* 2008	United States	Carbaryl	Toxicity	Mountain pine beetle, spruce beetle, bark beetles
Hardersen *et al.* 2000	New Zealand	Carbaryl	Toxicity and adult emergence	Damselflies *Xanthocnemis zealandica*

Marletto *et al.* 2003	Italy	Carbaryl, acephate, buprofezin, cartap hydrochloride, chlorpyrifos-methyl, cyfluthrin, cyromazine, dimethoate, heptenophos, imidacloprid, lamda-cyhalothrin, methomyl, phosalone, pirimicarb, quinalphos, rotenone, and teflubenzuron, of the acaricides fenazaquin, fenpyroximate, hexythiazox, propargite, and tebufenpyrad and of the insecticide-acaricides abamectin and amitraz	LD_{50}, LC_{50}	Bumblebees *Bombus terrestris*
Siegfried *et al.* 2004	Illions, Indiana	Carbaryl	Toxicity of carbaryl	Western corn rootworm, *Diabrotica virgifera*
Pilotos *et al.* 2020	Phillippines	Carbaryl	Larvicidal activity	Rice meal moth *Corcyra cephalonica*
Overmyer & Noblet 2003	United States	Carbaryl, chlorpyrifos, and malathion	Bioavailability of insecticides	Black fly *Simulium vittatum*
Feng & Zhang 2017	United States	Carbaryl, λ-cyhalothrin, acetamiprid, methyl benzoate (MB)	Ovicidal toxicity	Diamondback moth, Tobacco hornworm, Brown marmorated stink bug,

				spotted-wing drosophila
Wang *et al*. 2012	China	Carbaryl and 30 others	LC_{50}	Rice meal moth *Corcyra cephalonica*, Parasitiod wasp *Trichogramma ostriniae*
Wang *et al*. 2013	China	Carbaryl and 29 others	LC_{50}	Rice meal moth *Corcyra cephalonica*, Parasitiod wasp *Trichogramma evanescens*
Wu *et al*.2020	China	Carbaryl, malathion, chlorpyrifos, deltamethrin	*LmCYP4G* gene's function	Oriental migratory locust *Locusta migratoria*
Vonesh & Kraus 2009	United States	Carbaryl, acetone	Toxicity, abundance, oviposition	*Culex* mosquitoes, *Anopheles* mosquitoes, beetles, chironomid midge
Pervez & Manzoor 2021	Pakistan	Carbaryl, chlorpyrifos, Imidacloprid	LC_{50} of insecticides, feeding behaviour	Honeybee (*Apis mellifera*)
Bacci *et al*. 2009	Brazil	Carbaryl, cartap, deltamethrin, methamidophos, methyl parathion, permethrin and trichlorfon	LC_{50}	Predatory wasps, Brazilian wasp, Camoati wasp, Diamondback moth

Tong & Bloomsquit 2013	United States	Carbaryl, permethrin, and 14 plant essential oils	Toxicities and synergistic effects	Yellow fever mosquito *Ae. aegypti*
Kishk *et al.* 2017	United States, Egypt	Carbaryl, chlorpyrifos, fenpropathrin and imidacloprid	Mortality, susceptibility	Asian citrus psyllid, *Diaphorina citri*
Efrom *et al.* 2011	Not stated	Carbaryl, Neem oil, Lime sulfur, Pyroligneous extract, Rotenone, Pyroligneous extract	Toxicity	Mealybug ladybird *Cryptolaemus montrouzieri*
Hardersen & Wratten 2000	New Zealand	Carbaryl, azinphos-methyi	Sensitivity	Redcoat damselfly *Xanthocnemis zealandica*
Wei *et al.* 2004	China	Carbaryl, deltamethrin, endosulfan, malathion	Reproductive behaviour	Tobacco cutworm *Spodoptera litura*
Yeary *et al.* 2018	Tenessee	Carbaryl	Insect survival	lady beetle, Red-lipped lacewing
Wang *et al.* 2012	China	Carbaryl & 29 others	Susceptibility	Parasitic wasp *Trichogramma nubilale*

Shaw & Wallis 2010	New Zealand	Carbaryl, chlorantraniliprole, spirotetramat, emamectin benzoate and methoxyfenozide, indoxacarb, thiacloprid, spinosad and diazinon	Susceptibility	European earwig
Beuter *et al.* 2019	Germany	Carbaryl	Toxicity	Mayfly, Caddisfly
Rahardjo *et al.* 2021	Indonesia	Carbaryl	Toxicity	Diamondback moth *Plutella xylostella,* Parasitoids *Diadegma sp.*
Islam & Talukder 2005	Bangladash	Carbaryl, malathion, neem seed extract and marigold leaf powder	Toxicity	Red flour beetle (*Tribolium castaneum*)
Akhtar *et al.* 2021	China, United States	Carbaryl, chlorpyrifos, triazophos, profenofos, nitenpyram, acetamiprid and imidacloprid	Toxicological risk assessment	Lepidopteran parasitic wasp *Cotesia flavipes*
Gao *et al.* 2018	China	Carbaryl, carbofuran, dichlorvos and malathion	Susceptibility	Red flour beetle *Tribolium castaneum*
Yang *et al.* 2016	China, Chile	Carbaryl, deltamethrin, cypermethrin, methomyl, imidacloprid	Toxicity	Codling moth *Cydia pomonella*
Kim *et al.* 2022	Korea	Carbaryl, acetamiprid, imidacloprid, flupyradifurone, fenitrothion, amitraz, and bifenthrin	Toxicity	Honeybee *Apis mellifera*
Goa et al. 2022	China	Carbaryl and acetamiprid	Transcriptome and metabolome	Honeybee *Apis mellifera*
Fettig *et al.* 2006	United States	Carbaryl, bifenthrin	Susceptibility	Western pine beetle, *Dendroctonus brevicomis*

Abbate *et al.* 2022	Uruguay	thiamethoxam+lambda-cyhalothrin, imidacloprid+beta-cyfuthrin, acetamiprid+cypermethrin, imidacloprid+carbaryl, trichlorfon, thiamethoxam, imidacloprid and carbaryl trichlorfon	Emergence of eggs	Stink bugs
Urio *et al*. 2022	Tanzania	Carbaryl, lambda-cyhalothrin, chlorpyrifos	Susceptibility, fitness parameters	African malaria mosquito *Anopheles arabiensis*
Goblirsch & Adamczyk 2023	United States	Carbaryl, λ-cyhalothrin, imidacloprid, acephate	Assessment of cell viability	Honeybee
Yu *et al.* 2003	United States	Carbaryl, propoxur, carbofuran, bendiocarb, thiodicarb, methyl paraoxon, paraoxon, dichlorvos	Susceptibility, resistance	Fall armyworm, *Spodoptera frugiperda*
Raj Boina *et al*. 2009	United States	Carbaryl, chlorpyrifos and dimethoate, abamectin, bifenthrin, zeta-cypermethrin, fenpropathrin, and lambda-cyhalothrin, acetamiprid, imidacloprid, and thiamethoxam	Susceptibility	Asian citri psyllid *Diaphorina citri*
Rodríguez *et al*. 2012	Spain	Carbaryl and azinphos-methyl	Resistance	Codling moth *Cydia pomonella*
Qin *et al.* 2012	China	Carbaryl, DDT, chlorpyrifos, deltamethrin, and malathion	Insecticide detoxification, mortality	Oriental migratory locust *Locusta migratoria*

Liang *et al.* 2007	Australia	Carbaryl, horticultural mineral oil and methidathion	Toxicity	Rove beetle
Overmyer *et al.* 2008	United States	Carbaryl, sertraline and diazinon	Toxicity	Black fly
Abdullah *et al.* 2006	India	Carbaryl, quinalphos, acephate, endosulfan and fenvalerate	Oviposition preference	Whitefly *Bemisia tabaci*
Kang & Jung 2017	Korea	Carbaryl, cyhexatine, carbosulfan and fenpyroximate diflubenzuron, tebufenpyrad, and acrinathrin, bifurcated, thiacloprid, acetamiprid, imidacloprid, thiamethoxam and dinotefuran,abamectin, fenthion, amitraz and acequinocyl, fungicide of fenarimol, acaricides of acrinathrin and phosphamidon, diflubenzuron	Avoidance behaviour	Honeybees *Apis mellifera*
Nayak *et al.* 2003	Australia	Carbaryl, deltamethrin and permethrin	Mortality	Booklice
Mahdavi *et al.* 2011	Iran	Carbaryl and abamectin	Lethal and sublethal effects	Braconid wasp
Behle 2001	Illions	Carbaryl, cidetrak	Toxicity	Western Corn Rootworm

Chen 2006	Mississippi	Carbaryl, acephate, bifenthrin, cyßuthrin, deltamethrin, -cyhalothrin, permethrin, and pyrethrin.	Digging behaviour	Red imported fire ants *Solenopsis invicta*
Merdan& Ghareeb 2022	Egypt	Carbaryl, malathion, bacillus thurengiens	Resistance	Common house mosquito *Culex pipiens*
Margus *et al.* 2021	Not stated	Carbaryl, azinphos-methyl	Resistance	Colorado potato beetles
Zhu *et al.* 2001	United States	Carbaryl	Resistance	Western corn rootworm, *Diabrotica virgifera*
Moura *et al.* 2012	Brazil	abamectin, carbaryl, fenitrothion, methidathion, sulfur, and trichlorfon	Toxicity	Green lacewings *Chrysoperla externa*
Wang *et al*. 2014	China	Carbaryl and 29 other insecticides	Toxicity	Egg parasitoid *Trichogramma evanescens*
Yang *et al.* 2020	Beijing	carbaryl, deltamethrin, cypermethrin, acetamiprid	Acute and chronic toxicity	Honeybee
Gao *et al.* 2022	China	carbaryl and acetamiprid	Chronic toxicity	Honeybee
Wang *et al.* 2022	China	chlorantraniliprole and carbaryl	Toxicity	Fall armyworm

Table 4: Kruskal-Wallis test results of the analysis of survival of *Culex* sp. larvae after exposure to carbaryl and pymetrozine for 24 hours.

Insecticide	Carbaryl	Pymetrozine
P value	0,0059	0,0168
Exact or approximate P value?	Gaussian Approximation	Gaussian Approximation
P value summary	**	*
Do the medians vary significant (P < 0.05)	Yes	Yes
Number of groups	5	5
Kruskal-Wallis statistic	14,49	12,07

Table 5: Dunn’s multiple comparison test results of the analysis of survival of *Culex* sp. larvae after exposure to carbaryl for 24 hours.

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Control vs 12.5	3,750	No	ns
Control vs 25	6,500	No	ns
Control vs 50	11,00	No	ns
Control vs 100	13,75	Yes	**
12.5 vs 25	2,750	No	ns
12.5 vs 50	7,250	No	ns
12.5 vs 100	10,00	No	ns
25 vs 50	4,500	No	ns
25 vs 100	7,250	No	ns
50 vs 100	2,750	No	ns

Table 6: Dunn’s multiple comparison test results of the analysis of survival of *Culex* sp. larvae after exposure to pymetrozine for 24 hours.

Dunn's Multiple Comparison Test	Difference in rank sum	Significant? P < 0.05?	Summary
Control vs 6.25	0,000	No	ns
Control vs 12.5	0,000	No	ns
Control vs 25	6,750	No	ns
Control vs 50	8,250	No	ns
6.25 vs 12.5	0,000	No	ns
6.25 vs 25	6,750	No	ns
6.25 vs 50	8,250	No	ns
12.5 vs 25	6,750	No	ns
12.5 vs 50	8,250	No	ns
25 vs 50	1,500	No	ns

Table 7: Dunn's multiple comparison test results of the analysis of breathing time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
12.5 µg/L vs. Control	-19.17	No	ns	0.1601
25 µg/L vs. Control	-6.933	No	ns	> 0.9999
50 µg/L vs. Control	-24.83	Yes	*	0.0180
100 µg/L vs. Control	-23.23	Yes	*	0.0350
25 µg/L vs. 12.5 µg/L	12.23	No	ns	> 0.9999
50 µg/L vs. 12.5 µg/L	-5.667	No	ns	> 0.9999
100 µg/L vs. 12.5 µg/L	-4.067	No	ns	> 0.9999
50 µg/L vs. 25 µg/L	-17.90	No	ns	0.2448
100 µg/L vs. 25 µg/L	-16.30	No	ns	0.4052
100 µg/L vs. 50 µg/L	1.600	No	ns	> 0.9999

Table 8: Descriptive statistics of the analysis of breathing time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

	Control	12.5 µg/L	25 µg/L	50 µg/L	100 µg/L
Number of values	15	15	15	15	15
Minimum	282.0	0.0	0.0	0.0	19.00
25% Percentile	358.0	94.00	357.0	53.00	46.00
Median	501.0	356.0	488.0	182.0	104.0
75% Percentile	583.0	478.0	524.0	477.0	513.0
Maximum	600.0	554.0	580.0	538.0	546.0
Mean	467.3	317.2	407.4	238.9	243.9
Std. Deviation	113.5	183.8	174.5	198.1	224.2
Std. Error of Mean	29.29	47.45	45.06	51.16	57.89
Lower 95% CI	404.5	215.4	310.7	129.1	119.7
Upper 95% CI	530.2	419.0	504.1	348.6	368.0
Mean ranks	52.83	33.67	45.90	28.00	29.60

Table 9: Dunn’s multiple comparison test results of the analysis of swimming time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
12.5 µg/L vs. Control	7.700	No	ns	> 0.9999
25 µg/L vs. Control	-2.867	No	ns	> 0.9999
50 µg/L vs. Control	18.80	No	ns	0.1816
100 µg/L vs. Control	25.37	Yes	*	0.0143
25 µg/L vs. 12.5 µg/L	-10.57	No	ns	> 0.9999
50 µg/L vs. 12.5 µg/L	11.10	No	ns	> 0.9999
100 µg/L vs. 12.5 µg/L	17.67	No	ns	0.2642
50 µg/L vs. 25 µg/L	21.67	No	ns	0.0648
100 µg/L vs. 25 µg/L	28.23	Yes	**	0.0039
100 µg/L vs. 50 µg/L	6.567	No	ns	> 0.9999

Table 10: Descriptive statistics of the analysis of swimming time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

	Control	12.5 µg/L	25 µg/L	50 µg/L	100 µg/L
Number of values	15	15	15	15	15
Minimum	0.0	26.00	11.00	33.00	40.00
25% Percentile	17.00	54.00	28.00	63.00	73.00
Median	43.00	78.00	46.00	171.0	353.0
75% Percentile	143.0	133.0	94.00	289.0	422.0
Maximum	283.0	570.0	250.0	435.0	501.0
Mean	91.80	125.4	70.87	187.5	269.1
Std. Deviation	91.74	143.5	69.95	125.6	182.6
Std. Error of Mean	23.69	37.04	18.06	32.42	47.15
Lower 95% CI	41.00	45.96	32.13	117.9	168.0
Upper 95% CI	142.6	204.8	109.6	257.0	370.3
Mean ranks	28.20	35.90	25.33	47.00	53.57

Table 11: Dunn’s multiple comparison test results of the analysis of resting time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
12.5 µg/L vs. Control	21.83	No	ns	0.0605
25 µg/L vs. Control	12.10	No	ns	> 0.9999
50 µg/L vs. Control	26.40	Yes	**	0.0090
100 µg/L vs. Control	12.33	No	ns	> 0.9999
25 µg/L vs. 12.5 µg/L	-9.733	No	ns	> 0.9999
50 µg/L vs. 12.5 µg/L	4.567	No	ns	> 0.9999
100 µg/L vs. 12.5 µg/L	-9.500	No	ns	> 0.9999
50 µg/L vs. 25 µg/L	14.30	No	ns	0.7217
100 µg/L vs. 25 µg/L	0.2333	No	ns	> 0.9999
100 µg/L vs. 50 µg/L	-14.07	No	ns	0.7694

Table 12: Descriptive statistics of the analysis of resting time of *Culex* sp. larvae after exposure to carbaryl for 10 minutes.

	Control	12.5 µg/L	25 µg/L	50 µg/L	100 µg/L
Number of values	15	15	15	15	15
Minimum	0.0	0.0	0.0	0.0	8.000
25% Percentile	0.0	37.00	27.00	60.00	35.00
Median	37.00	114.0	44.00	136.0	53.00
75% Percentile	76.00	245.0	172.0	311.0	115.0
Maximum	101.0	553.0	554.0	430.0	319.0
Mean	40.87	157.4	119.1	175.0	87.00
Std. Deviation	38.49	145.3	158.7	129.7	82.45
Std. Error of Mean	9.937	37.52	40.99	33.50	21.29
Lower 95% CI	19.55	76.93	31.16	103.2	41.34
Upper 95% CI	62.18	237.9	207.0	246.8	132.7
Mean ranks	23.47	45.30	35.57	49.87	35.80

Table 13: Dunn’s multiple comparison test results of the analysis of breathing time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
6.25 mg/L vs. Control	-19.00	No	ns	0.1683
12.5 mg/L vs. Control	-20.67	No	ns	0.0932
25 mg/L vs. Control	-13.27	No	ns	0.9510
50 mg/L vs. Control	-26.90	Yes	**	0.0071
12.5 mg/L vs. 6.25 mg/L	-1.667	No	ns	> 0.9999
25 mg/L vs. 6.25 mg/L	5.733	No	ns	> 0.9999

50 mg/L vs. 6.25 mg/L	-7.900	No	ns	> 0.9999
25 mg/L vs. 12.5 mg/L	7.400	No	ns	> 0.9999
50 mg/L vs. 12.5 mg/L	-6.233	No	ns	> 0.9999
50 mg/L vs. 25 mg/L	-13.63	No	ns	0.8630

Table 14: Descriptive statistics of the analysis of breathing time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

	Control	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L
Number of values	15	15	15	15	15
Minimum	289.0	0.0	0.0	0.0	0.0
25% Percentile	462.0	35.00	241.0	369.0	0.0
Median	506.0	423.0	420.0	442.0	194.0
75% Percentile	571.0	498.0	493.0	535.0	468.0
Maximum	596.0	555.0	543.0	570.0	573.0
Mean	494.7	339.4	343.5	412.4	243.0
Std. Deviation	91.01	216.0	193.8	151.6	229.9
Std. Error of Mean	23.50	55.77	50.04	39.15	59.35
Lower 95% CI	444.3	219.8	236.2	328.4	115.7
Upper 95% CI	545.1	459.0	450.9	496.4	370.3
Mean ranks	53.97	34.97	33.30	40.70	27.07

Table 15: Dunn’s multiple comparison test results of the analysis of swimming time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
6.25 mg/L vs. Control	18.70	No	ns	0.1877
12.5 mg/L vs. Control	25.37	Yes	*	0.0143
25 mg/L vs. Control	16.27	No	ns	0.4093
50 mg/L vs. Control	25.33	Yes	*	0.0145
12.5 mg/L vs. 6.25 mg/L	6.667	No	ns	> 0.9999
25 mg/L vs. 6.25 mg/L	-2.433	No	ns	> 0.9999
50 mg/L vs. 6.25 mg/L	6.633	No	ns	> 0.9999
25 mg/L vs. 12.5 mg/L	-9.100	No	ns	> 0.9999
50 mg/L vs. 12.5 mg/L	-0.03333	No	ns	> 0.9999
50 mg/L vs. 25 mg/L	9.067	No	ns	> 0.9999

Table 16: Descriptive statistics of the analysis of swimming time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

	Control	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L
Number of values	15	15	15	15	15
Minimum	4.000	27.00	57.00	30.00	27.00
25% Percentile	19.00	49.00	80.00	44.00	42.00
Median	43.00	107.0	122.0	83.00	135.0
75% Percentile	88.00	177.0	163.0	158.0	278.0
Maximum	159.0	542.0	233.0	309.0	380.0
Mean	55.80	133.7	126.7	107.7	166.8
Std. Deviation	43.04	130.2	53.64	76.69	123.6
Std. Error of Mean	11.11	33.61	13.85	19.80	31.91
Lower 95% CI	31.96	61.66	97.03	65.26	98.35
Upper 95% CI	79.64	205.8	156.4	150.2	235.2
Mean ranks	20.87	39.57	46.23	37.13	46.20

Table 17: Dunn's multiple comparison test results of the analysis of resting time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value
6.25 mg/L vs. Control	1.367	No	ns	> 0.9999
12.5 mg/L vs. Control	9.033	No	ns	> 0.9999
25 mg/L vs. Control	4.400	No	ns	> 0.9999
50 mg/L vs. Control	18.53	No	ns	0.1902
12.5 mg/L vs. 6.25 mg/L	7.667	No	ns	> 0.9999
25 mg/L vs. 6.25 mg/L	3.033	No	ns	> 0.9999
50 mg/L vs. 6.25 mg/L	17.17	No	ns	0.2984
25 mg/L vs. 12.5 mg/L	-4.633	No	ns	> 0.9999
50 mg/L vs. 12.5 mg/L	9.500	No	ns	> 0.9999
50 mg/L vs. 25 mg/L	14.13	No	ns	0.7371

Table 18: Descriptive statistics of the analysis of resting time of *Culex* sp. larvae after exposure to pymetrozine for 10 minutes.

	Control	6.25 mg/L	12.5 mg/L	25 mg/L	50 mg/L
Number of values	15	15	15	15	15
Minimum	0.0	0.0	0.0	0.0	0.0
25% Percentile	0.0	0.0	3.000	2.000	23.00
Median	27.00	19.00	62.00	35.00	128.0
75% Percentile	59.00	90.00	155.0	132.0	377.0
Maximum	207.0	570.0	541.0	298.0	558.0
Mean	49.47	127.1	129.7	79.87	190.2
Std. Deviation	66.54	220.5	175.9	102.3	173.1
Std. Error of Mean	17.18	56.93	45.41	26.40	44.70
Lower 95% CI	12.62	4.960	32.33	23.23	94.34
Upper 95% CI	86.31	249.2	227.1	136.5	286.1
Mean ranks	31.33	32.70	40.37	35.73	49.87