Integrated control of water hyacinth [*Pontederia crassipes* Mart. (Solms) (Pontederiaceae)]: Interactions between the biological control agent, *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), and selected sublethal glyphosate-based herbicide applications, in South Africa

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Abstract

Pontederia crassipes Mart. (Pontederiaceae) poses serious ecological and economic problems in freshwater ecosystems. Herbicidal and biological control are two conventional methods of managing this aggressive weed. When used separately, these strategies have drawbacks: biological control alone is not perceived to work quickly enough, and herbicides present non-target environmental hazards. Careful integration of herbicides with biocontrol agents may potentially lead to synergistic effects in managing this invasive aquatic weed. Herbicide compatibility with biological control agents remains unclear and warrants further investigation. The current study investigated the impact of integrating a biocontrol agent*,* *Megamelus scutellaris* Berg (Hemiptera: Delphacidae), and sublethal concentrations of selected glyphosate-based herbicides for *P. crassipes* control.

Firstly, laboratory experiments were carried out to identify sublethal concentrations of three routinely used glyphosate-based herbicides (GBH) on *P. crassipes* plants. The results showed that sublethal concentrations of Kilo Max (0.4%) and Seismic (0.75%) herbicides reduced the vegetative growth and biomass of *P. crassipes* plants. The effect of these sublethal concentrations on *M. scutellaris* populations was then assessed by recording the number of surviving insects on treated plants, where significantly more insects were recorded from the plants treated with sublethal concentration of Kilo Max (0.4%). These results highlighted that the sublethal herbicide concentrations allowed *P. crassipes* to persist but not reproduce and suggested that biochemical changes in the sprayed plants might be favouring the population growth of *M. scutellaris*.

The effects of sublethal concentrations of herbicides on the biochemistry of *P. crassipes* plants were therefore assessed by isolating the nutritional plant metabolites in sprayed and unsprayed plants. Colorimetric analyses of herbicide-treated and untreated leaves were performed to quantify carbohydrate, protein, and phenolic contents from the extracted compounds. The accumulation of carbohydrates and proteins in *P. crassipes* leaves increased in the first week following the application of a sublethal concentration of Kilo Max (0.4%), which could explain the increase in insect populations from the sprayed plants. The accumulation of metabolites in the sprayed plants suggests that the primary metabolism is

induced by the sublethal herbicide application on *P. crassipes* leaves. Notably, the accumulation of carbohydrates such as sucrose is important for reducing the damage caused by herbicide stress in plants, as these soluble sugars act as osmoprotectants.

The accumulation of metabolites in *P. crassipes* plants following sublethal herbicide sprays may affect the foraging and dispersal behaviour of *M. scutellaris*, thereby altering population dynamics where integrated control is used as a weed management tool. Mesocosm experiments investigated the dispersal patterns of *M. scutellaris* during sublethal herbicide treatments of Kilo Max (0.4%) and Seismic (0.75%) herbicides, where tubs containing sprayed and unsprayed *P. crassipes* plants were placed near mesocosms containing plants inoculated with *M. scutellaris*. *M. scutellaris* persisted on the sprayed plants for the first five weeks of the experiments and then dispersed to unsprayed plants when the conditions of the sprayed plants deteriorated. From these results, we can deduce that sublethal herbicide concentrations supported the development of *M. scutellaris* populations, however, the death of the host plant caused their dispersal to neighbouring unsprayed plants. Sprayed plant populations decreased significantly, suggesting that the combined effects of sublethal herbicide stress and herbivory by *M. scutellaris* reduced *P. crassipes* vigour. Similar results were observed under field conditions, where *M. scutellaris* were released in herbicide-sprayed sites that were paired with unsprayed sites. The insects migrated from herbicide sprayed to unsprayed areas, however, their efficacy on unsprayed *P. crassipes* plants was inadequate as the insect population numbers were reduced.

These studies provided important insights into the potential safe integration of sublethal herbicide concentrations and biocontrol agents for weed management, indicating that sublethal herbicide concentrations can enhance the efficacy of biocontrol agents and promote effective weed suppression while minimising environmental pollution. However, small biocontrol populations in the field suggest that multiple releases of *M. scutellaris* in large numbers to inundate *P. crassipes* infestations should be implemented in future integrated management studies involving sublethal herbicide applications, as this is most likely to lead to yield successful control of the plants.

Preface

This thesis is presented in six chapters and is outlined as follows:

Chapter 1: This chapter reviews the literature on the control of water hyacinth (*Pontederia crassipes*), using physical, chemical, and biological methods. The section further emphasises herbicide use and biological control strategies. After navigating through the merits and demerits of the two methods, integrated control using sublethal herbicide concentrations and biological control agents is discussed. This section also identifies that research in this integrated approach is necessary as an alternative in *P. crassipes* control. The chapter concludes by outlining the rationale, overall aim and specific study objectives.

Chapter 2: This chapter identifies the sublethal concentrations of three glyphosate-based herbicides through experimental manipulation and evaluates their effects on the nutrient status of *P. crassipes* plants. Further, the section reports on laboratory-based herbicide bioassays toxicity tests on *M. scutellaris*. The chapter also assesses insect performance and behaviour in response to sublethal herbicide applications to *P. crassipes* plants by monitoring their survival and oviposition on treated plants.

- **Chapter 3:** The effects of the identified sublethal concentration of Kilo Max (0.4%) herbicide on the physiology of *P. crassipes* plants were investigated here, by analysing the macronutrient composition and secondary metabolites of the herbicide treated plants. These macronutrients and metabolites were analysed using colourimetry assays and High-Performance Liquid Chromatography (HPLC). Also, the chapter outlines how this sublethal concentration affected the macronutrient or metabolite profile of *M. scutellaris*.

- **Chapter 4:** This chapter demonstrates the effect of sublethal concentrations of Kilo Max (0.4%) and Seismic (0.75%) on *M. scutellaris* populations. Mesocosm experiments were conducted and the effect of sublethal concentrations of herbicides on the populations of *M. scutellaris* was tested. This was done by monitoring the survival, fecundity, and dispersal of the insect from herbicide-treated plants to healthy plants. The combined effect of *M. scutellaris* and sublethal herbicide concentrations on *P. crassipes* plants was also determined.

- **Chapter 5:** This chapter presents field investigations where biocontrol agent populations and plant parameters at the Bronkhorstspruit dam (Gauteng Province, South Africa) were assessed on the sites sprayed with selected sublethal concentration of Kilo Max herbicide (0.4%) and unsprayed areas. The effectiveness of *M. scutellaris* herbivory combined with the sublethal concentration of Kilo Max herbicide on *P. crassipes* control was reported.

- **Chapter 6:** This chapter links all the experimental chapters (Chapters 2-5) by interpreting the main findings to answer the objectives of the study. The section also answers the main aim of the study and subsequently gives recommendations for further studies in the advocacy of biological control and integrated (sublethal herbicide and biological) control.

Some aspects of the thesis will be published in peer-reviewed articles.

Declaration

I declare that this thesis submitted for a Doctor of Philosophy in Science degree at Rhodes University has not been submitted for a degree at this University or any other university. The work entailed in the document is my own in design and execution. Also, all the reference material in this thesis has been duly acknowledged.

Name & Surname: Siyasanga Tracy Mnciva

Date: 12 December 2024

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CHAPTER 1: General introduction

1.1. Introduction

Aquatic ecosystems in South Africa have been susceptible to invasion by introduced macrophytes since the early 1900s. The increased pollution of water bodies through urban runoff, agricultural practices, informal development, and industrial waste that leads to alteration of water flow regimes and eutrophication causes aquatic ecosystems to be prone to biological invasion. Water hyacinth, *Pontederia crassipes* Mart. (Pontederiaceae), was the first recorded invasive aquatic weed in South Africa (Cilliers 1991) and was found naturalised in the Western Cape province in the early 1900s. Several other species of freshwater aquatic plants, all notorious weeds in other parts of the world, have also become invasive in many of the rivers, human-made impoundments, lakes, and wetlands of South Africa (Hill & Coetzee 2017). These aquatic weeds include *Pistia stratiotes* L. (Araceae) (water lettuce); *Salvinia molesta* D.S. Mitch. (Salviniaceae) (Kariba weed); *Myriophyllum aquaticum* (Vell. Conc.) Verd. (parrot's feather); and *Azolla filiculoides* Lam. (Azollaceae) (red water fern) (Hill 2003), which along with *P. crassipes,* comprise the 'Big Bad Five' (Henderson & Cilliers 2002). All these weeds, except *P. crassipes*, have been brought under complete control by a single biocontrol agent in as little as two years, to a point that they are no longer a threat to our aquatic ecosystems (Hill et al. 2020).

In recent years, new aquatic plant invasions, which are still in their early stages of invasion, have been recorded in the country (Hill & Coetzee 2017). These include the submerged species, *Egeria densa* Planch. (Hydrocharitaceae) (Brazilian water weed) and *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae); the emergent species, *Sagittaria platyphylla* (Engelm.) J.G.Sm. and *S. latifolia* Willd. (Alismataceae); *Lythrum salicaria* L. (Lythraceae) (purple loosestrife), *Nasturtium officinale* W.T. Aiton. (Brassicaceae) (watercress); *Iris pseudacorus* L. (Iridaceae) (yellow flag); and *Hydrocleys nymphoides* (Humb. & Bonpl. Ex Willd.) Buchenau (Alismataceae) (water poppy); the floating weeds, *Salvinia minima* Baker

(Salviniaceae) and *Azolla cristata* Kaulf. (Azollaceae) (Mexican azolla); and the rooted floating *Nymphaea mexicana* Zucc. (Nymphaeceae) (Mexican water lily) (Coetzee et al. 2011). The introduction and dispersal of these newly invasive plants into aquatic ecosystems via the horticultural and aquarium trade pose severe environmental and economic threats in South Africa (Martin & Coetzee 2011).

Of all the aquatic plant invaders in South African freshwater ecosystems, *P. crassipes* remains the worst aquatic weed in terms of its widespread distribution, economic and environmental impacts, and difficulties experienced with its control. This chapter, therefore, reviews the origin and distribution of the problematic *P. crassipes*, the ecological and socio-economic impacts associated with the plant, and the management strategies employed to control the plant. The literature review deals with the integration of two control strategies, biological and herbicidal control, for the management of *P. crassipes*. The toxic impacts posed by herbicides on biological control agents are also reviewed.

1.2. Origin and distribution of *Pontederia crassipes*

Pontederia crassipes is indigenous to the Amazon basin of South America, and it is considered one of the world's worst aquatic invasive weeds. This aquatic macrophyte was cultivated as a greenhouse and landscape exotic plant shortly after the Civil War in the USA (Penfound & Earle 1948). However, the first authentic account details its introduction as an ornamental plant in the United States in 1884 at the Cotton Centennial Exposition in New Orleans, Louisiana (Gopal & Sharma 1990). The weed was similarly introduced in many parts of the world as an ornamental plant and then spread by gardeners, aquarium owners and boating enthusiasts (Cilliers 1991). Today, this plant has been found in more than 50 countries on five continents, including North America, Australia, China, Japan, Indochina and Africa from the 19th and early 20th centuries (Penfound & Earle 1948, Téllez et al. 2008, Villamagna & Murphy 2010). *Pontederia crassipes* has invaded most tropical and subtropical regions globally (Figure 1.1), becoming a major conservation and economic problem since the 1940s (Téllez et al. 2008, Coetzee et al. 2017).

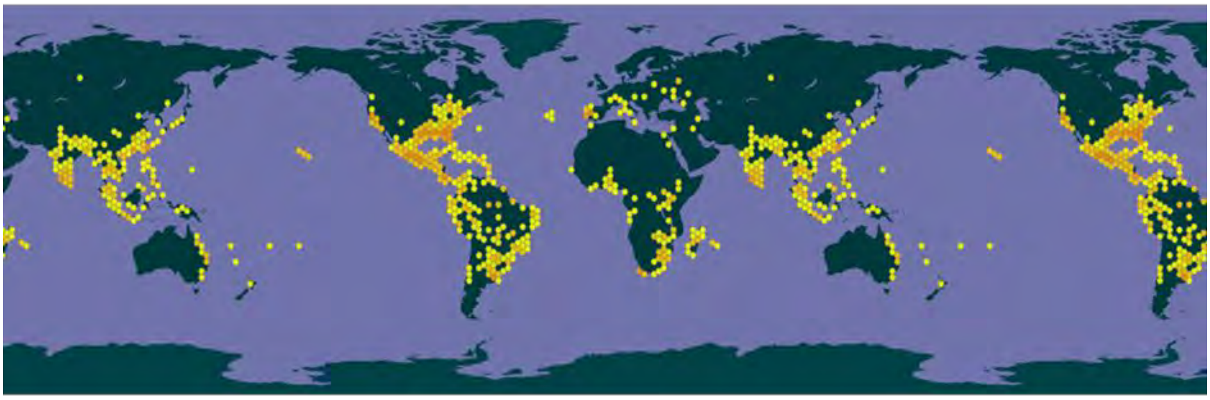

Figure 1.1: Global distribution of *Pontederia crassipes* (GBIF database, 2022). The different coloured points indicate multiple sites in close proximity.

In the last two centuries, this macrophyte has become one of the most damaging invasive species in Africa, including South Africa. The first record of *P. crassipes* in South Africa was on the Cape Flats in 1908 (Scott et al. 1979, Gopal 1987), followed by records of introduction to KwaZulu-Natal around 1910 (Byrne et al. 2010, Hill et al. 2020). The plant has since spread across the country and is now found in water bodies in sub-tropical and temperate areas (Figure 1.2). Under South African legislation, the National Environmental Management: Biodiversity Act (NEMBA) (10/2004), *P. crassipes* is declared a category 1b species, which requires control or eradication where possible (NEMBA 2014). In many countries such as Australia, Botswana, Burma, China, the European Union, New Zealand, and the United States, appropriate legislation has also been put in place to curb the further spread of the weed (Coetzee et al. 2017).

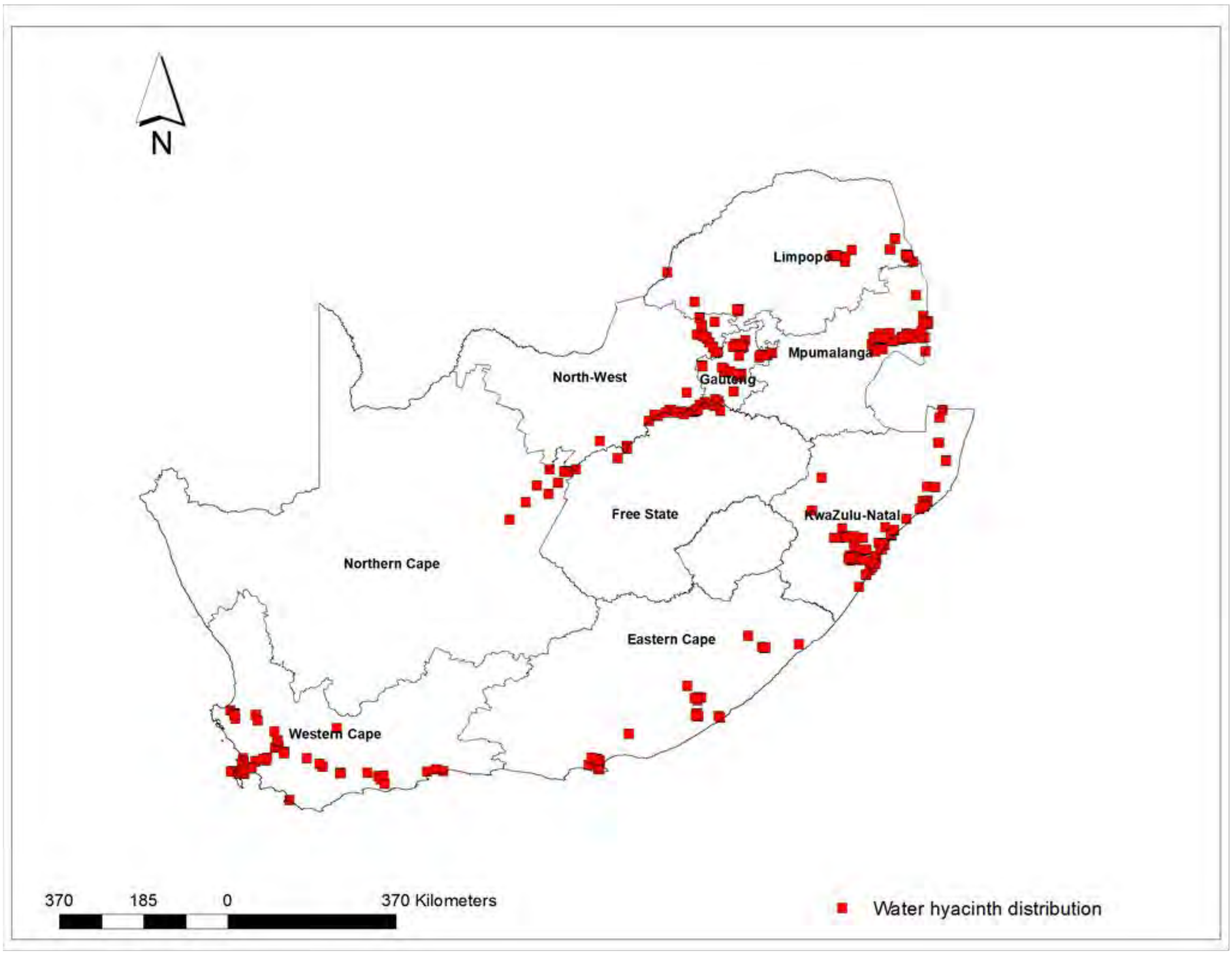

Figure 1.2: Distribution of *Pontederia crassipes* in South Africa (Data obtained from GBIF database, 2022).

1.3. Description and biology of *Pontederia crassipes*

Pontederia crassipes is a perennial, herbaceous, free-floating aquatic macrophyte (Penfound & Earle 1948, Coetzee et al. 2017). The plant's inflorescence produces about 6–10 shiny green leaves arranged in basal rosettes, borne on bulbous or elongated petioles, with attractive flowers with violet-blue petals (Figure 1.3). Each plant inflorescence can produce over 3000 seeds, and a single rosette can produce several inflorescences each year (Barrett 1980). The small, long-lived seeds sink and remain viable in sediments for 15–20 years (Matthews 1967, Gopal 1987, Van Wyk & Van Wilgen 2002). The persistence of these seeds revives, enhances, and sustains field populations of *P. crassipes* whenever the conditions become favourable for germination and growth (Julien et al. 2001, Sullivan & Wood 2012). Seeds germinate on moist sediments or in warm shallow waters, and flowering can occur 10–15 weeks thereafter (Barrett 1980). *Pontederia crassipes* is one of the most rapidly growing aquatic macrophytes (Albano Pérez et al. 2011, Coetzee et al. 2017); under ideal growth conditions, this plant has a growth rate of up to 6% per day (Ashton et al. 1981). The plant reproduces primarily by vegetative propagation, but seeds are a major source of reinfestation. New daughter plants are produced via the plant's stolons; the ability of *P. crassipes* to reproduce both sexually and asexually results in rapid growth and spread, allowing it to cover a wide range within a short time (Ndimele & Jimoh 2011, Elenwo & Akankali 2018). The plant develops a large leaf canopy, which may provide a competitive edge over other floating aquatic plants growing in the same system (Reddy & Sutton 1984) (Figure 1.3).

Figure 1.3: *Pontederia crassipes* infestation in a dam in Roodeplaat dam, Gauteng, South Africa (Courtesy of Julie Coetzee).

1.4. Factors influencing the growth of *Pontederia crassipes*

Pontederia crassipes tolerates a wide range of nutrients, temperature, and pH levels and grows in a wide variety of ecosystem types (Malik 2007). Environmental factors such as temperature, pH, solar radiation, and salinity of the water can influence the growth and performance of *P. crassipes* (Gupta et al. 2012). pH levels between 6 and 8 are optimal conditions for growth of *P. crassipes* (Malik 2007), and temperatures that range between 28 and 30℃ are ideal for *P. crassipes* growth (Gupta et al. 2012). Wilson et al. (2007) reported that temperature and nutrient concentrations are two of the most important factors determining *P. crassipes* growth and reproduction. Temperatures ranging from 28 to 30℃ encourage rapid growth of *P. crassipes,* resulting in dense mats of interwoven plants blanketing the water bodies (Reddy & Tucker 1983, Malik 2007, May & Coetzee 2013). The plant is thought to be cold-sensitive (Wilson et al. 2005); the growth stops if temperatures fall below 10℃. Frost is the major cause of *P. crassipes* leaf mortality in temperate regions (Wilson et al. 2005, Byrne et al. 2010). Owens & Madsen (1995) investigated the limits of low temperature on *P. crassipes* growth and reported a steady decline in regrowth potential of *P. crassipes* plants exposed to near-freezing temperatures (<5℃).

Pontederia crassipes growth is positively correlated with the high levels of nutrients in aquatic bodies, especially nitrogen and phosphorus concentrations (Reddy et al. 1989, 1990, Xie et al. 2004). Previous studies found that a high-nutrient supply can improve the photosynthetic capacity, resource-use efficiency, and competitiveness of *P. crassipes* (Ripley et al. 2006, Fan et al. 2013). Yu et al. (2019) found that the biomass, length, and stem diameter of *P. crassipes* plants were higher in the nutrient-rich treatments than in low-nutrient treatments. In South Africa, many rivers receive run-off, which is highly polluted with nitrates and phosphates arising from agricultural activities, which enhances the rapid growth of *P. crassipes* (Hill & Olckers 2001) and boosts its competitive advantages. Salinity, on the other hand, is a major constraint for *P. crassipes* growth (Olivares & Colonnello 2000). Several studies suggest that salinity imposes a limit on *P. crassipes* growth (Mangas-Ramírez & Elías-Gutiérrez 2004, Villamagna & Murphy 2010). NaCl-induced mortality of *P. crassipes* has been demonstrated from concentrations as low as 0.2% (Olivares & Colonnello 2000, Guezo et al. 2017). Bick et al. (2020) suggested that salinity is the primary driver of *P. crassipes* survival and, therefore, should be included on the list of its growth determinants. Other abiotic impacts include sunlight shading, wind direction, disturbance, and reproduction systems of the invasive weed (Dersseh et al. 2019). Understanding these factors will help to identify suitable management practices for this problematic macrophyte.

1.5. Impacts of *Pontederia crassipes* invasion

Invasion by *P. crassipes* mostly occurs in slow-moving or still water (Coetzee et al. 2007, Malik 2007), and invasion is successful owing to its reproductive strategies via clonal growth as well as seedling recruitment, with clonal growth being more important (Malik 2007, Téllez et al. 2008). Vegetative reproduction enables new plants to disperse from their parent plants via fragmentation where a complete fragment can give rise to new plants, which eventually form a mat (Greco & Freitas 2002). During times of stress, such as droughts and herbicidal applications, seeds germinate, giving rise to new plants despite the perturbations; thus, the plant poses a continual problem wherever it invades (Center & Spencer 1981, Midgley et al. 2006). Most dispersal is via direct anthropogenic means, but accidental introductions also occur via fishing boats and fishing gear (Eiswerth & Johnson 2002). Additionally, efforts to mechanically control *P. crassipes* cause fragmentation of the weed, facilitating spread.

Pontederia crassipes presents wide-ranging ecological and socio-economic impacts in its invaded range. Several studies in the literature have provided valuable information on the ecological impacts of *P. crassipes* on aquatic ecosystems around the world (Center et al. 1999, Schmitz et al. 2008, Hill et al. 2020). The ecological impacts caused by the infestations of this plant include water loss, suppression of natural aquatic vegetation and obstruction of river or canal flows, which may cause or aggravate flooding and increased siltation (Van Wyk & Van Wilgen 2002, Villamagna & Murphy 2010). The floating mats of *P. crassipes* block sunlight penetration into the waterbody, which depletes oxygen for aquatic communities, thereby reducing submerged indigenous flora and altering aquatic biodiversity (Julien et al. 2001, Jafari 2010, Villamagna & Murphy 2010). Coetzee et al. (2014) reported that *P. crassipes* invasion results in a reduction of benthic macroinvertebrate diversity. Similar findings were previously reported by Midgley et al. (2006) in New Year's Dam, South Africa, where there was higher benthic macroinvertebrate diversity in open water than in *P. crassipes*-infested waters. Dense mats of this plant block the light from reaching deep into waterbodies causing anoxia, thereby leading to declines in invertebrate diversity, and negatively affecting overall primary production (Toft et al. 2003, Malik 2007, Schultz & Dibble 2012). Gezie et al. (2018) discovered that dissolved oxygen and pH are lower in the habitats covered with *P. crassipes* mats than in open water and cleared habitats. *Pontederia crassipes* mats provide a habitat for invertebrate predators such as odonates and malaria-bearing mosquitos, as they search for food around the roots and leaves of macrophytes (Portilla et al. 2020), which may lead to the decline of aquatic biodiversity and pose a potential health risk to humans.

The infestations of *P. crassipes* have greater socio-economic impacts when the waterbody supports several human uses. For a system that is primarily used as a water source for human use, impacts could be measured in terms of changes to water quality and quantity (Villamagna & Murphy 2010). *Pontederia crassipes* has high evapotranspiration rates that lead to water loss from dams, rivers, and lakes. For example, evapotranspiration from *P. crassipes* can exceed open-water evaporation rates by a factor of 10 in some areas (Gopal 1987). A study by Van Der Weert & Kamerling (1974) reported that water lost by evapotranspiration of this weed is about 3.2 to 3.7 times more than the evaporation from a free water surface. More recently, Stan et al. (2016) reported that open water evaporation was an average of 4.3 mm/day while water invaded by aquatic invasive plants recorded evapotranspiration of 7.8 mm/day in Caldarusani Lake in Romania. After accounting for normal water evaporation, Arp et al. (2017) found that water loss by *P. crassipes* was estimated to be 5.6 million m^3 to 30.6 million m^3 from an irrigation dam under the area 100% covered by *P. crassipes*, depending on the season. This can be a serious concern in water-limited areas and small waterbodies that support several human uses.

1.6. Beneficial attributes of *Pontederia crassipes*

Some studies have demonstrated positive attributes associated with *P. crassipes*, including nutrient control, phytoremediation, and increased biodiversity in certain environments. A few studies have shown that *P. crassipes* infestations can positively alter the biodiversity of invertebrate assemblages mainly due to the complex structures of *P. crassipes* morphology that provide better habitat conditions for epiphytic invertebrates (Schramm et al. 1987, Masifwa et al. 2001, Rocha-Ramírez et al. 2007, Schultz & Dibble 2012). Gezie et al. (2018) reported that a *P. crassipes*-covered habitat had higher macroinvertebrate family richness and relative abundance than open habitat, mainly due to the high affinity of most of the macroinvertebrate taxa to aquatic vegetation.

Pontederia crassipes has been useful in reducing excess nutrients, pollutants, heavy metals, organic compounds, and pathogens from aquatic ecosystems (Newete et al. 2016). Ali et al. (2013) reported that heavy metals are taken up by the roots of the *P. crassipes* plant, translocated to the shoots and other plant tissues, where they are concentrated; harvesting the plant permanently removes these contaminants from the water system. Moreover, Rezania et al. (2015) reported that the rate of photosynthetic activity and plant growth of *P. crassipes* have a key role during the implementation of phytoremediation technology for the removal of water pollutants. Some studies have reported that dried *P. crassipes* can be used for manufacturing briquettes, which is used for co-firing in coal power plants (Ighodalo et al. 2011, Rezania et al. 2016). Recently, Tshithukhe et al. (2021) reported similar findings, where *P. crassipes* mats reduced concentrations of heavy metals in the Swartkops River, in South Africa.

Pontederia crassipes plants can be useful in the production of biogas and biofuel since they possess high amounts of hemicellulose contents (Nugraha et al. 2018). A potential of *P. crassipes* to produce biogas was studied by Fadairo & Fagbenle (2014) who revealed that about three litres of biogas was produced when 2.5 kg of dried *P. crassipes* was mixed with cow dung and poultry droppings. Njogu et al. (2015) also reported a biogas yield of between 70%-75% using the *P. crassipes*, which is said to be high enough to power internal combustion engines coupled with an electricity generator. The dry matter of *P. crassipes* contains high protein and mineral content, which makes it a useful source of animal feed and roughage (Su et al. 2018). The presence of important phytochemicals, such as alkaloids and saponins has been reported in *P. crassipes*, suggesting that they can be considered as a good feed supplement (Adelakun et al. 2016). Inhabitants of communities occurring around water bodies where *P. crassipes* grows can begin to process the plant into useful products for income. Ropes and yarn can be made from the stem of *P. crassipes* (Ayanda et al. 2020). This can be further processed into baskets, hats, mats, and even furniture (Harun et al. 2021).

Although studies have reported the positive attributes of *P. crassipes*, the costs associated with its presence in the majority of systems in South Africa and around the world far outweigh its benefits (Van Wyk & Van Wilgen 2002, Jafari 2010, Honlah et al. 2019). Therefore, to mitigate the negative impacts of *P. crassipes* invasion on the environment and the country's economy, its control is mandatory.

1.7. Management and control measures of *Pontederia crassipes*

The management of *P. crassipes* outside its native range requires diligent control efforts due to its long-lived seed bank, high vegetative growth rates, and ability to produce large amounts of viable seed within 12 weeks of germination. Initial management of this weed has focused on eradication. However, due to difficulties associated with this approach, including the plant's rapid growth rates and longevity of the seed bank, management has shifted towards reducing plant density to levels that minimise economic and ecological impacts (Villamagna & Murphy 2010). Methods of controlling *P. crassipes* usually include the use of physical barriers, manual, mechanical, chemical, and biological control. Each of these has its own benefits and drawbacks (Charudattan et al. 1995, Mujere 2015).

1.7.1. Mechanical control

Controlling *P. crassipes* by means of mechanical removal involves complete or partial removal of plants by mechanical means, including shredding, harvesting, and mowing. It can also include manual removal through hand-pulling handling, the use of pitchforks to rake the plants, and the use of motor-driven machinery (Hill & Coetzee 2008, Gettys et al. 2021). This management technique does not result in complete eradication of the weed, but it can reduce the plant abundance to non-nuisance levels in small systems. Mechanical control of *P.*

crassipes is known to be environmentally friendly, posing minimal harmful threats to aquatic life, and since the plant removes nutrients from the water, mechanical harvesting may improve water quality (Jones 2009, Auchterlonie et al. 2021). This method immediately opens physical space, such as habitat for fish, open water for boat traffic, fishing, and recreation (Villamagna & Murphy 2010, Carvalho & Cerveira Junior 2019), but control is often temporary if not maintained.

Mechanical shredding of *P. crassipes* can lead to increased dissolved oxygen in the system. Greenfield et al. (2007) evaluated the effect of *P. crassipes* mechanical shredding on water chemistry and nutrients in the Sacramento-San Joaquin River Delta, California. Their study indicated that dissolved oxygen concentrations were low during *P. crassipes* invasion, but the concentrations increased after mechanical shredding of the plant. The effectiveness of *in situ* shredding of *P. crassipes* on water quality was also investigated by Tung (2004) in the Dow Wetlands Preserve, a naturally restored wetland habitat located in Antioch, California. This study observed increased values of dissolved oxygen following mechanical shredding, while water nutrients decreased.

Mechanical harvesting has been effective for *P. crassipes* control to some extent; however, it is costly and is often ineffective for large infestations where expensive cutting material is required. Some studies have shown that after mechanical removal of *P. crassipes*, water transparency and dissolved oxygen decreased, while pH, phosphorus, phytoplankton, and cyanobacteria biomass increased (Mangas-Ramírez & Elías-Gutiérrez 2004, Bicudo et al. 2007, Villamagna & Murphy 2010). Another concern with mechanical control is that it is labour-intensive, and it is only effective for small infestations (Hill & Coetzee 2008). Disposal of *P. crassipes* from polluted water bodies also becomes an important health and ecological consideration owing to its capacity to absorb contaminants. In some cases, the cost of an offsite disposal area can be more expensive than removing the weed (Villamagna & Murphy 2010). This control method is not sustainable because it does not maintain *P. crassipes*-free water surfaces, as *P. crassipes* mat removal exposes the seeds at the bottom of the waterbody to sunlight, creating favourable conditions for seed germination and allowing for reinfestations.

1.7.2. Chemical control

Diquat, Paraquat, 2,4 – D and glyphosate herbicides are amongst the suite of herbicides that have been used worldwide for the reduction of *P. crassipes* populations (Gopal 1987, Hill & Coetzee 2008). These herbicides have been successful in controlling *P. crassipes* in small-scale water systems such as irrigation canals and small dams of around one hectare in size (Hill & Coetzee 2008). Herbicidal control is less labour-intensive than mechanical control, more so at large scales (Gutierrez-Lopez 1993). This method also removes plant barriers and opens physical space for fish movement and population. A significant increase in fish abundance was observed in a Nigerian creek 14 days after *P. crassipes* herbicide control (Villamagna & Murphy 2010). In South Africa, in the late 1970s, a severe *P. crassipes* infestation on the Hartebeespoort Dam was brought under control using terbutryn (Ashton et al. 1981).

However, herbicides are associated with significant socio-economic and ecological impacts, especially if beneficial uses of the waterbody are affected. For example, herbicide formulations are less selective than mechanical control strategies, as they attack non-target algae, which is a critical foundation of aquatic food webs. Herbicides also degrade ecosystem functioning by reducing species diversity, changing community structure, modifying food chains, altering patterns of energy flow and nutrient recycling, and reducing the resilience of ecosystems (Perez et al. 2011). Also, in developing countries, many *P. crassipes* infested waterbodies are used for drinking water, washing and fishing, and so the use of herbicide sprays contaminates the water, thereby threatening human health (Julien et al. 2001, Hill & Coetzee 2008). At large scales, herbicides can be expensive, especially if repeated applications are required. The cost of herbicidal control depends heavily on the locality and equipment used to administer the herbicide (for example, backpack sprayer, helicopter, or airboat) (Villamagna & Murphy 2010, Hill et al. 2020). From 1986 to 1999, the Department of Water Affairs and Forestry (DWAF) of South Africa spent between $507 143,11– 760 714,65 on aquatic weed control operations, of which $304 424,82 was on herbicide applications alone (Van Wyk & Van Wilgen 2002). Recently, the Department of Fisheries, Forestry and the Environment (DFFE) of South Africa spent a total of $2325570,24 on chemical control of *P. crassipes* at a cost of $996,67 per hectare (Hill et al. 2020). In addition to their expense, herbicides are deemed poisonous, as they degrade water quality.

All herbicide products have chemical properties that influence their ability to suppress growth or kill plants (Marin-Morales et al. 2013, Van Bruggen et al. 2021). While some of these properties are inherent in the chemical nature of the herbicides, others are added to enhance their efficacy (Mensah et al. 2015). Herbicides have chemical properties that influence their use; some of these include their chemical structure, solubility and polarity in water, volatility, and formulations (Mendes et al. 2020, Krähmer et al. 2021). The biologically active portion of a herbicide product is the 'active ingredient' which is the fundamental molecular composition and configuration of the herbicide (Mensah et al. 2013, Baer & Marcel 2014) and can determine the method by which herbicide should be applied (Mensah et al. 2013). Herbicides produced as salts dissolve quite well in water and are usually formulated to be applied in water, while non-polar herbicide sources are not (Mensah et al. 2013, 2015). Water is the main substance used to disperse (spray) herbicides; hence the water solubility of a herbicide influences the type of product that is formulated and how it is applied (Gao et al. 2019).

Commercial herbicide products contain an active ingredient and "inert" ingredients. An "inert" ingredient could be a carrier that is used to dilute and disperse the herbicide (e.g., water, oil, certain types of clay, vermiculite, plant residues, starch polymers, certain dry fertilizers) or an adjuvant (e.g., activator, additive, dispersing agent, emulsifier, spreader, sticker, surfactant, thickener, wetting agent) that enhances the herbicide's performance, handling, or application (Radosevich et al. 2007). In recent years, carriers and adjuvants have been implicated in adding to the toxicity of the active ingredients and in some cases, have been even more toxic, especially to insect herbivores, than the active ingredient alone (Radosevich et al. 2007). Of all the herbicides used in aquatic systems, glyphosate-based herbicides gained popularity for their effectiveness in controlling invasive competing vegetation and for their low animal toxicity (Busse et al. 2001) and are used most widely in South Africa for *P. crassipes* control (Jadhav et al. 2008, Hill et al. 2012).

1.7.2.1. Glyphosate-based herbicides

Glyphosate-based herbicides are the world's leading post-emergent, organophosphate, systemic, broad-spectrum, and non-selective herbicides for controlling annual and perennial weeds (Woodburn 2000, Perez et al. 2011). Commercial formulations of glyphosate contain glyphosate in the form of salt, which have high water solubility compared to technical grade glyphosate (Perez et al. 2011) but do not lose any of the herbicidal properties of the parent compound. Formulations of glyphosate in salt form include monoammonium salt, diammonium salt, isopropyl amine salt, potassium salt, sodium salt, and trimethyl sulfonium or trimesium salt. Of these, the isopropyl amine salt is the most commonly used in formulated commercialised herbicide products (Sihtmäe et al. 2013). Several studies have demonstrated that inert ingredients in glyphosate formulations are several folds higher in toxicity to non-target organisms than glyphosate alone (Cedergreen & Streibig 2005). For example, the surfactant polyoxyethylene amine (POEA) is thought to be responsible for the relatively high toxicity of Roundup to several freshwater invertebrates and fishes, although isopropylamine (IPA) salt of glyphosate also contributes its share (Giesy et al. 2000, Tsui & Chu 2003).

Glyphosate herbicides are the most widely used herbicides for controlling aquatic macrophytes (de Souza et al. 2020). In South Africa, the Working for Water programme of the Department of Fisheries, Forestry and the Environment (DFFE) recommends using glyphosate-based herbicides such as Mamba (Dow AgroSciences, Southern Africa (Pty) Ltd), Tumbleweed (Enviro Industries, South Africa (Pty) Ltd), Touchdown (Syngeta, South Africa (Pty) Ltd), Roundup (Monsanto, South Africa (Pty) Ltd) and recently, Kilo Max (Arysta LifeScience, South Africa (Pty) Ltd) and Seismic (Arysta LifeScience, South Africa (Pty) Ltd) to control invasive alien aquatic and terrestrial plant species (Mensah et al. 2013). The herbicides are often sprayed directly onto the problematic plants at the recommended label rate. Currently, manufacturers of registered glyphosate-formulated herbicides in South Africa recommend between 2-4% of the active ingredient as the application rate. Kilo Max and

Seismic are routinely used by DFFE, at the recommended-label doses of 2% and 3%, respectively (Abbie Heunis, DFFE, Pers. Comm. 2021).

The phytotoxic effects of glyphosate on plants usually start gradually, becoming visible within two to four days in most annual weeds, but may not occur until after seven days in most perennial weeds (Mensah et al. 2015). Physical phytotoxic symptoms include progress from gradual wilting and chlorosis to complete browning, total deterioration and finally, death (Perez et al. 2011). The glyphosate mode of action is confined to the shikimate pathway of aromatic amino acid biosynthesis, a pathway that links primary and secondary metabolism. The biosynthesis of the aromatic amino acids is promoted by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target enzyme of glyphosate (Schönbrunn et al. 2001). This enzyme is one of the seven enzymes that catalyse a series of reactions, which begins with the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). The shikimate (shikimic acid) pathway is an important biochemical intermediary in plants, and its pathway accounts for about 35% of the plant's dry weight; therefore, any interference in the pathway is highly detrimental to the plant. The shikimate pathway is absent in animals, which may account for the low toxicity of glyphosate to animals.

In South Africa, literature on glyphosate-based herbicides is scarce. Glyphosate has been found in high concentrations in the Hex River Valley, an agriculturally intensive grape-farming area in the Western Cape, since the early 1990s (London et al. 2000, Dalvie et al. 2003, Mensah et al. 2013). Glyphosate-based herbicides have strong adsorption to soil and sediments and, therefore, can be detected in surface waters long after being used to kill aquatic weeds (Glusczak et al. 2007). This has raised concerns among aquatic ecotoxicologists regarding the adverse impacts that glyphosate formulations might have on aquatic non-target animals and non-target native flora since they have high water solubility (Tsui & Chu 2003). Consequently, more pristine, and environmentally friendly methods of controlling aquatic weeds are required.

1.7.3. Biological control

Biological control is a widely used, long-term method for controlling alien invasive species because it is environmentally friendly and safe if practised appropriately, and it remains economical and sustainable (Wittenburg & Cock 2001, Moran 2006). This method involves using host-specific natural enemies that are either herbivorous arthopods (insects and mites) or naturally occurring plant pathogens, which reduce the field population densities and the biomass of alien invasive plant species (Firehun et al. 2013).

The use of biological control has successfully reduced the abundance of *P. crassipes* in several countries worldwide, including Papua New Guinea (Julien et al. 2007), India (Jayanth 1988), Lake Victoria, Africa (Mailu 2001), Benin (De Groote et al. 2003), Mexico (Aguilar et al. 2003), in Louisiana, Florida, and Texas, USA (Cofrancesco et al. 1985). The mite *Orthogalumna terebrantis* Wallwork (Acarina: Sarcoptiformes: Galumnidae), the moth *Niphograpta albiguttalis* Warren (= *Sameodes albiguttalis* (Warren)) (Lepidoptera: Crambidae), the Miridae *Eccritotarsus catarinensis* Carvalho and *Eccritotarsus eichohrniae* Henry (Hemiptera: Miridae) (Coetzee et al. 2008), and the weevils, *Neochetina eichhorniae* Warner and *N. bruchi* Hustache (Coleoptera: Curculionidae) (Center et al. 1999, Ogwang & Molo 2004, Moran et al. 2005), and more recently the plant hopper, *Megamelus scutellaris* Berg. (Hemiptera: Delphicidae) (Sosa et al. 2005, Tipping et al. 2011, Hill & Coetzee 2017) are among the insects that have been successfully explored for the control of *P. crassipes* around the world (Table 1.1).

Research into the biological control of *P. crassipes* was initiated by the United States Department of Agriculture (USDA) in 1962. In 1970, two weevil species *N. eichhorniae* and *N. bruchi*, and the moth *N. albiguttalis* were released in the United States Gulf Coast states such as Louisiana, Texas, and Florida where thousands of hectares were infested by *P. crassipes* (Gupta & Yadav 2020). The coverage of *P. crassipes* mats was decreased by 33% in 1980 (Gupta & Yadav 2020). The *P. crassipes* moth, *N. albiguttalis* was first released in Zambia in 1971, and to date, it has established, and it contributes to *P. crassipes* control in 13 countries

(Table 1.1). Damage by the larvae of the moth causes necrosis and waterlogging of internal tissues of the plant, leading to wilting and eventually death of the ramets (Julien et al. 2001). The mite, *O. terebrantis* was also released in Zambia in 1971 (Julien et al. 1996) and in India in 1986 (Jayanth & Visalakshy 1989).

These biocontrol agents have provided excellent control in some habitats; however, varying levels of success of these agents have been attained in different parts of the world.

1.7.3.1. Factors influencing the success of biological control

Abiotic factors, including temperature, nutrients, and salinity, affect the growth of *P. crassipes* plants and are known to have an impact on the survival and establishment of their biocontrol agents. In South Africa, the success of biocontrol initiatives has been variable due to several abiotic factors that constrain the impact of biocontrol agents. Hill & Olckers (2001) reviewed the factors that contribute to the limited success and establishment of *P. crassipes* biocontrol agents in South Africa. These factors include fluctuating temperatures, which include cold winters and extremely hot summers; nutrient-enriched water systems in which *P. crassipes* thrives; periodic removal of the weed and natural enemy populations through flooding and drought; and interference from other control methods, notably herbicide applications.

Table 1.1: Biological control agents released against *Pontederia crassipes*, with the countries and years of introduction (adapted from Julien & Griffiths 1998, Julien 2001, Byrne et al. 2010, Coetzee et al. 2011, Tipping et al. 2014b)

Species										
	Neochetina bruchi	*Neochetina eichhorniae*	*Niphograpta albigutallis*	*Eccritotarsus catarinensis*	*Eccritotarsus eichhorniae*	*Orthogalumna terebrantis*	*Xubida infusellus*	*Cercospora piaropi*	*Cornops aquaticum*	*Megamelus scutellaris*
Countries										
Australia	1990	1975	1977				1981;1996			
Benin	1992	1991	1993	1999						
China	1996	1996		2000						
Congo	1999	1999								
Cuba	1995	1976	1995			1977				
Egypt	2000	2000								
Fiji		1977								
Ghana	1994	1994	1996							
Honduras	1989	1990								
India	1984	1983				1986				
Indonesia	1996	1979								
Ivory Coast	1997	1997								
Jamaica						1969				
Kenya	1995	1993								
Malawi	1995	1995	1996	1996		1991;1996				
Malaysia	1992	1983	1996							
Mexico	1995	1967	1993							

Mozambique	1972	1972;1985				1971				
Myanmar		1980								
Niger		1993								
Nigeria	1995	1993;1984	2008							
Panama	1977									
Philippines	1992	1992								
PNG	1993	1986	1994							
Puerto Rico			1995							
Rwanda	2000	2000								
Solomon Islands		1988								
South Africa	1989	1974;1996	1990	1996	1999			1987	2011	2013
Sri-Lanka		1988								
Sudan	1979	1978	1980							
Taiwan	1993	1992								
Tanzania	1995	1995								
Thailand	1991	1979	1995				1999			
Uganda	1993	1993								
USA	1974	1972	1977			1968				2010
Vietnam	1996	1984								
Zambia	1997	1971;1996	19971;1997	1997		1971				
Zimbabwe	1996	1971	1994	1999		1996				

Numerous biological control agents have failed to establish in areas of introduction due to climate incompatibility (Byrne et al. 2002, 2003). *Pontederia crassipes* grows in a wide variety of climatic regions in South Africa, including high altitudes, where the plants and insects remain dormant for most of the cold months (Miller et al. 2020) which lowers the build-up of biocontrol populations, thus impacting the levels of biocontrol. In South Africa, the majority of biological control agents for *P. crassipes* are adapted to low altitude, warm climates; this is because of their warm tropical origin (Amazon Basin, South America) (May & Coetzee 2013). Additionally, *P. crassipes* growth is directly correlated with water nutrient concentrations, particularly nitrogen and phosphorus (Heard & Winterton 2000, Coetzee et al. 2011, Coetzee et al. 2022). Due to rising agricultural activities in South Africa, as well as failing wastewater treatment plants, most dams and rivers receive run-off, which is highly polluted by phosphates and nitrates (Hill & Olckers 2001). Research has proven that *P. crassipes* biomass can increase eight-fold in nutrient-rich sites when compared to sites that are nutrient poor (Reddy et al. 1990), leading to the proliferation of *P. crassipes* plants beyond the rate of insect reproduction and feeding intensity (Hill & Olckers 2001, Coetzee & Hill 2008, Coetzee et al. 2011). For example, in South Africa, the Hammarsdale Dam in the KwaZulu-Natal Province receives a nutrient-rich effluent from the wastewater treatment plant (Hill & Olckers 2001), resulting in a highly eutrophicated water system. However, this leads to high plant productivity, causing reduced damage impact by *Neochetina* weevils despite their high population numbers (Coetzee & Hill 2012). Salinity is also a major determinant of *P. crassipes* growth and biocontrol establishment in estuaries. A study by Bick et al. (2020) revealed that even though *N. bruchi* tolerates high NaCl concentrations; its host plant, *P. crassipes*, has a much lower tolerance of NaCl. However, studies suggest that when the host plant perishes, the egg, larval and pupal stages of *N. bruchi* also perish due to the lack of food source (Thielen et al. 1994). It is important to select optimal biocontrol agents that suit the environmental conditions that the weed has invaded.

1.7.3.2. Benefits of biological control

Biological control of *P. crassipes* has numerous benefits, including increased biodiversity (Hosking & du Preez 2004), various water benefits which include improved water quality and water savings (Hosking & du Preez 2004) and economic benefits. The water-saving benefits of *P. crassipes* biocontrol are associated with the increase in water available for recreational activities in recreational dams and rivers and the increased water supply for agricultural production where the dam is used for agricultural purposes (Arp et al. 2017). The water-saving effects of the biocontrol programme were observed at the New Year's Dam in the Eastern Cape, South Africa, where the implementation of the programme successfully saved about 2 million m^3 of water over a 20-year period (Fraser et al. 2016). Increased native fauna and flora biodiversity has also been reported following weed biocontrol programmes. However, weed biological control has suffered from a lack of quantitative post-release evaluation studies that show economic or ecological benefits.

Benefit-cost analysis is a commonly used approach to determine the feasibility of control programmes (Van Wilgen et al. 2004). Where the benefits of a biological control programme have been measured, it has focussed on economic benefits (Hill & Coetzee 2017). For example, Van Wyk & Van Wilgen (2002) compared the costs and benefits of three control interventions for *P. crassipes* and showed that biological control, along with integrated control, offered the best return on investment. Moreover, De Groote et al. (2003) demonstrated that the successful biological control of *P. crassipes* in southern Benin significantly increased the yearly income of the population of this region through increased crop and fish production. A thorough analysis of control options for invasive weeds is important to assist land managers in making better-informed and cost-effective decisions for weed management and control.

1.7.3.3. Biological control of *Pontederia crassipes* in South Africa

Biological control of *P. crassipes* in South Africa was initiated in the early 1970s with the introduction of the South American weevil, *N. eichhorniae,* into quarantine in 1973, and release in 1974 (Cilliers 1991). It is notable that it was re-released in 1977 and re-introduced in 1985, which suggests that the establishment had been poor and possibly the release effort had been inadequate. Since 1974, nine species of biological control agents have been introduced (deliberately or inadvertently) against *P. crassipes* in South Africa (Coetzee et al. 2011, Hill & Coetzee 2017, Paterson et al. 2019). Two weevil species, *N. eichhorniae* in 1974 and *N. bruchi*, in 1990; the moth, *N. albiguttalis*, in 1990; the mite, *O. terebrantis*, in 1989; two mirid bugs, *E. catarinensis* in 1996 and *E. eichhorniae*; the pathogenic fungus, *Cercospora piaropi Tharp*. (Mycosphaerellales: Mycosphaerellaceae), in 1987 (Hill & Cilliers 1999), and the grasshopper, *Cornops aquaticum* (Orthoptera: Acrididae) in 2005 (Bownes et al. 2011). Recently, a planthopper, *M. scutellaris*, was screened and approved for release in 2013 (Coetzee et al. 2014, Heard et al. 2014, Miller et al. 2019). Since its release, this agent has established around the country across various climatic regions such as the Mediterranean climate zones of the Western Cape with hot dry summers and winter rainfall, the humid subtropical zones of the KwaZulu-Natal east coast with very hot summers and mild winters, and the drier subtropical highlands with cold and dry winters of North West and Gauteng provinces (Miller et al. 2020). *Megamelus scutellaris*, which is the subject of the present study, is showing much promise as one of the most effective insect agents released in South Africa as it causes significant damage potential to *P. crassipes* in the field (Coetzee et al. 2022, Moffat et al. 2024).

1.7.3.4. Description and life history of *Megamelus scutellaris*

Megamelus scutellaris is native to parts of South America where *P. crassipes* occurs, including Argentina, Brazil, Peru, and Uruguay (Sosa et al. 2005, 2007). This species is host-specific to *P. crassipes,* and it is a multivoltine species capable of producing multiple, overlapping generations in a short period of time (Tipping et al. 2011). Adults exhibit wing dimorphism, a short-winged, non-flying form (brachypterous) (Figure 1.4A) and a long-winged dispersal form which is capable of flight (macropterous) (Figure 1.4B) (Fitzgerald & Tipping 2013). The wing form is determined within the insect generation and is dependent on environmental cues, such as crowding and host plant quality. The macropterous form enables the insect to disperse from overcrowded or poor-quality plants to find better quality *P. crassipes* plants (Fitzgerald & Tipping 2013).

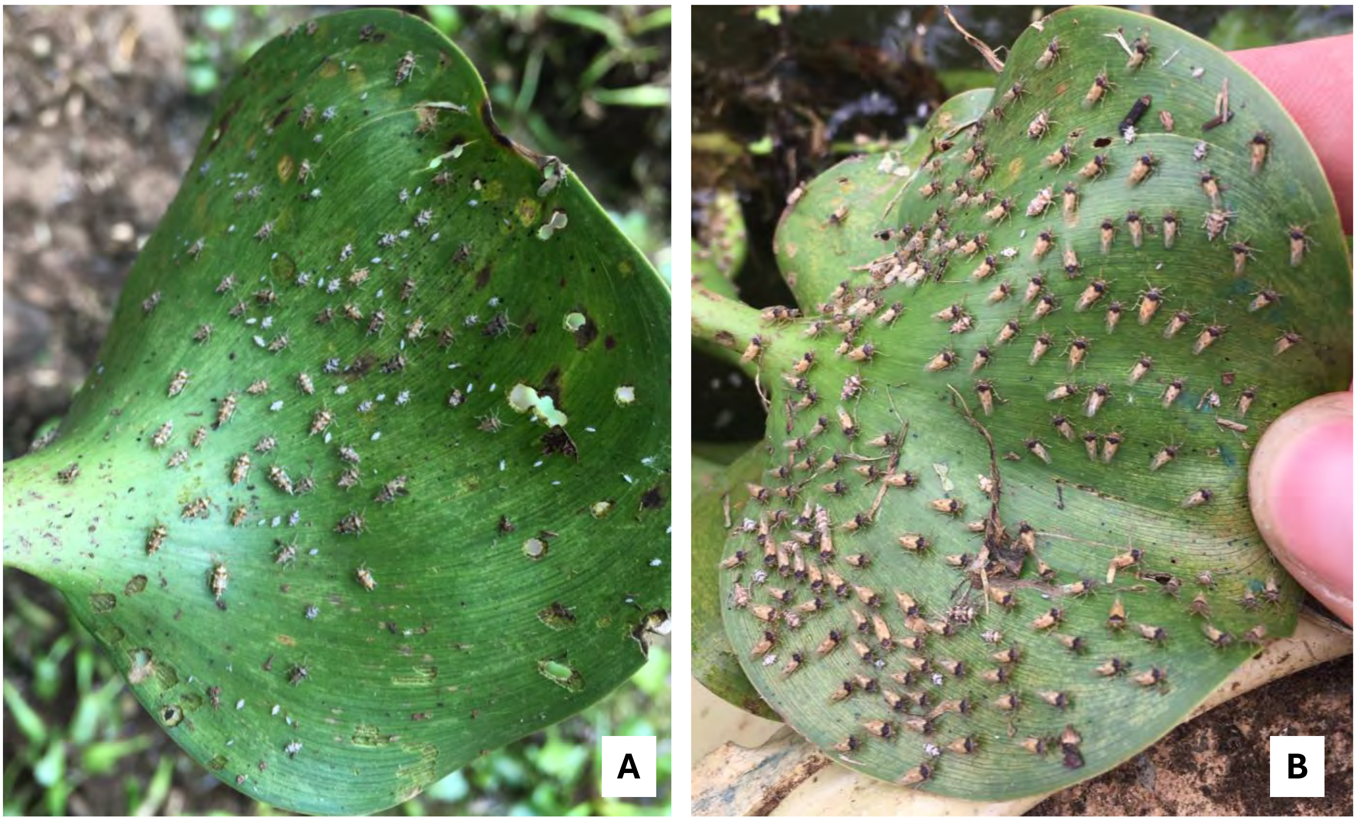

Figure 1.4: Adult *Megamelus scutellaris*, A. Brachypterous adults, B. Macropterous adults (Courtesy of Benjamin Miller).

Megamelus scutellaris utilises specific microhabitats on *P. crassipes* to satisfy its nutritional requirements and to reproduce. Adult insects mate at the bottom and upper leaves of *P. crassipes* plants, and adult females oviposit in the petiole and lamina of *P. crassipes*. Nymphs (immature insect stage) emerge 7 to 13 days after oviposition (Sosa et al. 2005, Tipping et al. 2008), depending upon the temperature. Development of nymphs to mature adults is through five instars, and they feed on adaxial and abaxial lamina surfaces and petioles. Development of the entire immature stage in outdoor conditions takes about 25 days (Sosa et al. 2005). *Megamelus scutellaris* is a phloem-feeding phytophagous insect, it can reduce *P. crassipes* growth rates, induce significant tissue damage, and increase plant mortality rates (Tipping et al. 2011). This planthopper feeds by inserting its stylets into the host plant and sucking the sap. Saliva of the planthoppers is secreted during penetration and forms a stylet sheath which acts to hold the stylets together and facilitates lubrication and movement toward food sources (Sogawa 1982). According to Spiller et al. (1990), the stylet probing pierces the plant tissues, causing significant damage to cell organs, and eventually leads to cell death.

The behaviour and performance of *M. scutellaris* may be limited by a number of factors such as high ambient temperatures, plant quality (i.e., nutritional value, Nitrogen (N) content) and competitive interactions with other biological control agents (Center & Dray 2010, Freedman et al. 2017). Temperature significantly affects the survival and performance of *M. scutellaris* and both high and low temperatures have strong negative influences on the survival and establishment of this insect (Grodowitz et al. 2017). In a report by Grodowitz et al. (2017), significantly lower populations of *M. scutellaris* were observed at the 18℃ and 33℃ treatment regimens than at an optimum temperature of 25℃. Competition between *M. scutellaris* and other insects may exert positive or negative impacts on the plant's feeding partners. In South Africa, there have been no negative competitive interactions between *M. scutellaris,* and other control agents (Petela 2017). Plant nutritional quality is crucial to herbivorous insects' fecundity (Awmack & Leather 2002), and most biocontrol programmes have even promoted agent establishment by increasing plant quality at release sites through fertilisation (Room & Thomas 1985). Despite these limiting factors, *M. scutellaris* has acclimatised and established across South Africa, even in the country's temperate regions (Miller et al. 2020).

1.8. Integrated control of *Pontederia crassipes*

In some regions, acceptable levels of *P. crassipes* control by biological control methods alone have not been achieved, as it is perceived as slow acting (Hill & Coetzee 2008, Hill et al. 2012). In the face of confounding factors in the management of *P. crassipes* by a single method, integrated control may prove effective (Center et al. 1999). The integration of biological and herbicide control is currently the most widely advocated method for the control of problematic weeds (Byrne et al. 2010, Hill et al. 2012). In the past, scientists assumed that biocontrol and chemical control techniques were incompatible, but it has been recognised that their integration results in improved weed control programmes (Paynter 2003).

Integration of herbicides with insect biocontrol agents may provide the most satisfactory control method by reducing weed density below the economic threshold more quickly than a biocontrol insect working alone (Figure 1.5.).

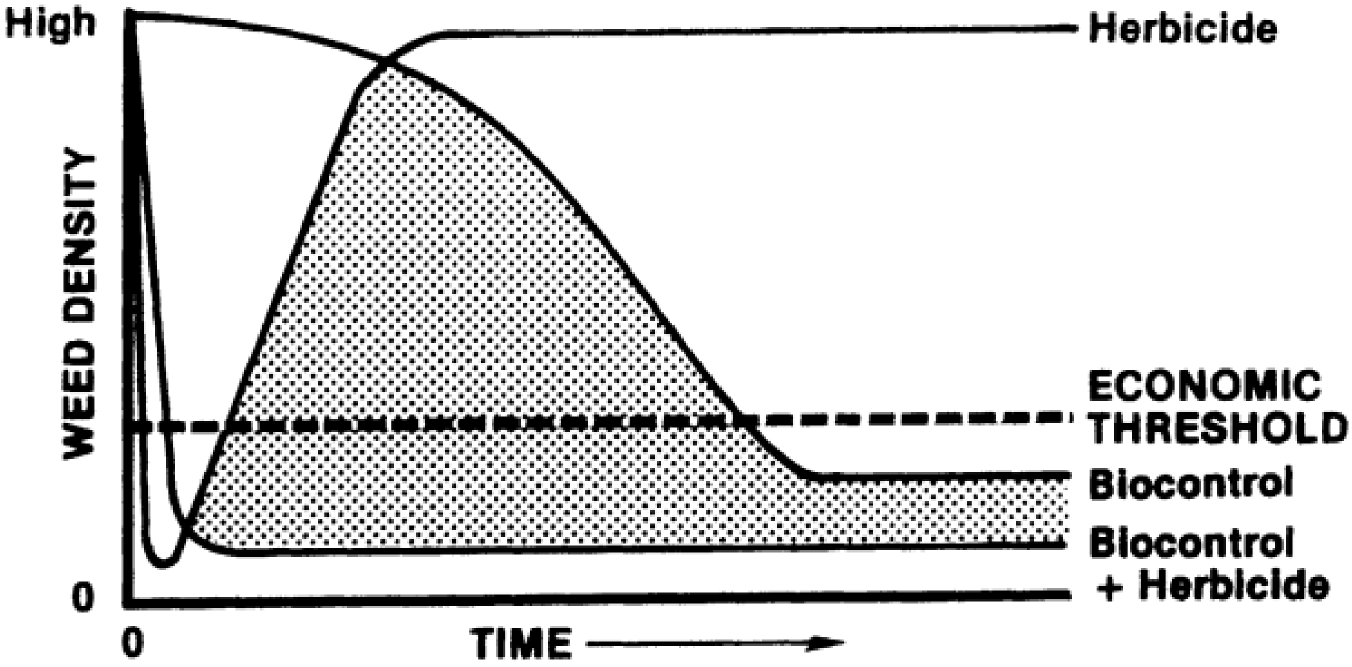

Figure 1.5: Relative efficacy of herbicide, biocontrol agent, herbicide, and biocontrol agent for weed control over time. The shaded area represents the potential gain in control from integrating biocontrol agent plus herbicide, and its size will vary with the efficacy of the herbicide and rate of weed reestablishment, rate of biocontrol agent establishment and total efficacy, and continuing benefit of the integrated control programme (Adapted from review by Messersmith & Adkins 1995).

A successful integrated management programme was developed for the Nseleni River system in South Africa (Jones 2001). Using the integrated method, *P. crassipes* coverage at Nseleni River was decreased from 100% by herbicide applications and is maintained at 5% by biological control with occasional follow-up herbicide around the sensitive sites when necessary. There are three ways in which biocontrol may be integrated with herbicidal control programmes that were described by Cullen (1996); these are, ‘purpose-specific approaches’, ‘ecological integration’ and ‘physiological integration’. The purpose-specific approach is the method where a single control method is used for each site based on the required level of control. For example, hand-pulling or herbicidal control is used for small infestations, fire followed by herbicides for medium infestations, and biological control for large infestations.

Thus, although biological control and herbicides are integrated on a landscape scale to manage the weed, at a given site only one is used (Ainsworth 2003). In ecological integration, chemical and biological control are used to control the same weed infestation at the same time, or almost at the same time. The objective of ecological integration is to achieve a rapid and large decline in weed infestations using herbicides, after which the biological control agents are expected to maintain the weed density at acceptable levels. This type of integration requires information on factors such as the toxicity of herbicides to biocontrol agents, timing of herbicide application in relation to the life cycle of the agents, and the mobility of biocontrol agents. Physiological integration refers to the interactions between biocontrol agents with sublethal herbicides. The use of sublethal or retardant concentrations of herbicides suppresses the growth of weed populations but has no detrimental effect on the biological control agents. Research questions relevant to physiological integration include the direct toxic effects of herbicides, the responses of different life stages of the biocontrol agent to altered food quality, and herbicide-induced changes in the selection of oviposition sites by biocontrol agents (Ainsworth 2003). Preserving the resident biocontrol agent populations is of utmost necessity and importance; therefore, for integration programmes, the use of herbicides should be regulated to reduce the abundance of weed infestations while leaving the biological control agent populations intact, enabling them to inflict proportionately more damage on the weed and reduce it to acceptable levels.

Biological control agents have the capacity to reduce the invasive qualities of *P. crassipes* significantly by slowing vegetative growth and reducing seed production. However, eutrophic waters allow *P. crassipes* plants to grow rapidly, and therefore, the plant densities become too great for biological control agents to make any significant reduction in the infestation (Coetzee et al. 2007). Eutrophic water facilitates the growth of *P. crassipes*, increasing the production of new leaves at a faster rate than the insects can damage them, allowing plants to persist, often despite the presence of biological control agent populations (Coetzee et al. 2022). As biocontrol is perceived to be too slow because of eutrophication in South Africa, recent emphasis has been placed on developing strategies for the integrated control of *P. crassipes*, integrating biological control and herbicidal application programmes.

1.9. Biochemical and physiological effects of glyphosate herbicides on invasive plants and their biological control agents

Glyphosate has been shown to affect several plant physiological processes, which could be linked to glyphosate herbicidal effects (Gomes et al. 2014). These physiological mechanisms include photosynthesis, carbon and nitrogen metabolism, mineral nutrition, and oxidative events. The disruptions of physiological mechanisms caused by herbicides may have an impact on insect survival and performance (Ainsworth 2003). Disruptions to the normal physiology of plants caused by herbicides with different modes of action may be comparable with those caused by other stresses. Physiological effects in the plants can have different consequences depending upon how a particular insect species encounters and uses the plant (Larsson 1989). There has been considerable interest in how insect herbivores respond to stress-induced changes in their host plants.

The plant stress hypothesis proposes that stress causes changes in plants which improves their quality as a resource for herbivores (White 1974). However, the actual responses of insects to plant physiological changes often appear to be unpredictable owing to insufficient knowledge of which plant properties are relevant to each insect, and how herbicides affect those properties over time. For example, the synthetic auxin herbicides that disrupt plant cell growth (2,4–D, MCPA and other phenoxy compounds) have frequently been associated with improved performance of aphids and other sap feeders (Yardim & Edwards 1998). It has been reported that sublethal concentrations of these herbicides stimulate growth in certain tissues, and this appears sometimes to enhance the nutritional value of phloem sap. There have also been a few cases that have reported that the use of glyphosate herbicide, including both deliberate and unintentional sublethal application, is possibly associated with the increase in insect herbivory or insect performance. Atkinson and Nuss (1989) found that glyphosate used as an aid to ripen sugarcane caused changes in stem composition that were similar to those caused by water stress, and which were beneficial to the stem-boring larvae of the moth *Eldana saccharina* Walker (Lepidoptera: Pyralidae). Increased amino acid levels in this study were associated with improved larval performance; in another case, amino acids in oat seedlings were increased by a low glyphosate treatment but not by a higher one (Falco et al.

1989). Therefore, positive responses of insects may be common in sublethal concentrations of glyphosate. There has been interest in the use of glyphosate to preserve the nutritional value of senescing annual grasses for herbivores, sometimes referred to as chemical curing. However, some studies found contrasting results; for example, Campbell et al. (1991) reported that glyphosate treatment of tomato plants reduced weight gain of tomato fruit worm larvae *Helicoverpa zea* Boddie. (Lepidoptera: Noctuidae) by 75% compared to controls. No change in simple carbohydrates or secondary compounds was identified that could explain the effect. Also, Dreyer et al. (1984) found decreased levels of phenolics, including known feeding deterrents, in glyphosate-treated sorghum. The evident unpredictability of plant-herbicide-insect interactions suggests that there is a challenge in identifying integrated control treatments that will have a useful positive effect on the biocontrol agents. Therefore, when developing an integrated management strategy, it is important to establish the biochemical and physiological effects of herbicides on plants and how those affect the biocontrol insect performance.

1.10. Study Rationale and Aim

Pontederia crassipes infestations in South Africa are considered to be most serious in the high altitudes and nutrient-rich sites (Hill & Olckers 2001, Byrne et al. 2010). In view of the fact that infestations of *P. crassipes* pose serious economic, social, and ecological impacts, it is absolutely necessary to control and reduce its proliferation. Biological control is the preferred strategy of control for large infestations of *P. crassipes* (Ray & Hill 2016). Biological control agents reduce many of the more invasive qualities of *P. crassipes* by slowing vegetative growth and seed production. Nevertheless, biological control is considered slow–acting and in some areas, surface coverage remains unacceptably high. Moreover, several factors hinder the successful establishment of biological agents in South Africa, including cold winters, highly nutrient-enriched waters, hydrology of the water bodies, and the excessive use of herbicides (Hill & Coetzee 2008). In addition to biological control, a variety of other methods have been explored to reduce the invasiveness of this plant, with a variety of results (Byrne et al. 2010). Herbicidal control has been successfully used to mitigate the invasiveness of *P. crassipes* infestations in small-scale water systems, such as irrigation canals and small dams of around one hectare in size (Hill & Coetzee 2008). However, spraying plants with herbicides may indirectly destroy the insect populations by destroying their food source, which eventually leads to their death (Jadhav et al. 2008) or by directly killing them. Herbicidal control is often too expensive or difficult to apply, mainly on a large scale where repeated applications are required.

Integrated control of *P. crassipes*, combining herbicides and biological control agents, has proved to be more effective where careful herbicide application that suppresses the growth of the plant with a minimal impact on the biocontrol agents and the environment is applied (Center et al. 1999, Byrne et al. 2010). Several studies have identified the utility of keeping a population of untreated plants near herbicide-treated areas to act as a reservoir or refuge for biological control agent populations. This way, biological control agents could continue to live and reproduce during the decline and ensuing regrowth of the treated mat and recover rapidly once sufficient plant material regrows (Tipping et al. 2017, Goode et al. 2019, 2021). Other studies have proposed the use of sublethal concentrations of herbicides to minimise their negative impacts on the biocontrol agents (Jadhav et al. 2008, Katembo et al. 2013). Sublethal herbicide concentrations retard the growth of *P. crassipes* plants in terms of ramets and leaf production, which will maintain the survival and growth of biological control agents (Jadhav et al. 2008). In addition, it has been reported that sublethal concentrations of glyphosate increase the carbohydrate content in sprayed *P. crassipes* plants, making them more palatable to insect biocontrol agents (Jadhav et al. 2008). It is of paramount necessity to develop integrated management programmes where herbicides complement and do not antagonise biological control agents. This study was initiated in an attempt to develop such a system for use against *P. crassipes* in South Africa, using the latest released biological control agent, *M. scutellaris*. This insect was chosen partly because, unlike other *P. crassipes* biocontrol agents, all its life stages feed externally, therefore immatures would not be killed with the dying sprayed plant (Tipping et al. 2011).

The overall aim of this study is to develop an integrated management approach that maximises the impact of two management strategies (i.e., biological control and herbicidal control) for *P. crassipes* control and provide a more sustainable way of managing this plant and potentially improving water quality.

1.11. Objectives

The objectives of this study were to:

1. Identify a sublethal concentration of herbicide that suppresses the growth of *P. crassipes* without killing it,

2. Determine the toxicity profile of a range of herbicidal applications on the survival of *M. scutellaris*, and

3. Assess the performance and behaviour of *M. scutellaris* insects exposed to sublethal herbicide-treated plants.

4. Determine the effect of sublethal concentrations of glyphosate herbicide on the physiological properties of *P. crassipes*, and its biocontrol agent, *M. scutellaris.*

5. Monitor the effects of sublethal herbicide applications on *M. scutellaris* population densities,

6. Quantify the impact of the integration of *M. scutellaris* and sublethal herbicide on *P. crassipes* populations, and

7. Investigate dispersal patterns of *M. scutellaris* from herbicide-treated plants to untreated plants.

8. Carry out field-based studies following on the mesocosm studies where the effects of sublethal concentrations of herbicide on *P. crassipes* populations and the dispersal behaviour of *M. scutellaris* insects will be studied.

CHAPTER 2: Identifying sublethal concentrations of glyphosate-based herbicides and their effects on *Pontederia crassipes* and its biocontrol agent, *Megamelus scutellaris*

2.1. Introduction

The economic and ecological damage associated with *P. crassipes* has necessitated the implementation of extensive control measures to manage the plant, and chemical control has been the most commonly used method (Gettys et al. 2014, Goode et al. 2020). However, the high costs and unsustainable results associated with herbicides, and the risks they pose to the environment and non-target species have led to changes in management strategies that focus on long-term, safe and sustainable control of *P. crassipes* using classical biological control (Villamagna & Murphy 2010, Hill & Coetzee 2017, Coetzee et al. 2021). Nine biological control agents have been established on *P. crassipes* in South Africa (Hill & Coetzee 2017), with *M. scutellaris* successfully established at several sites where *P. crassipes* has invaded South Africa since its first release in 2013 (Miller et al. 2023). Feeding damage by *M. scutellaris* causes plant tissue death, thereby weakening the plants, reducing their growth rates, and eventually killing them (Tipping et al. 2011, Heard et al. 2014). *Megamelus scutellaris* reduces the invasive qualities of *P. crassipes,* including the reduction of plant biomass, production of ramets, and production of flowers (Goode et al. 2020). However, even though biological control is the more sustainable option, this method can be perceived to take too long, and this often results in biological control release sites being treated with herbicides by the land managers.

Herbicides have been shown to negatively affect biological control agents (Wyss & Müller-Schärer 2001, Goode et al. 2020) either by directly killing them, or indirectly by destroying their food source, which eventually leads to their death. As such, including biocontrol in integrated management strategies requires that the agents are compatible with herbicides used in the target weed (Hill et al. 2012). Hence, to achieve a successful, integrated management programme, it is important to develop a spraying regime that will cause minimal disruption to biological control agent populations (Affeld et al. 2003, Deguine et al. 2021, Das et al. 2024). Previous studies examining the effects of herbicides on insects have suggested that the use of sublethal concentrations of herbicides might be compatible with biological control. Sublethal concentrations of herbicides will retard the growth of plants without killing them, thereby preserving the habitats that will support the development of insect populations. Research on the effects of sublethal herbicide application to broom plants, *Cytisus scoparius* L. (Fabaceae) by Affeld et al. (2003) reported that glyphosate, at sublethal concentrations, was less toxic to *Arytainilla spartiophila* Foerster (Hemiptera: Psyllidae) (broom psyllid) than triclopyr and picloram but caused significant mortality at recommended field concentrations. These studies further showed that *Bruchidius villosus* Fabricius (Coleoptera: Chrysomelidae) (broom seed beetle) was more tolerant of all herbicide formulations. In the case of *P. crassipes,* a study by Kumar et al. (2008) reported that a glyphosate herbicide caused low *Neochetina* spp. weevil mortality compared to 2,4 – D at three sublethal concentrations, and at sublethal concentrations, the herbicide did not affect the virulence of the biocontrol fungus, *Alternaria alternata* to *P. crassipes*. In a similar study by Hill et al. (2012), the *P. crassipes* mirid, *E. catarinensis* was more susceptible to the toxic effects of herbicide formulations than the weevil *N. eichhorniae.* This suggests that some insect species may be more sensitive to certain herbicides than others and that, at sublethal concentrations, herbicides are less toxic to biocontrol agents than lethal concentrations.

The direct effects of herbicides on biocontrol agents of *P. crassipes*, including two *Neochetina* spp. weevils and the mirid, *E. catarinensis* have been thoroughly investigated (Haag 1986a, Jadhav et al. 2008, Hill et al. 2012), but, except for recent studies by Moran et al. (2023), there is no research on the direct and indirect toxic impacts of sublethal herbicide concentrations and formulations on *M. scutellaris*. The current study assessed the direct and indirect effects of different concentrations of glyphosate herbicides on *M. scutellaris* to determine a sublethal concentration which will be compatible with the insect. I hypothesised the following: 1. Recommended concentrations of glyphosate herbicides will increase the mortality of the insects via direct toxic effects, while concentrations below that recommended for field application may not show direct toxic effects, but possible changes in physiology may indirectly decrease survival, and 2. *Megamelus scutellaris* prefers to feed and oviposit on plants treated with sublethal concentrations of glyphosate herbicides. The objectives of this study were to:

1. Determine a sublethal concentration of herbicide that suppresses the growth and reproductive output of *P. crassipes* without killing it.
2. Determine the toxicity of a range of herbicidal applications on the survival of *M. scutellaris*.
3. Assess the behaviour of *M. scutellaris* insects exposed to sublethal herbicide-treated plants.

2.2. Materials & Methods

Integration of herbicides and biocontrol agents to control invasive weeds relies on the assumption that these two methods are compatible. This study was conducted to determine the impact of three commonly used glyphosate herbicides on a biocontrol agent *M. scutellaris* and its host plant, *P. crassipes,* at sublethal (below recommended field application rates) concentrations.

2.2.1. Herbicides

The herbicides used for these experiments were all glyphosate-based herbicides (GBHs) containing a range of active ingredients. Kilo Max (700 g a.i./L active ingredient) (ARYSTA LifeScience South Africa (Pty) Ltd), Seismic (480 g a.i./L active ingredient) (Volcano AgroScience South Africa (Pty) Ltd), and Roundup (360 g a.i./L active ingredient (Monsanto South Africa (Pty) Ltd) are registered and currently used to control *P. crassipes* in South Africa (Debbie Muir, DFFE, Pers. Comm.). The chemical classes and properties of these herbicides are provided in Table 2.1.

Table 2. 1: Herbicides used in control of weeds in South Africa assessed to verify their toxicity against a biocontrol agent, *Megamelus scutellaris* used for control of *Pontederia crassipes*.

Herbicide	**Active Ingredient**	**Chemical group**	**Mode of action (on target plants)**	**Recommended application dose for *P. crassipes***	**Dosages used (% Conc.)**	**Other target weeds**
Kilo Max	700 g a.i./kg	Glyphosate-Sodium (Na) salt	Systemic and non-selective, active when applied to the green foliage	3.1 kg/ha or 1.57 % solution	0.4,0.6,0.8,1.2,1.4, 1.6	*Typha capensis*, *Arundo donax, Acacia mearnsii*
Seismic	480 g a.i./L	Glyphosate Isopropylamine (IPA) salt	Systemic, non-selective and actively absorbed through immature bark and leaves of most plants and trees.	6 *l*/ha or 3 *l* / 100 *l* [3 % solution]	0.35,0.75,1.5,2,2.5, 3	*Panicum maximum*, *Malva parviflora*, *Solanum nigrum*
Roundup	360 g a.i./L	Glyphosate-Potassium (K) salt	Systemic, foliar absorbed, works best on lush, vigorously growing weeds which are actively translocating nutrients into roots, bulbs, rhizomes and stolons.	3 % (30 ml / 1*l* water)	0.1,0.3,0.5,1,1.5,3	*Chromolaena odorata*, *Cynodon dactylon*, *Cirsium arvense*

2.2.2. Test plants and insects

Stock cultures of *P. crassipes* and *M. scutellaris* were maintained at the Centre for Biological Control's (CBC) Waainek Mass Rearing Facility, Rhodes University, Makhanda, South Africa. A consignment of *P. crassipes* plants was collected from the Kubusi River (32º.5926 S; 27º.4218 E) near Stutterheim, South Africa, and used to cultivate plants for rearing biological control agents in 3000 L plastic pools housed in polyethylene greenhouse tunnels. Pools were supplied with a constant-release nutrient supply from two perforated plastic bottles suspended in the water column, which were replenished approximately every six months. *Megamelus scutellaris* (ex. Argentina via USDA, Fort Lauderdale) adult insects used for these experiments were collected from cultures initiated in 2008 and maintained on *P. crassipes* plants. The insects were collected into a large, clean plastic container containing freshly cut *P. crassipes* leaves using an electric aspirator. The insects were divided into groups of ten adults (1:1) sex ratio and put in plastic tubs (65 mm diameter) lined with damp filter paper one day before treatment to allow them to acclimatise. The insects were kept at 25 °C with 65% relative humidity, and 12:12 (Light: Dark) photoperiod.

2.2.3. Experimental design

Mesocosm experiments were set up in polyethylene tunnels at the Waainek Mass Rearing Facility. In each of the experiments described below, three medium-sized *P. crassipes* plants were placed in each cylindrical 25 L (52 cm diameter) plastic tub, containing 15 L of tap water supplemented with Culterra Multisol 'N' fertiliser (6N:1P:3K) (Culterra Pty Ltd) at a concentration of 10 mg N/L fertiliser, and iron chelate 0.9 g/L (Miller et al. 2020). This was done to mimic the high eutrophic conditions of South African water systems (Sutton et al. 2016). Five replicates of each treatment were arranged in a completely randomised design. Herbicide treatments consisted of applications of three glyphosate-based herbicides: Kilo Max, Seismic, and Roundup at three different concentrations each identified as Low, Medium and High.

2.2.3.1. Identifying sublethal herbicide concentrations for management of *Pontederia crassipes*

To identify the sublethal concentrations of glyphosate herbicides for *P. crassipes*, a range of herbicide concentrations were tested on the growth of *P. crassipes* plants. For this experiment, fifty tubs were divided into ten groups of five replicates. Nine of the groups were treated with different herbicide formulations and concentrations; Kilo Max (0.4%, 0.6%, 0.8%) (400 mg/L, 600 mg/L, 800 mg/L), Seismic (0.35%, 0.75%, 1.5%.) (350 mg/L, 750 mg/L, 1500 mg/L) and Roundup (0.1%, 0.3%, 0.5%) (100 mg/L, 300 mg/L, 500 mg/L), with active ingredients (g a.i./m^2) of Kilo Max (0.04, 0.06, 0.08), Seismic (0.025, 0.040, 0.108), and Roundup (0.005, 0.016, 0.027), respectively. Each herbicide treatment had five replicates, including the control treatment, that received no glyphosate spray, but was sprayed with distilled water. Plants were weighed before the start of the experiment to obtain the initial biomass. The experiments ran for six weeks, and ramet production, leaf production, maximum petiole length and the length of petiole two were measured and recorded at weekly intervals. After the end of six weeks, plants were weighed to obtain final biomass. The concentrations of each glyphosate herbicide that slowed the growth of *P. crassipes* plants without causing death were considered sublethal and were used for further experiments.

2.2.3.2. Effect of sublethal herbicide concentrations on plant quality of *Pontederia crassipes* plants

Nitrogen and carbon content were quantified to determine the effect of sublethal herbicide sprays on the quality of *P. crassipes*. These included Kilo Max (0.4%, 0.6%, 0.8%) (400 mg/L, 600 mg/L, 800 mg/L), Seismic (0.35%, 0.75%, 1.5%) (350 mg/L, 750 mg/L, 1500 mg/L) and Roundup (0.1%, 0.3%, 0.5%) (100 mg/L, 300 mg/L, 500 mg/L), and control plants that were not treated with herbicide, but with distilled water. *Pontederia crassipes* leaves were harvested from the plants and oven-dried for ~5 days. After drying, leaves were ground into a homogenous powder using a mortar and pestle, weighed, and transferred into Eppendorf tubes and labelled before being sent for analysis. Leaf samples were analysed for Carbon and Nitrogen content, and Carbon: Nitrogen ratio at the Stable Isotope Laboratory, Mammal Research Institute, University of Pretoria, South Africa. Flash EA 1112 Series coupled to a Delta V Plus stable light isotope ratio mass spectrometer via a ConFlo IV system (all equipment supplied by Thermo Fischer, Bremen, Germany) was used for these assays.

2.2.3.3. Statistical analysis

All analyses were performed using R v4.2.2 (R Development Core Team, 2022). Data were checked for normality using Shapiro-Wilk's test, and Levene's test was used to test for homogeneity. One-way-ANOVA was used to analyse the effect of different levels of concentrations of three glyphosate herbicides tested on growth parameters of *P. crassipes* measured at the end of a six-week period, and Kruskal-Wallis ANOVA was used if the data were not normally distributed. Tukey's Honest Significant Difference (HSD) post-hoc tests were carried out to compare the means. Means and standard error (SE) were determined from five independent replicates of each treatment using Bonferroni adjustment. Results were considered significant at $P < 0.05$. A Generalised Linear Model (family= gaussian, link= "log") compared the effect of Nitrogen content and C: N ratio in sprayed and unsprayed plants. The best-fit model was chosen based on collinearity and dispersion that was close to 1. Variable 'Concentration M' did not meet these requirements and was excluded from the model.

2.2.4. Toxicity of glyphosate-based herbicides on *Megamelus scutellaris*

For the development of successful and sustainable integrated management strategies for invasive weeds, it is important to evaluate the effects of herbicides on biological control agents. Therefore, I tested the toxic effects of three herbicides, Kilo Max, Seismic, and Roundup on the biological control agent, *M. scutellaris* of *P. crassipes*. The sublethal effects (survival and fecundity) of herbicides were also tested on *M. scutellaris*.

2.2.4.1. Toxicity assays

The toxicity of the three selected glyphosate-based herbicides to *M. scutellaris* was evaluated using two methods, oral and topical application, under controlled laboratory conditions. Insects were exposed to a range of herbicide concentrations to establish the dose-mortality relationship. The insects were exposed to six concentrations from concentrations equal to recommended field application rates, to concentrations causing less than 50% mortality rates. The concentrations of each herbicide included 0.4%, 0.6%, 0.8%, 1.2%, 1.4%, 1.6% (400, 600, 800, 1200, 1400, 1600 mg/L) (0.04, 0.06, 0.08, 0.12, 0.14, 0.16 g a.i./m^2) of Kilo Max, 0.35%, 0.75%, 1.5%, 2%, 2.5% to 3% (350, 750, 1500, 2000, 2500, 3000 mg/L) (0.025, 0.040, 0.108, 0.144, 0.180, 0.216 g a.i./m^2) of Seismic, and 0.1%, 0.3%, 0.5%, 1%, 1.5%, 3% (100, 300, 500, 1000, 1500, 3000 mg/L) (0.005, 0.016, 0.027, 0.054, 0.081, 0.162 g a.i./m^2) of Roundup to calculate the LC_{50} values. Each treatment of each concentration was replicated five times. Ten adult insects were placed in a plastic tub (110 mm diameter × 80 mm length, 500 ml), covered with an aerated lid (five replicates with 10 insects/tub). All herbicide dilutions were prepared in pure distilled water from a Mili-Q (≥99.9 % Sigma- Aldrich, St. Louis, MO, USA) an hour before application.

For topical application, herbicide concentrations were applied to the cuticle of the insects using a handheld atomiser spray, which produces freely atomised small uniform droplets. Insects sprayed with pure distilled water served as the control. Treated planthoppers were then transferred to the plastic tubs and covered with aerated lids and fed with fresh *P. crassipes* leaves. For the oral administration assay, herbicide solution was uniformly applied to a *P. crassipes* leaf and placed inside the plastic tub and insects were released and allowed to feed on the treated leaves. Insects fed with leaves treated with distilled water solution only were used as the control. The insects were observed for mortality every 24 hours for 168 hours (seven days). Mechanical stimuli were applied by gently touching the body of each insect upon each observation, using a thin paintbrush. *Megamelus scutellaris* that did not respond to mechanical stimuli were scored as dead.

2.2.4.2. Effect of sublethal concentrations of glyphosate herbicide on *Megamelus scutellaris* behaviour

To assess the behaviour of *M. scutellaris* exposed to sublethal/reduced herbicide-treated plants, tub experiments were set up. Fifty tubs were divided into ten groups of five replicates arranged in a completely randomised design. Nine groups were treated with different concentrations of different herbicide treatments, in three concentrations for each that were regarded as less toxic during toxicity tests: Kilo Max (0.40%, 0.60%, 0.80%) (400 mg/L, 600 mg/L, 800 mg/L), Seismic (0.35%, 0.75%, 1.50%) (350 mg/L, 750 mg/L, 1500 mg/L) and Roundup (0.10%, 0.30%, 0.50%) (100 mg/L, 300 mg/L, 500 mg/L). One group served as the control group, where plants were not treated with herbicide, but with *M. scutellaris* release. Thirty *M. scutellaris* insects (1:1 sex ratio) were released into each tub. Each experiment was then covered with nylon mesh netting to prevent the insects from escaping the experiments, and the insects were allowed to acclimatise for a week before the experiment started.

Thirty adult brachypterous planthoppers at a 1:1 female: male sex ratios were released into each tub and were allowed to feed, mate, oviposit, and develop for three weeks prior to herbicide applications. Plants with established *M. scutellaris* populations were then treated with different concentrations of herbicides as described above. Spraying was completed during daylight hours, before noon, to approximate field practices. Herbicides were applied to the plants using an atomiser spray. Insect development and mortality were recorded daily. At the end of the six-week experiment, the following insect population parameters were recorded; insect development (number of adult insects) and fecundity (number of eggs and nymphs).

2.2.4.3. Statistical analysis

All analyses were performed using R v4.2.2 (R Development Core Team, 2022). Mortality percentages were calculated for each treatment in both bioassay methods and corrected using Abbott's formula, which takes into account control mortality (Abbott 1925). The dose-response data were analysed by Generalised Linear Model, using the function 'dose. P' from the MASS library, to generate LC_{50} values for toxicity bioassay, along with slopes of the regression curves according to probit analysis (Finney 1971, Wheeler et al. 2006). Generalised linear model with Poisson distribution was used to analyse the impact of sublethal herbicide concentrations on *M. scutellaris* insect survival.

2.3. Results

2.3.1. Identifying a sublethal concentration of glyphosate herbicides for *Pontederia crassipes* plants

The influence of different concentrations of herbicides on plant growth parameters varied between the herbicide treatments. However, plants treated with higher concentrations of all three tested glyphosate herbicides lost all ramets and leaves; and died by the last week of the experiments.

Kilo Max

Plants treated with 0.40% concentration of Kilo Max herbicide produced 1.00 ± 0.135 (Mean ± SE) ramets per plant by the end of the 6-week experiment. This was significantly fewer than the ramets produced by the unsprayed control plants (2.75±0.315) (χ^2= 12.79, DF = 3, $P <$ 0.05), but more than the ramets produced by plants treated with 0.60% and 0.80%, as there were no ramets produced in either of these treatments at the end of the experiment (Figure 2.1A). Unsprayed control plants produced significantly more leaves (8.50±0.451) than all the plants treated with different Kilo Max herbicide concentrations on week 6 (χ^2= 12.95, DF = 3, P = 0.004744). Plants treated with 0.40% continued to produce green leaves. The mean number of leaves produced by plants sprayed with 0.60% and 0.80% Kilo Max was significantly lower than leaves produced by unsprayed plants and plants treated with 0.40% herbicide (Figure 2.1B). Biomass was significantly different between concentrations in Kilo Max ($F_{3,12}$ = 41.08, $P < 0.05$). The control treatment yielded significantly more biomass (270g±17.7) (Mean ± SE) than the plants treated with herbicide (Figure 2.2). There was no difference between the herbicide treatments. However, plants treated with a low concentration yielded more biomass (60.5g±17.7), than a high concentration (48.5g±17.5) and a medium concentration (27.5g±17.5) (Figure 2.2).

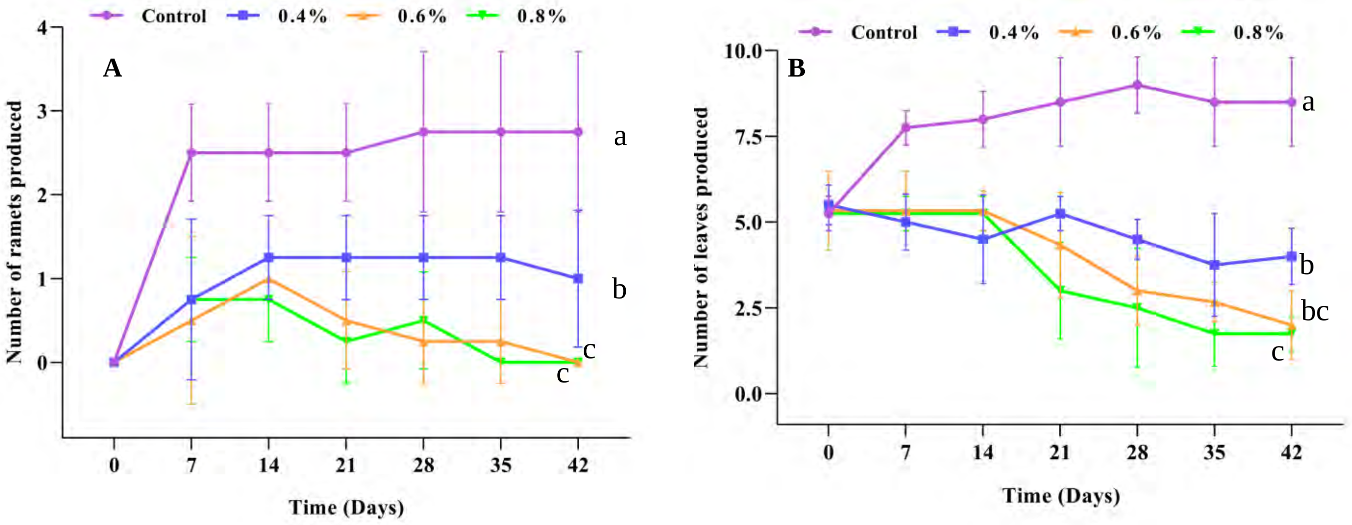

Figure 2.1: Mean number of (A) ramets and (B) leaves produced by *Pontederia crassipes* plants sprayed with different concentrations of the glyphosate herbicide, Kilo Max, at 150 L/ha. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at the end of the experiment, at $P < 0.05$.

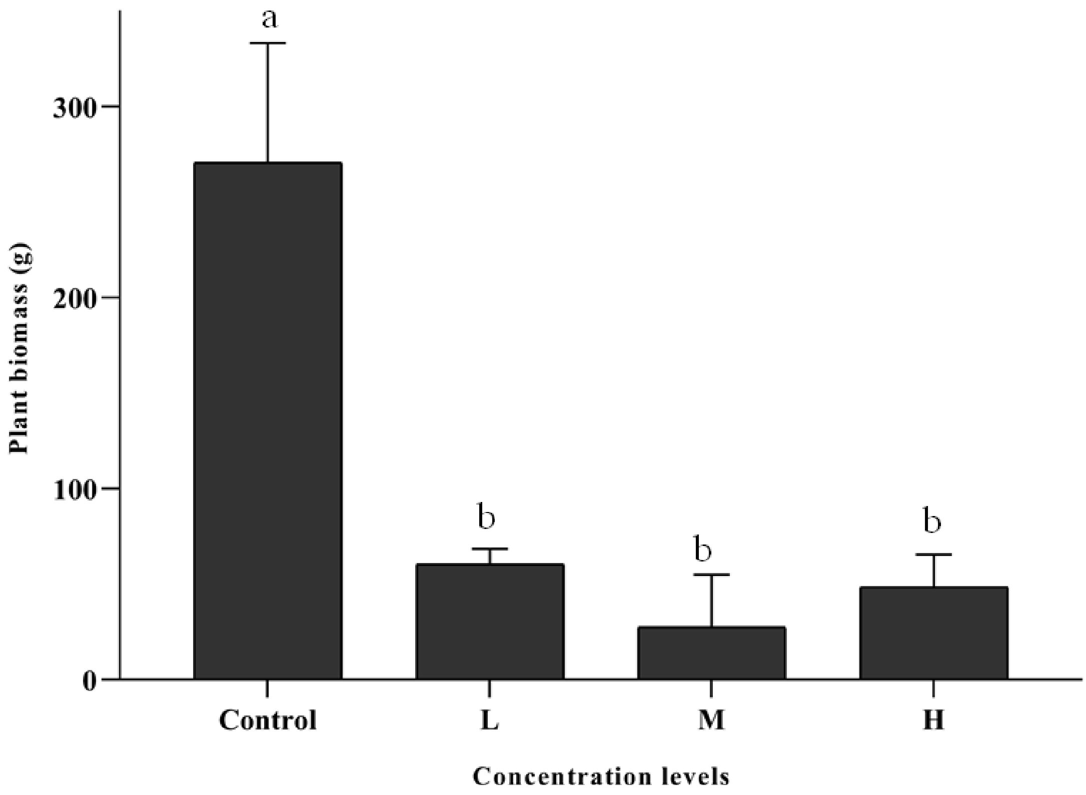

Figure 2.2: Plant biomass (g) response to Kilo Max herbicide. Mean (±SE) change in biomass of *Pontederia crassipes* when exposed to different concentration levels of the herbicide. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at $P < 0.05$.

Seismic

Significant differences were found in the production of ramets of the plants treated with different concentrations of Seismic ($F_{3,9} = 9.611$, $P = 0.00363$). However, there was no significant difference in the number of ramets produced by plants sprayed with 0.35% Seismic and unsprayed control plants. Plants treated with 1.50% concentration Seismic did not produce ramets in the final week of the experiment (Figure 2.3A), ramet production declined over the weeks as the health of the mother plant declined. Plants sprayed with 0.75% herbicide produced 1.00±0.448 ramets during week 6. This was significantly lower than ramets produced by control plants and plants treated with 0.35% herbicide (Figure 2.3A), but more than those produced by plants with 1.50% herbicide. Significant differences were observed among the leaves produced by unsprayed control plants and plants treated with different concentrations of Seismic herbicide ($F_{3,9} = 61.06$, $P < 0.05$) (Figure 2.3B). A mean of 3.33 ± 0.484 leaves was produced by plants treated with 0.75% Seismic herbicide by the end of the experiment. This was lower than leaves produced by plants sprayed with 0.35% (4.67±0.419), although not significantly different (Figure 2.3B). No leaves were produced on plants treated with 1.5% of Seismic herbicide in the last week of the experiment. Significant differences were observed in plant biomass between Seismic concentration treatments ($F_{3,12} = 3.63$, $P = 0.045$). Control plants yielded significantly more biomass (270.8g±41) than the plants treated with high herbicide concentration treatment (88.8g±41) (Figure 2.4). Plant biomass of the plants treated with low and medium concentrations of Seismic herbicide was not significantly different from the control plants or plants treated with high concentrations (Figure 2.4).

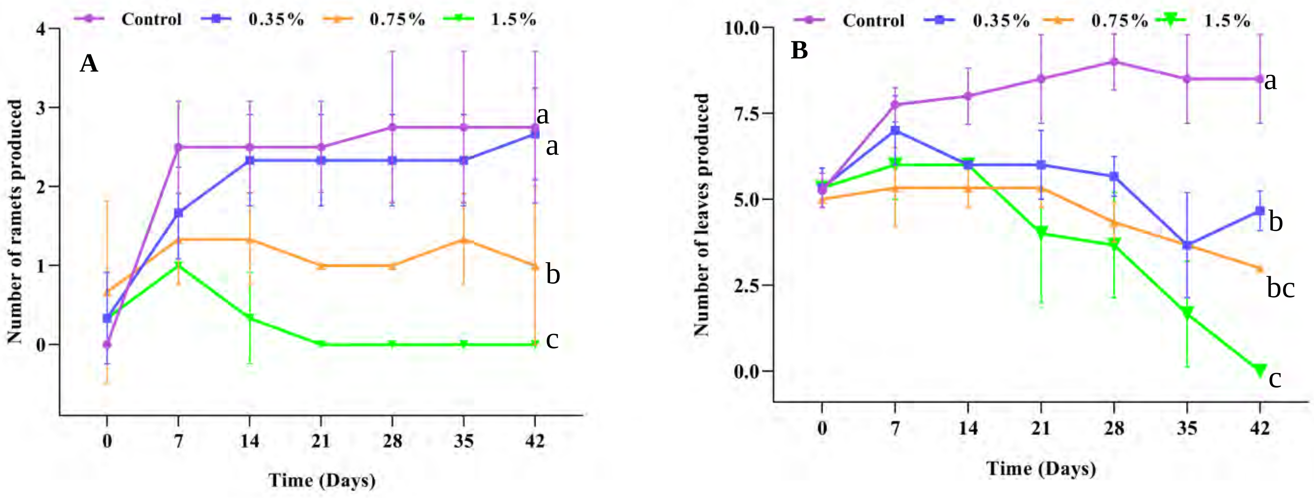

Figure 2.3: Mean number of (A) ramets and (B) leaves produced by *Pontederia crassipes* plants sprayed with different concentrations of the glyphosate herbicide, Seismic, at 150 L/ha. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at the end of the experiment, at $P < 0.05$.

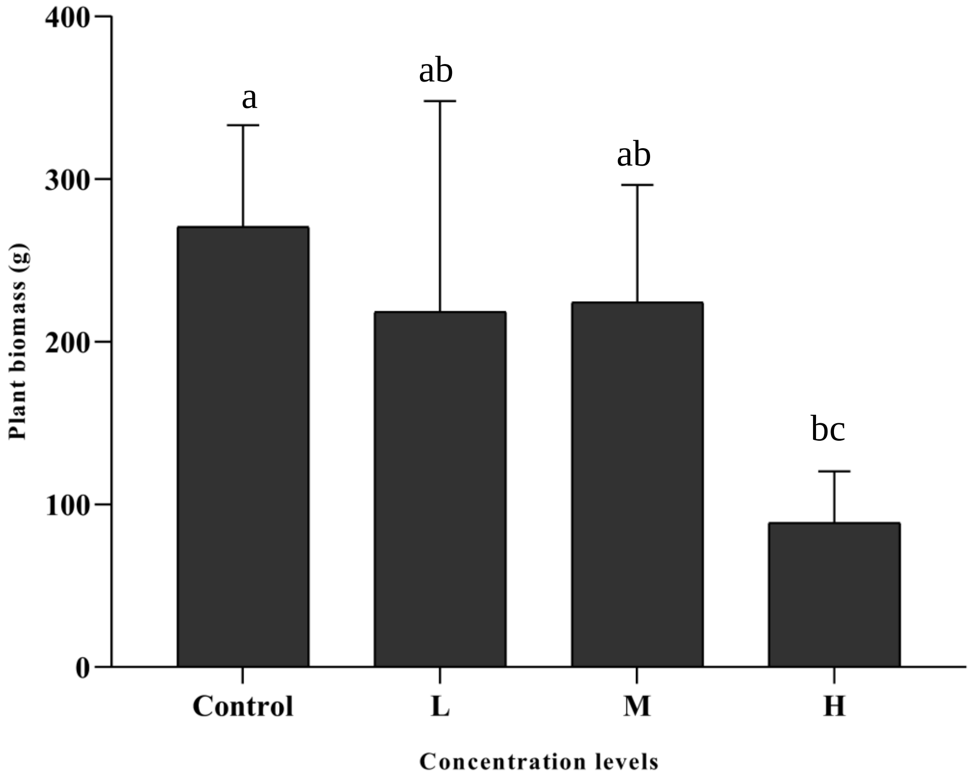

Figure 2.4: Plant biomass (g) response to Seismic herbicide. Mean (±SE) change in biomass of *Pontederia crassipes* when exposed to different concentration levels of the herbicide. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at $P < 0.05$.

Roundup

Significantly more ramets ($F_{3,12} = 10.19$, $P = 0.00128$) and leaves ($\chi^2 = 13.467$, DF = 3, $P = 0.003727$) were produced by control plants than the plants treated with 0.10%, 0.30% and 0.50% Roundup (Figure 2.5A & B). By week 6, plants sprayed with 0.5% Roundup did not produce ramets, as they were dying as a result of the herbicide. Plants treated with 0.10% and 0.30% concentrations of herbicide produced 2.00 ± 0.375 and, 1.00 ± 0.375 ramets, respectively, which is significantly lower than the ramets produced by the control plants (2.75 ± 0.375) (Figure 2.5A). By week 6, control plants and plants treated with 0.10% and 0.30% Roundup still had green leaves. Plants treated with 0.50% gradually wilted and by week 6 had lost all leaves and died. Plants treated with 0.30% continued to produce leaves until the experiment ended. Fresh weight biomass at the end of experiments was not significantly different amongst all the treatments, including the control ($F_{3,12} = 2.45$, $P = 0.114$). Although not significant, the unsprayed control treatment had more biomass (270g±47.3), followed by the medium (160g±47.3) and low concentration (116±47.3). High-concentration treatment of Roundup yielded less biomass (112g±47.3) than other treatments.

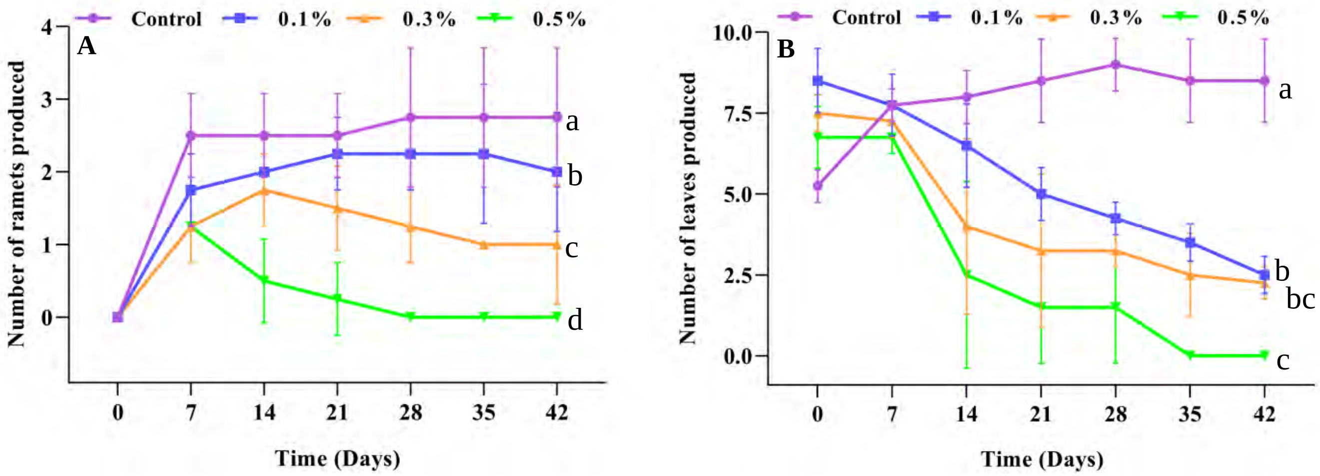

Figure 2.5: Mean number of (A) ramets and (B) leaves produced by *Pontederia crassipes* plants sprayed with different concentrations of the glyphosate herbicide, Roundup, at 150 L/ha. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at $P < 0.05$.

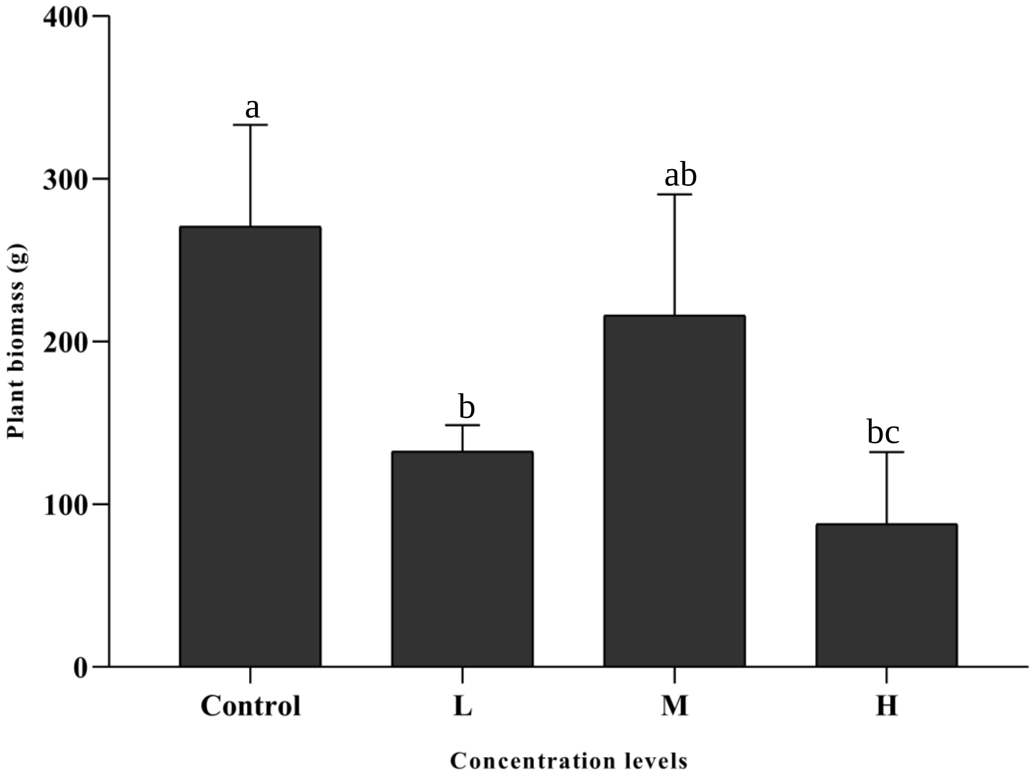

Figure 2.6: Plant biomass (g) response to Roundup herbicide. Mean (±SE) change in biomass of *Pontederia crassipes* when exposed to different concentration levels of the herbicide. Error bars = standard error of the mean, n = 5. Different letters indicate significant differences at $P < 0.05$.

2.3.2. Effect of sublethal concentrations of herbicides on *Pontederia crassipes* plant quality

The level of plant nutrients, nitrogen levels and carbon-to-nitrogen ratio were used to determine plant quality in the treated plants after sublethal herbicide applications. High N levels and lower C: N are indicators of good quality plants while higher C: N and lower N indicate poor plant quality. Significant differences were observed in the C:N ratio between herbicide treatments ($\chi^2 = 15.61$, DF = 2, $P = 0.00040$) and concentration levels ($\chi^2 = 224.33$, DF = 3, $P < 0.001$). Significantly lower C:N ratio was observed on plants treated with the herbicide Kilo Max on all concentration levels with 17.1±0.484, 17.0±0.484, and 18.7±0.484 C:N ratios (High, Medium, and Low concentrations), respectively, compared to other treatments (Figure 2.7). Plants sprayed with low concentrations of Seismic had significantly higher C:N ratio (21.5±0.52) than the other treatments, except for the control plants (25.8±0.69). All the herbicide treatments significantly influenced plant quality, with Kilo Max having the lowest C:N ratio (Figure 2.7). Interactions between the low concentrations and herbicide Roundup and Kilo Max significantly influenced the C:N ratio on plants (Table 2.2). High concentrations of herbicides did not have a significant influence on the C:N ratio. Owing to collinearity, medium concentrations were removed from the model.

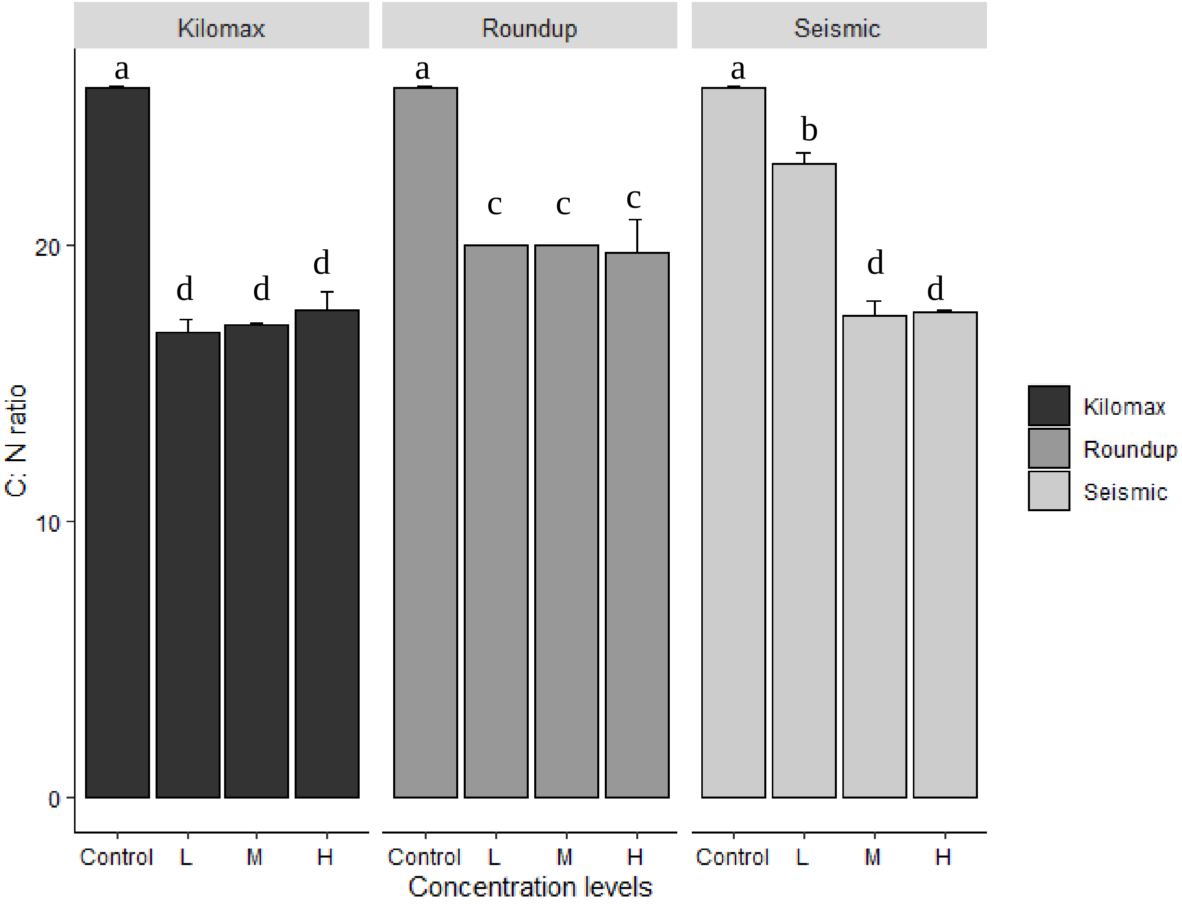

Figure 2.7: Effect of different glyphosate herbicides (150 L/ha spray volume) on C:N ratio of *Pontederia crassipes* four weeks after spraying. Error bars represent standard error of the mean. Means with different letters are significantly different from each other $P < 0.05$.

Table 2.2: GLM results showing the influence of herbicide treatments, concentration levels and their interactions on the plant quality (C:N ratio) of *Pontederia crassipes*. Bold values indicate significant differences in C:N ratios.

Coefficients:	**Estimate**	**Std Error**	**t value**	***P***
Intercept	**25.7584**	**0.1600**	**160.960**	**<2e-16**
Herbicide Kilo Max	**-8.6067**	**0.2263**	**-38.030**	**<2e-16**
Herbicide Roundup	**-5.7594**	**0.2263**	**-25.448**	**<2e-16**

Herbicide Seismic	**-8.2964**	**0.2263**	**-36.658**	**<2e-16**
Concentration H	0.1046	0.2263	0.462	0.646
Concentration L	**5.5376**	**0.2263**	**24.468**	**<2e-16**
Herbicide Kilo Max: Concentration H	0.4099	0.3201	1.281	0.205
Herbicide Roundup: Concentration H	-0.3264	0.3201	-1.020	0.311
Herbicide Kilo Max: Concentration L	**-5.8628**	**0.3201**	**-18.318**	**<2e-16**
Herbicide Roundup: Concentration L	**-5.5376**	**0.3201**	**-17.302**	**<2e-16**
AIC: 111.52 **Dispersion: 0.204876**				

Plant nitrogen levels were influenced by herbicide treatments ($\chi^2 = 21.99$, DF = 2, $P < 0.001$) and concentration levels ($\chi^2 = 43.36$, DF = 3, $P < 0.001$) (Table 2.3). There were significantly higher levels of nitrogen content from the leaves of the plants sprayed with the low concentration Kilo Max herbicide (2.89%±0.85) than the control unsprayed plants (1.92%±0.69) (Figure 2.8); spraying with low concentrations of Kilo Max herbicide increased nitrogen content in the leaves of treated plants (Figure 2.8). There were no significant differences between the nitrogen content of the plants treated with low concentrations of Roundup (2.08%±0.72) and Seismic (1.79%±0.68) herbicides and control plants (Figure 2.8).

Nitrogen content was significantly influenced by all the herbicide treatments (Table 2.3). Interactions between the Roundup with low and high concentrations had a significant influence on the nitrogen levels. High concentration levels alone did not have any influence on the nitrogen levels (Table 2.3). High concentrations of Kilo Max did not influence nitrogen levels, but low concentrations resulted in increased nitrogen content (Figure 2.8; Table 2.3). Contrary to the reduced C:N ratio in Kilo Max treated plants, N levels were significantly increased in herbicide-treated plants (Figure 2.7; Figure 2.8). Lowest C:N ratio and the highest

N levels were observed on the plants treated with lowest (sublethal) concentrations of Kilo Max (0.40%) than all the other treatments.

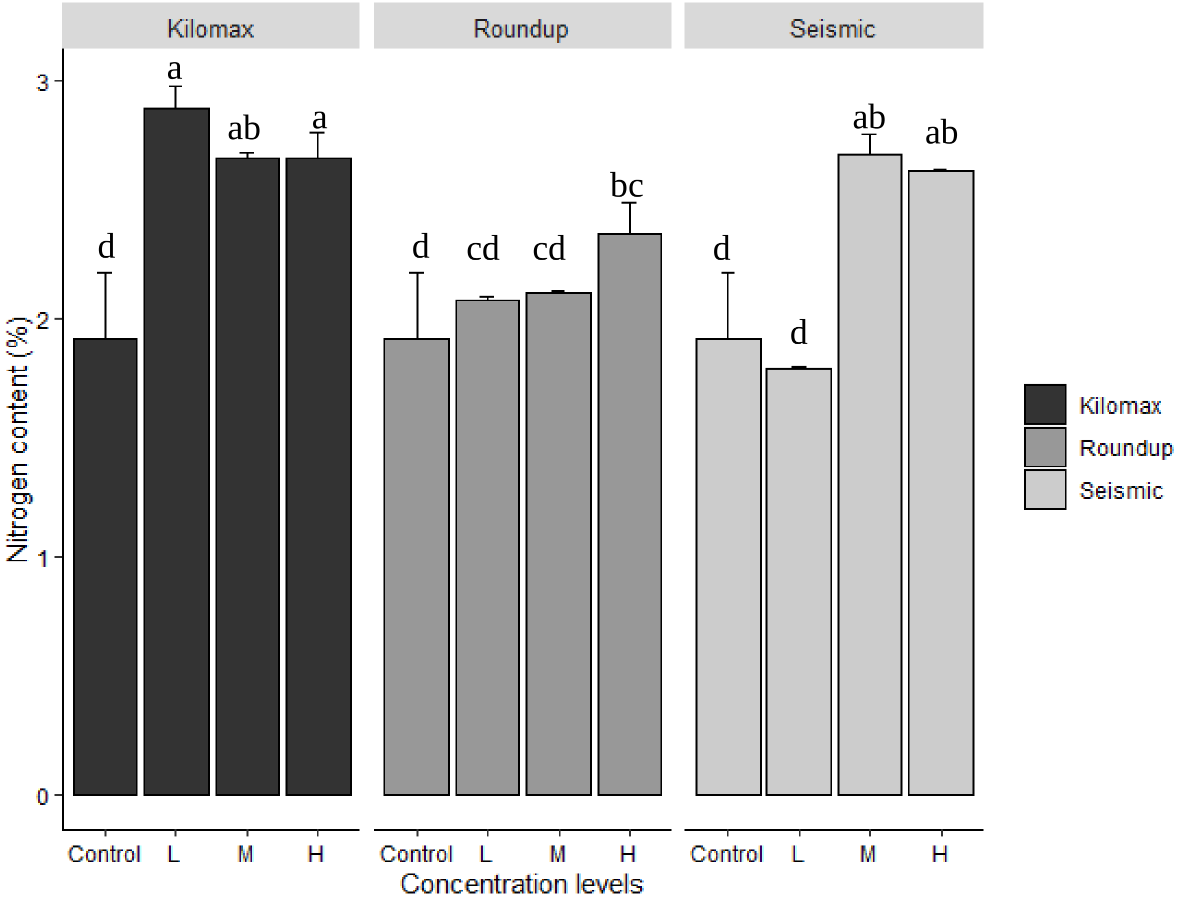

Figure 2.8: Nitrogen levels of *Pontederia crassipes* plant parts four weeks after spraying with different concentration levels of glyphosate herbicides (150 L/ha spray volume). Error bars represent the standard error of the mean. Means with different letters are significantly different from each other $P < 0.05$.

Table 2.3: GLM results illustrating the effect of glyphosate herbicides and herbicide concentration levels and their interactions on *Pontederia crassipes* plant quality (Nitrogen). Values in bold indicate significant differences in mean plant nitrogen.

Coefficients:	**Estimate**	**Std Error**	**t value**	***P***
Intercept	**0.64972**	**0.01913**	**33.958**	**< 2e-16**
Herbicide Kilo Max	**0.33610**	**0.02352**	**14.292**	**< 2e-16**
Herbicide Roundup	**0.09697**	**0.02584**	**3.753**	**0.000357**
Herbicide Seismic	**0.34168**	**0.02347**	**14.557**	**< 2e-16**

Concentration H	-0.02822	0.01950	-1.447	0.152342
Concentration L	**-0.40918**	**0.02457**	**-16.652**	**< 2e-16**
Herbicide Kilo Max: Concentration H	0.02636	0.02748	0.959	0.340737
Herbicide Roundup: Concentration H	**0.14020**	**0.03038**	**4.615**	**1.73e-05**
Herbicide Kilo Max: Concentration L	**0.48462**	**0.03085**	**15.711**	**< 2e-16**
Herbicide Roundup: Concentration L	**0.39486**	**0.03487**		**< 2e-16**
AIC: -124.35 **Dispersion: 0.01074**				

2.3.3. Toxicity of glyphosate-based herbicides on *Megamelus scutellaris*

The results of toxicity bioassays of formulated glyphosate herbicides, Kilo Max, Seismic, and Roundup, are summarised in Table 2.4 and Figures 2.9A, B &C for both the topical and oral application methods. *Megamelus scutellaris* responded differently to different herbicide formulations. Kilo Max showed less toxicity to the insects for both oral and topical applications, with LC_{50}s 13.8 g/L and 6.3 g/L, respectively, at 24 h. Roundup at 24 h also showed less toxicity (LC_{50} = 12.5 g/L) to the insects for oral application. Seismic was less toxic to *M. scutellaris* adults at 120 h (5 days) than either Kilo Max and Roundup with LC_{50}s 4.8 g/L orally and 1.6 g/L topically (Table 2.4). At 168 hours (7 days), insect mortality increased in all the herbicide treatments, more specifically for the topical application. However, Roundup proved to be the most toxic of the three herbicides after a week, both for oral (LC_{50} = 0.3 g/L) and topical (LC_{50} = 0.1 g/L) applications (Table 2.4 and Figure 2.9C). Kilo Max herbicide toxicity, even though lower than Roundup, increased with time, with low LC_{50} values at 168 h on both oral (1.1 g/L) and topical (0.6 g/L) application techniques (Table 2.4, Figure 2.9A). This was more toxic than Seismic with LC_{50} values of 1.7 g/L and 1.1 g/L, both for oral and topical application, respectively (Figure 2.9B). The mortality of *M. scutellaris* feeding on sprayed leaves was generally lower than the topically sprayed insects, meaning that the feeding method (oral) was less detrimental to insects. The mortality of insects increased with increasing herbicide concentrations and exposure time.

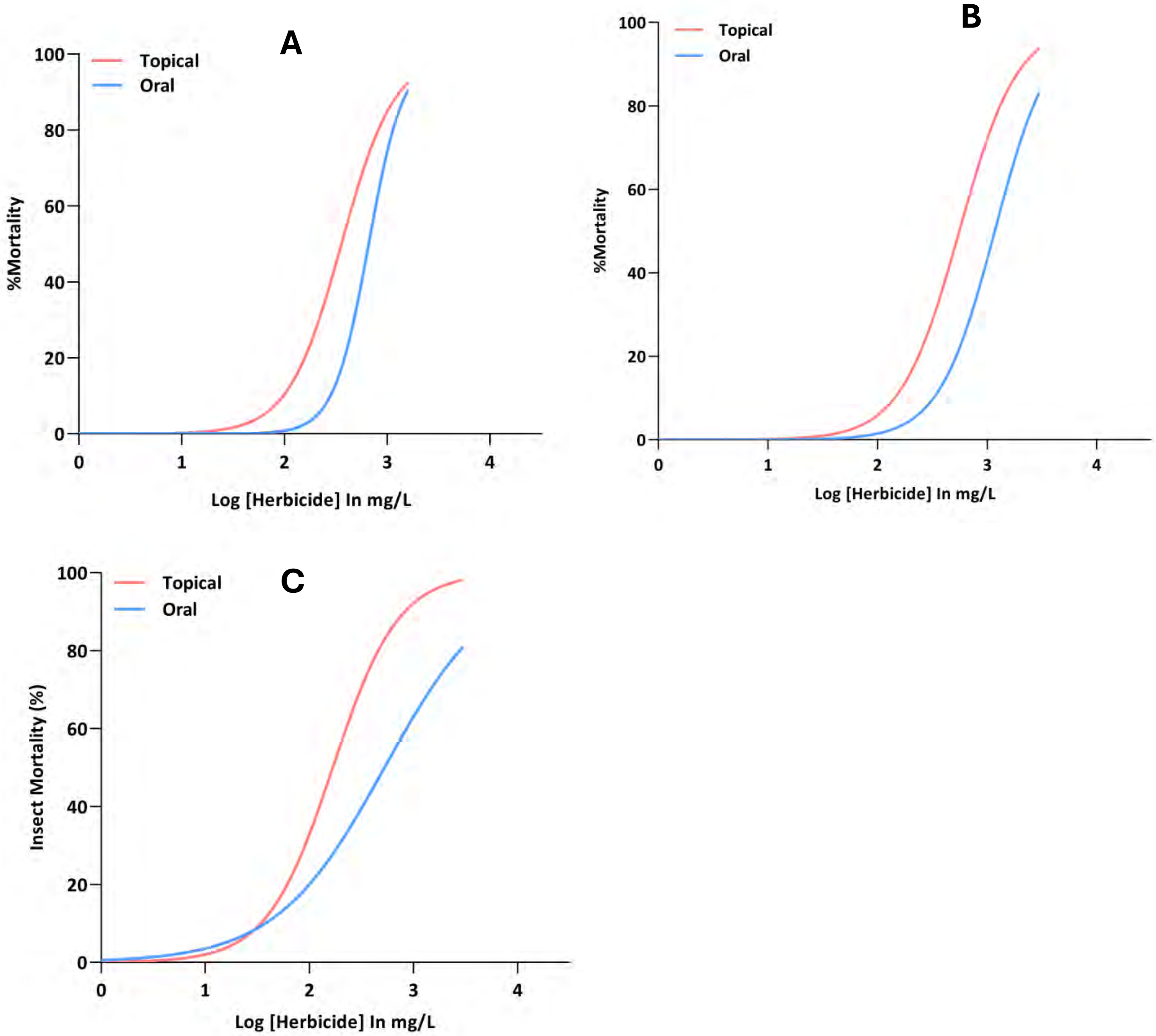

Figure 2. 9: Dose-response curve of *Megamelus scutellaris* insects to glyphosate-based herbicides, A. Kilo Max, B. Seismic and C. Roundup, exposed by direct contact (topical) and feeding on a sprayed leaf (oral).

Table 2.4: LC$_{50}$ values of *Megamelus scutellaris* after exposing adults to glyphosate-based herbicides (Kilo Max, Seismic, and Roundup) for 24h, 120h and 168h by topical and oral application at a range of concentrations to determine their dose-response mortality.

Herbicide	**[a]LC$_{50}$ with fiducial limits [b](χ^2±SE)**		**[a]LC$_{50}$ with fiducial limits [b](χ^2±SE)**		**[a]LC$_{50}$ with fiducial limits [b](χ^2±SE)**	
	24h		120h		168h	
	Topical	Oral	Topical	Oral	Topical	Oral
Kilo Max	6.3(3.00-4.21) χ^2=34.6±1.87	13.8(3.48-15.77) χ^2=36.5±1.49	1.1(0.83-2.02) χ^2=40.9±1.14	2.99(2.70-6.29) χ^2=36.3±1.49	0.6(0.30-0.72) χ^2=19.6±1.16	1.1(0.94-1.45) χ^2=18.1±1.11
Seismic	4.8(3.30-5.40) χ^2=45.6±1.23	4.1(3.34-7.12) χ^2=10.7±1.16	1.6(1.22-2.26) χ^2=29.8±1.15	4.8(3.10-12,0) χ^2=31.8±1.33	1.1(0.30-2.28) χ^2=14.4±1.14	1.7(1.02-3.51) χ^2= 16.8±1.11
Roundup	1.4(1.14-1.64) χ^2=68.3±1.06	12.5(5.76-10.90) χ^2=31.9±1.91	0.6(0.10-1.03) χ^2=83.5±1.23	1.7(1.44-1.95) χ^2=39.9±1.06	0.1(0.10-0.15) χ^2=15.9±1.27	0.3(0.23-0.46) χ^2=15.9±1.27

[a] Concentration causing 50% (LC$_{50}$) mortality of insects after 24, 120 and 168 hours.

[b] Chi-square ± Standard Error of the concentration-mortality regression line.

Megamelus scutellaris insects were more susceptible to glyphosate herbicides when applied topically than when they were feeding on the herbicide-sprayed leaves (χ^2= 29.36, DF = 1, $P <$ 0.001). This was consistent for all the herbicide formulations (Figures 2.12, 2.13, 2.14). *Megamelus scutellaris* exhibited different susceptibility to the tested glyphosate herbicides. The mortality results confirmed that Roundup was the herbicide most toxic to *M. scutellaris,* with a mean mortality of 72.2%± 5.94, followed by Seismic with a mean mortality of 54%±5.94. Although there was no significant difference to Seismic, Kilo Max was the herbicide least toxic to *M. scutellaris,* causing a mean mortality of 52±5.94. At the recommended application rate (3%), Roundup killed 100%±7.02 of the sprayed insects and the insects that ingested the treated leaves by day 7 (Figure 2.14). Kilo Max and Seismic herbicides caused 78%±3.75 and 87%±5.80 mortality, respectively, when sprayed directly to the insects, using recommended label doses. These herbicides caused slightly lower insect death when the insects ingested the sprayed leaf material, 62%±3.75 (Kilo Max) and 69%±5.80 (Seismic) (Figure 2.12 and 2.13, respectively). This shows that spraying herbicides directly onto the insects has more deleterious impacts on insect survival than when they feed on the herbicide-treated leaves. *Megamelus scutellaris* was more tolerant of Kilo Max and Seismic, particularly at lower concentrations. Insect mortality of 22%±14.56 for the control treatment, for both direct topical and indirect oral treatment, was observed after seven days of the experiment.

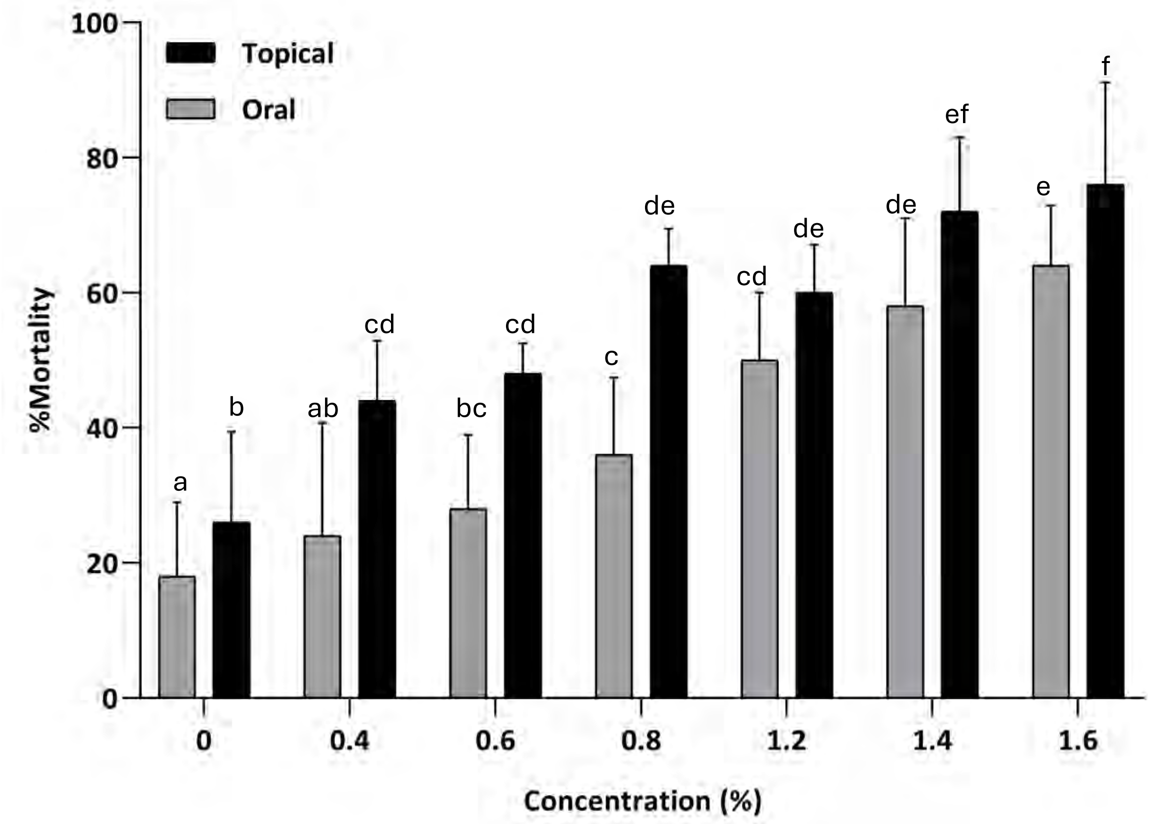

Figure 2.10: Mean mortality of *Megamelus scutellaris* adult insects exposed to a range a of Kilo Max herbicide concentrations via topical and oral applications after 168 hours (7 days). Error bars represent standard error of means (n = 10). Means with different letters are significantly different from each other $P < 0.05$.

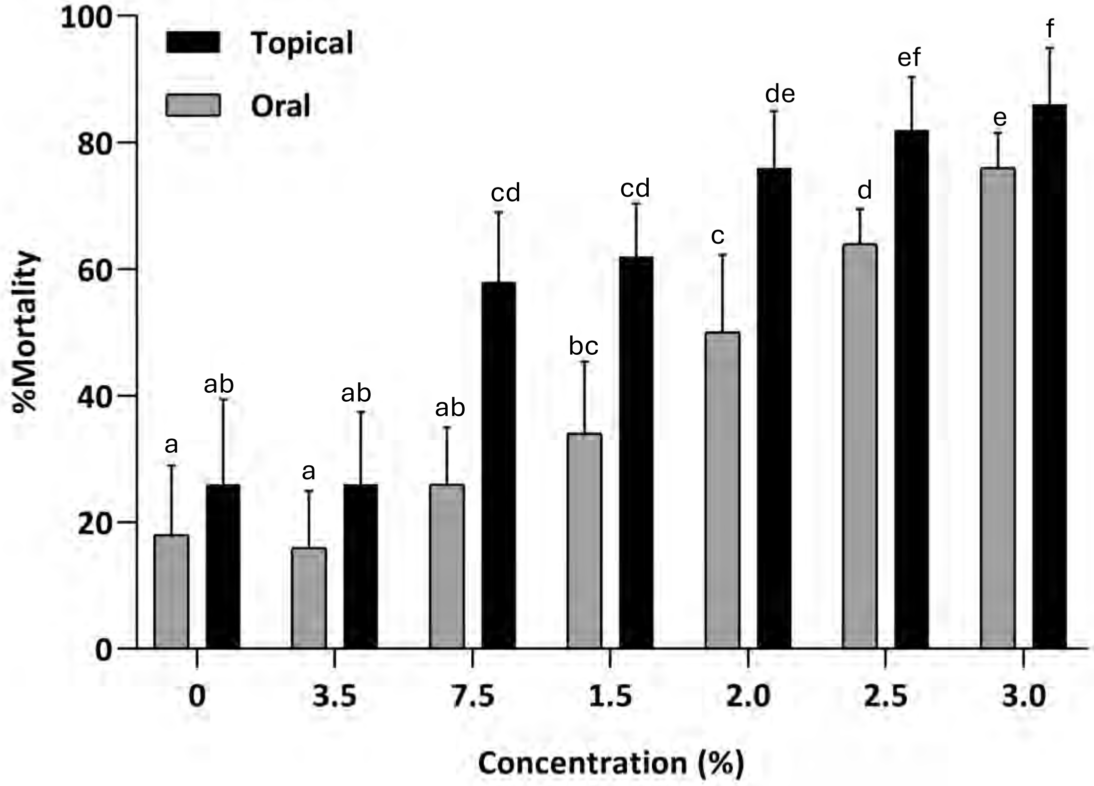

Figure 2.11: Mean mortality of *Megamelus scutellaris* adult insects exposed to a range of Seismic herbicide concentrations via topical and oral application after 168 hours (7 days). Error bars represent standard error of means (n = 10). Means with different letters are significantly different from each other *P* < 0.05.

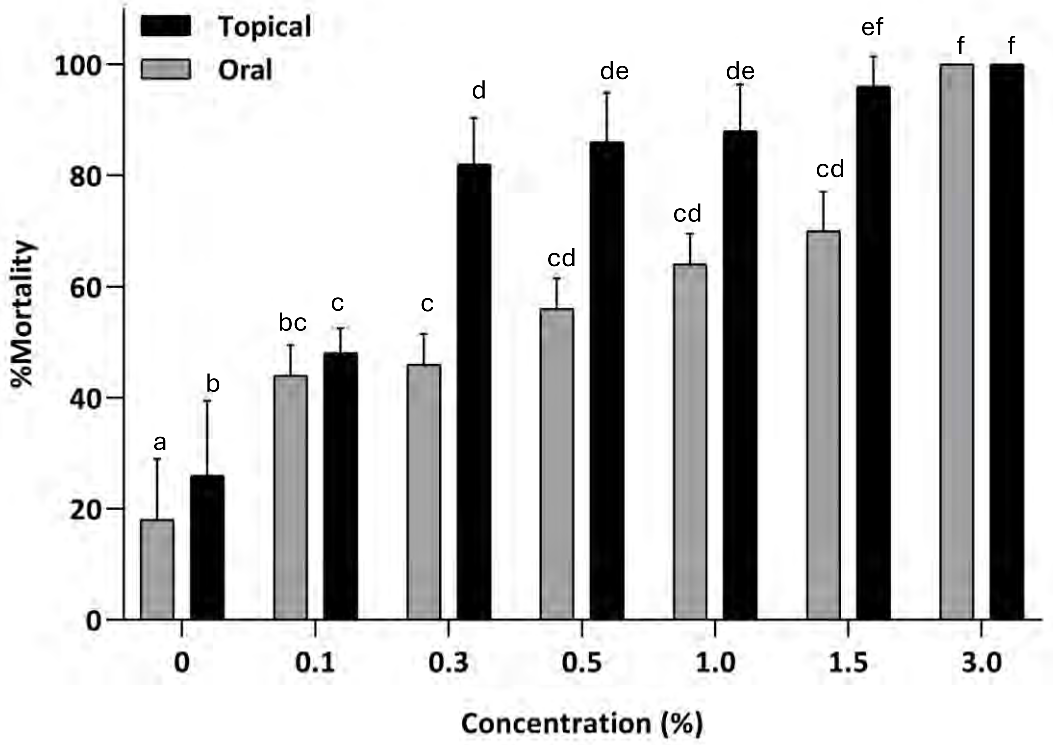

Figure 2.12: Mean mortality of *Megamelus scutellaris* adult insects exposed to a range of Roundup herbicide concentrations via topical and oral application after 168 hours (7 days). Error bars represent standard error of means (n = 10). Means with different letters are significantly different from each other $P < 0.05$.

During this study, none of the herbicides tested was regarded as safe for use at the recommended dose for *M. scutellaris* based on the insect mortality and LC_{50} values calculated at the recommended doses of each herbicide. Therefore, concentrations lower than recommended concentrations were used for the following experiments.

2.3.4. Insect behaviour following exposure to sublethal herbicide concentrations

The different concentration levels significantly influenced biocontrol agent densities (χ^2 = 42.25, DF = 3, $P < 0.001$), but herbicide treatments did not have a significant impact on insect densities (χ^2 = 0.973, DF = 2, $P = 0.614$). There were significant differences in the number of insects recorded in each herbicide treatment and concentration levels (Figure 2.15). High insect populations (436/tub±65.1) were recovered from plants treated with the lowest concentration (0.40%) of Kilo Max. Roundup had the lowest insect densities across concentration treatments, but the lowest densities (143/tub±44.1) were recorded at the high concentrations. Insects recovered from the Seismic treatment were high (351/tub±44.1) at the lowest concentration (0.35%), and low densities (150/tub±44.1) were recorded at the high concentration (1.50%). Unsprayed control plants yielded lower numbers of biocontrol insects (95/tub±59.2) than the treated plants, but these were not significantly different from the high Roundup and Seismic concentrations. Even though there were high insect densities (235/tub±44.1) recorded from the high Kilo Max concentration (0.80%), they were still significantly lower than those recorded at low concentrations (Figure 2.15). Applying glyphosate herbicides at higher concentrations reduced the biomass of *P. crassipes*, which resulted in the rapid death of biocontrol agents.

The generalised linear model (GLM) results confirmed that all herbicide applications significantly influenced the density of biocontrol insects. High and low concentrations of the herbicides also significantly affected the insect populations, medium concentrations were excluded from the model due to collinearity (Table 2.5). There were significant interactions between low herbicide concentrations and herbicides Kilo Max and Roundup. Interactions between herbicide Roundup and high concentrations also significantly influenced *M. scutellaris* populations. According to the results of this study, lower concentration levels did not have any detrimental effects on *M. scutellaris* and could be boosting their populations. Glyphosate-based herbicides obstruct amino acid synthesis, therefore low concentrations could potentially increase the availability of nitrogen to *M. scutellaris* feeding on treated plants, which in turn, supports increases in insect populations.

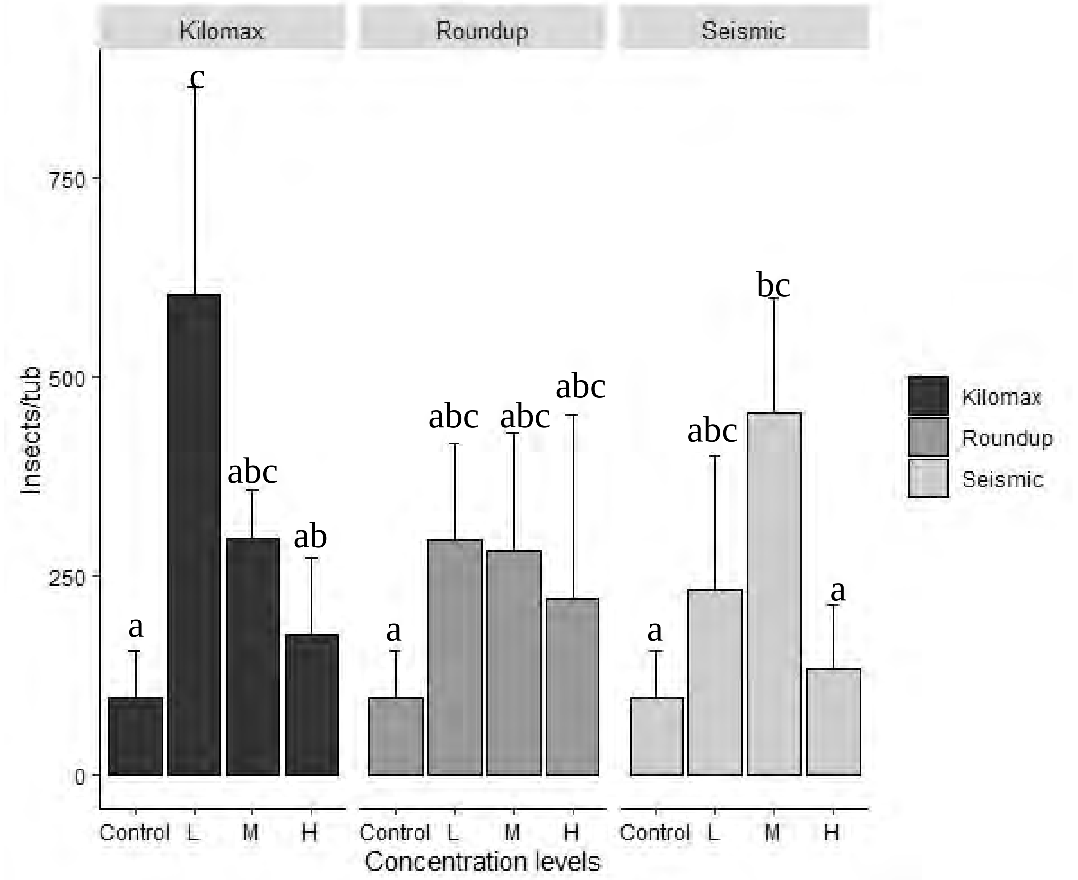

Figure 2.13: Populations of *Megamelus scutellaris* harvested from *Pontederia crassipes* plants in three herbicide treatments with three concentration treatments (Control, L =Low, M = Medium, and H= High). GLM was used to compare means between treatments; error bars represent standard error of means (n = 5), and different letters represent significantly different means.

Table 2.5: Generalised Linear Modelling for *Megamelus scutellaris* insect population response to herbicide treatments, concentration levels and their interactions. Values in bold represent significant differences at $P < 0.05$.

Coefficients:	**Estimate**	**Std Error**	**z value**	***P***
Intercept	**4.5617**	**0.1830**	**24.928**	**< 2e-16**
Herbicide Kilo Max	**1.1303**	**0.2571**	**4.397**	**1.10e-05**
Herbicide Roundup	**1.0784**	**0.2571**	**4.194**	**2.74e-05**
Herbicide Seismic	**1.5591**	**0.2568**	**6.071**	**1.27e-09**
Concentration H	**-1.2305**	**0.2561**	**-4.805**	**1.55e-06**
Concentration L	**-0.6752**	**0.2553**	**-2.645**	**0.008176**
Herbicide Kilo Max: concentration H	0.7032	0.3620	1.942	0.052089
Herbicide Roundup: concentration H	**0.9885**	**0.3619**	**2.732**	**0.006301**
Herbicide Kilo Max: concentration L	**1.3859**	**0.3608**	**3.841**	**0.000122**
Herbicide Roundup: concentration L	**0.7237**	**0.3611**	**2.004**	**0.045059**
Log likelihood: -985.499 **AIC: 1007.5** **Dispersion: 1**				

2.4. Discussion

The use of retardant/ sublethal concentrations of herbicide that complement biocontrol agents has been suggested to address the reliability of integrating herbicides and biocontrol agents for enhanced *P. crassipes* control (Ueckermann & Hill 2001, Byrne et al. 2010, Hill et al. 2012). Results from the present study showed that plants subjected to low concentration levels of the tested glyphosate herbicides were smaller and shorter, with lower ramet and leaf production, than control plants sprayed with pure distilled water. The loss of vigour and reduced reproduction of plants is attributed to herbicide-induced stress as glyphosate prevents plants from making proteins that are necessary for plant growth. After glyphosate enters the plant system, it is translocated to young developing tissues, where it can accumulate and hinder plant growth (Feng et al. 1999, Kanissery et al. 2019). Glyphosate inhibits the production of tryptophan, an amino acid that restricts the synthesis of the important growth stimulator, indole acetic acid (IAA) (Yamada et al. 2009), which can explain the reduced plant growth. Observed growth effects were generally similar to those reported earlier by Jadhav et al. (2008), as the treatments with sublethal concentrations of Roundup produced significantly fewer ramets and leaves than the unsprayed plants. Similarly, Van & Center (1994) showed that Paclobutrazol, a plant growth retardant reduced plant size, canopy height and daughter plants of *P. crassipes*. The current study determined that reduced/sublethal herbicide concentrations slow down aspects of *P. crassipes* production, which may then result in damaging numbers of biocontrol agents.

Application of glyphosate caused an increase in nitrogen (N) content, more so on the leaves treated with low Kilo Max (0.4%) concentration, which resulted in a decrease in the C:N ratio. A low C:N ratio and high N content in plants often results in high food quality for insect herbivores. Plant nutritional quality (i.e., nitrogen content) is crucial to insect herbivore survival and fecundity (Awmack & Leather 2002, Han et al. 2019). Leaf N content can be an indicator of host plant quality for phytophagous insects (Mattson 1980, Awmack & Leather 2002, Sarfraz et al. 2009). The high N content and low C:N ratio of the leaves may explain the high number of insects found in the plants treated with low glyphosate concentrations. *Megamelus scutellaris* insects feed and oviposit at the base of the adaxial and abaxial leaf surfaces and petiole lamina of *P. crassipes* plants (Tipping et al. 2014b). The reduced C:N ratios in the leaves often suggest lower structural compounds like cellulose and lignin, making the leaves softer and more palatable to the insects. These results agree with previous studies that found that nitrogen was more concentrated in *P. crassipes* leaves than in other parts of the plants, like the petioles and crowns of the plant, after exposure to sublethal herbicide concentrations (Spencer & Ksander 2004, Katembo et al. 2013). This is exhibited by the large numbers of insects found amassed in the leaf area of the treated plants. The distribution of *M. scutellaris* on *P. crassipes* plants may be related to their nutritional requirements (i.e., nitrogen-rich feeding sites).

In weed control systems, applications of herbicides are not expected to be toxic to biocontrol insects since their active ingredients are formulated to act only on plant pathways. However, it has been reported that herbicides may affect biocontrol agents directly by penetrating their cuticles or indirectly by eliminating their host plants (i.e., food source) (Kraus & Stout 2019). One of the objectives of this study was to determine if applications of three glyphosate-based herbicides caused mortality in *M. scutellaris* adults that were exposed to both topical and oral application. The results showed that high concentrations of herbicides tested increased mortality of *M. scutellaris* when applied topically, more so at maximum recommended label rates. However, insect mortality caused by the lowest concentrations of the tested herbicides did not significantly differ from the control treatment, where the insects were topically sprayed with pure distilled water. *Megamelus scutellaris* adults experienced reduced mortality when they ingested *P. crassipes* leaves sprayed with herbicides compared to direct topical treatment. This is an exception for the *M. scutellaris* adults that fed on leaves treated with Roundup herbicide at a recommended dose, which experienced 100% mortality. Similar findings were reported by Kraus and Stout (2019), where adult rice water weevils, *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae) directly exposed to herbicides 2,4 – D, Bolero, Propanil, and Ricebeaux experienced high mortality; while those fed leaf material that had been exposed to herbicides did not. Studies by Hill et al. (2012) also showed that the herbicides with 2,4-D and diquat as active ingredients were highly toxic to the *P. crassipes* weevil, *N. eichhorniae,* and the mirid, *E. catarinensis,* via direct topical contact. Ahn et al. (2001) reported that direct application of the herbicide glufosinate-ammonium caused significant mortality to the nymphal and adult stages of mite species *Tetranychus urticae* Koch (Acari: Tetranychidae), *Amblyseius womersleyi* Schicha (Acari: Phytoseiidae) and *Phytoseiulus persimili*s Athias-Henriot (Acari: Phytoseiidae) under laboratory conditions. The current study is one of the few studies that has simultaneously examined the direct and indirect impacts of herbicides on biocontrol insects of *P. crassipes*. The feeding assays with herbicide-treated leaves better represent the type of exposure that *M. scutellaris* is subjected to in field settings. The direct contact exposure of *M. scutellaris* to herbicides is unlikely in field settings as these insects inhabit the undersides of *P. crassipes* leaves and petioles and are also capable of dispersing from disturbed plants by jumping and flying. However, populations of *M. scutellaris* may be negatively impacted by herbicides through the loss of host plants.

A further objective of this study was to determine how the reduced glyphosate-based herbicide formulations influenced the behaviour of *M. scutellaris.* The results revealed that reduced herbicide concentrations had no negative impacts on the survival of the insects. More adult insects and nymphs were collected from low Kilo Max (0.4%) and Seismic (0.75%) concentrations. The reproductive capacity of the insects might have been affected by different glyphosate salt types, as insect populations on Roundup treatments were consistently low at all the concentrations. In the study by Zanuncio et al. (2018), insect reproduction was reduced by potassium (K) salt formulations of glyphosate, while the isopropyl amine (IPA) salt increased longevity and oviposition. Formulations based on K-salt could have caused more disruption to the shikimic acid pathway of the plants than IPA-salt-based formulations (Reddy & Zablotowicz 2003, Sihtmäe et al. 2013). The K-salt formulations may have decreased the nutrients that are essential for the diet of *M. scutellaris*. Several similar studies have reported that herbicides do not have damaging effects on insect herbivores used as biological control agents (Lindgren et al. 1999, Lym et al. 2002, Jadhav et al. 2008), instead their surfactants are toxic to the insects (Hill et al. 2012). Ueckermann & Hill (2001) reported that the herbicide Roundup herbicide without surfactant did not exhibit any lethal or feeding retardant for *N. eichhorniae* on *P. crassipes*, however, the surfactant Agral caused increased weevil mortality when combined with Midstream herbicide. Studies by Grodowitz & Pellessier (1990) also tested a range of surfactants on *N. eichhorniae* mortality and suggested that weevil mortality was significantly increased by the combination of surfactant and diquat herbicide.

2.5. Conclusion

The success of integrated management plans to control *P. crassipes* with biocontrol agents and herbicides relies on understanding the ecology of the weed, the insects used in its control, and the formulations of herbicides (Hill et al. 2012). The present study has demonstrated that a 0.4% concentration of a glyphosate herbicide, Kilo Max, and 0.75% of Seismic were sublethal to the *P. crassipes* plants, as they retarded their vegetative growth, did not kill the plants and were not deleterious to the biocontrol agent, *M. scutellaris*. Additionally, the nitrogen content, which is fundamental for plant quality, was increased by the application of a sublethal herbicide concentration of Kilo Max (0.4%). Nitrogen metabolism in plants is tightly linked with the fundamental physiological and biochemical pathways in plants. Herbicide application can disrupt physiological and biochemical processes in plants, potentially leading to changes in metabolite accumulation, which may affect the physiological functioning of herbivorous insect populations. The next chapter of this study (Chapter 3) determined the effects of sublethal concentrations of glyphosate herbicides on *P. crassipes* physiological and biochemical responses and the influence they have on the physiology of *M. scutellaris*.

CHAPTER 3: Biochemical effects of a sublethal concentration of glyphosate-based herbicide on *Pontederia crassipes* and *Megamelus scutellaris*

3.1. Introduction

Various biochemical and physiological effects may be evoked in plants when exposed to herbicides, including chlorosis, lipid peroxidation, and antioxidant responses (Kaya & Yigit 2014). In some plants, herbicides induce irreversible cellular damage that includes chloroplast rupture, cell plasmolysis, hyperplasia, cell proliferation, wax layer removal, and cuticle disruption, resulting in cell collapse and necrosis. Since the early 1900s, herbicides, including glyphosate-based herbicides (GBHs), have been frequently used to manage one of the major damaging aquatic invaders, *P. crassipes,* in South Africa (Ashton et al. 1981, Hill et al. 2012). Glyphosate is an important broad-spectrum, non-selective, post-emergence herbicide that is used worldwide for the management of problematic weeds. The mechanism of this herbicide is based on the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), preventing the synthesis of three essential aromatic amino acids phenylalanine, tryptophan and tyrosine (Gomes et al. 2014, Duke 2018). These three aromatic amino acids and other intermediate compounds of the shikimic acid pathway are precursors of a wide variety of plant primary and secondary metabolites (Dewick 1988, Parthasarathy et al. 2018). The disruption of the shikimic acid pathway by glyphosate can lead to primary effects and secondary consequences in plants that cause growth inhibition and eventually plant death. When any chemical inhibits a specific pathway, the consequences may result either from a direct depletion of the end products, from the depletion of intermediates of the pathway for some critical processes, or from a buildup of a toxic substrate. Thus, the final toxic effects can be derived from the variation in the levels of any of these metabolites and their effect on physiological processes that may be regulated by them (Singh & Tiwari, 2020).

Even though the application of sublethal herbicide concentrations reduces their toxic effects in several plant species (Chapter 2, Wiedman & Appleby 1972), they still disrupt the plant's biochemical pathways, which are essential for producing metabolites. Previous studies have identified the potential for using sublethal herbicide concentrations to control weeds (Vieira et al. 2019, Benedetti et al. 2020), with the hypothesis that they will increase essential metabolites that are beneficial to insect herbivores (Jadhav et al. 2008). Still, little information has been reported on the mechanism or mode of action of these herbicides in plants that underline herbicide-induced stress conditions. Jadhav et al. (2008) suggested that sublethal concentrations of glyphosate herbicides induce carbohydrate accumulation in *P. crassipes*, which could be beneficial to the insect biocontrol agents that feed on them, but this was never experimentally demonstrated. Also, Zabalza et al. (2006) reported that the soluble protein content of imazethapyr herbicide–treated plants was not affected by exposure to the herbicide. A few recent studies, however, report contrasting findings, for example, Royuela et al. (2000) reported that sublethal imazethapyr caused a decrease of 30% in soluble protein levels, while soluble carbohydrate content of *Vitis vinifera* L. (Vitaceae) leaves was significantly lower in plants treated with sublethal flazasulfuron herbicide in a study by Magnè et al. (2006).

Herbicide–induced stress influences the palatability of plants to insect herbivores by altering the production of metabolites involved in nutrition (Kraus & Stout 2019, Divekar et al. 2022). Larsson (1989) reported that biochemical effects caused by herbicides in plants can have different consequences on insect herbivores. Many studies have suggested that exposure to herbicides can also affect the metabolic physiology of insects (Katagi & Tanaka 2016, Bantz et al. 2018). Exposure to glyphosate herbicide can affect the accumulation of secondary metabolites in insect herbivores (Rainio et al. 2020, Fuchs et al. 2021); the metabolism of important macronutrients, carbohydrates proteins and lipids in insects is particularly responsive to pesticides. A study by Liu et al. (2020) found that panaxadiol saponin (PDS) consumption changed the metabolomic profiles of free amino acids (FAAs), free fatty acids (FFAs) and carbohydrates and altered the activities of metabolic-related enzymes of *Ostrinia furnacalis* (Guenée) (Crambidae: Lepidoptera) third-instar larvae. Also, Giglio et al. (2021) reported that exposure to the recommended field rate of pendimethalin herbicide has effects on the most predicted functional categories of gut microbiota related to metabolic functions, including carbohydrate, amino acid and lipid metabolism.

Chapter 2 showed that sublethal concentrations of glyphosate herbicides decreased biomass and reproductive outputs (daughter plants and leaf production) of the plant, suggesting alterations in the plants' physiological functioning post-exposure. In this study, we aimed to investigate the physiological and bio-compound profile changes of *P. crassipes* caused by a sublethal concentration of the glyphosate herbicide Kilo Max. The changes in the physiology of plants may potentially disrupt the metabolic functioning of herbivorous insects feeding on them; hence, the effects of exposure to sublethal herbicide on the physiological aspects and bio-compound profile of *M. scutellaris* were assessed. The objectives of the current study were to:

1. Investigate the effects of sublethal concentration of Kilo Max on *P. crassipes* macronutrient composition and secondary metabolites (carbohydrates, proteins and phenolic compounds) and physiological functioning of *M. scutellaris* adult insects.
2. Analyse metabolomic profiles (monosaccharides and phenolics) on *P. crassipes* plants exposed to sublethal herbicide concentration using High-Performance Liquid Chromatography (HPLC).

3.2. Materials and Methods

3.2.1. Biochemical effects of Kilo Max herbicide on *Pontederia crassipes*

3.2.1.1. Plant growth and glyphosate herbicide application

Pontederia crassipes plants were collected from insect-free plants maintained at the Waainek Rearing Facility at Rhodes University. The plants were grown in 25L buckets in water fertilised with Culterra Multisol 'N' fertiliser as described in Chapter 2. The plants were allowed to grow and acclimatise for a week, and thereafter, a sublethal concentration of Kilo Max (0.4% w/v) was sprayed onto the leaves of the plants. The control plants were sprayed with distilled water and allowed to grow under the same conditions. The experiment was replicated three times and randomly set up.

3.2.1.2. Plant collection and preparation

Leaves from a single plant per replicate of each treatment were collected at 1, 4, 8, 16, and 32 days after treatment application, always at the same time. The plant material was washed three times in 50 ml of distilled water following collection to remove any unabsorbed herbicide residues deposited on leaves. All plant material was stored in paper bags and dried in a forced-air oven at 40°C for 72 hours. The dried leaf material was macerated using a coffee grinder, and the resulting powder was stored at room temperature until further use.

3.2.1.3. Pre-treatment and extraction of bio-compounds from *Pontederia crassipes*

Ten grams of dried *P. crassipes* leaf per treatment was added to a 50% ethanol-water solution in a ratio of 1:30 (w/v). The suspension was incubated overnight in a shaking incubator (Biobase BJPX-100, Shandong, China) at 50°C, shaking at 150 rpm. The hydrolysate was collected the following day, the ethanol evaporated at 50°C, and the extract was lyophilised (Labconco Corp, Alaska). The freeze-drying conditions were −50°C and a low vacuum pressure of 40 Pa. After approximately 72 h, the lyophilised powder was collected, and the sample was stored at room temperature.

Total carbohydrate content was determined by using a modified phenol-sulfuric acid method (Dubois et al. 1956). The amount of total carbohydrate was interpolated using a D-glucose standard curve. Briefly, 300 µl of concentrated sulphuric acid (95-97%) was added to 100 µl of 1 mg/ml *P. crassipes* sample. Subsequently, 50 µl of 5% (w/v) phenol was added, and the mixture was vortexed and then heated at 90°C for 10 minutes. After cooling to room temperature, total sugars were quantified by reading the absorbance at 490 nm using a Hidex microplate reader (Bio-Tek instruments with KC Junior software®).

Protein content was also measured using bovine serum albumin (BSA) as a standard (Bradford 1976). An amount of 10 µl of leaf samples and BSA standards were mixed briefly with 230 µl of Bradford's reagent. The mixture was then incubated at room temperature for 10 minutes. The absorbance values of the samples were read at 595 nm using a Hidex microplate reader (Bio-Tek instruments with KC Junior software®), and the protein concentrations were interpolated using the BSA standard curve.

A modified Folin–Ciocalteu method was used to determine the total polyphenols (Huang et al. 2005). In summary, 30 µl of 1 mg/ml leaf sample, 540 µl of ddH_2O, and 60 µl of the Folin-Ciocalteu reagent were combined and incubated for 10 minutes at room temperature in a low-light setting. After adding an aliquot of 50 µl 2M sodium carbonate (Na_2CO_3), the reaction mixture was incubated at 37℃ for 30 minutes. After that, absorbance was measured at 765 nm using a Hidex microplate reader (Bio-Tek instruments with KC Junior software®), and a gallic acid standard curve was used to calculate the total amount of polyphenols.

3.2.1.4. Metabolomic profiles of *Pontederia crassipes* plants using High-Performance Liquid Chromatography (HPLC)

The monosaccharide profiles of the leaf extracts were further analysed using a Shimadzu HPLC (RID) instrument (Mabate et al. 2021). Before being injected into the HPLC run, all samples and standards underwent filtration via a 0.22 µm PES membrane and were diluted. With a small adjustment to the supplier's suggested procedure, the sugars were subjected to an HPLC analysis using a Fortis Amino column (Fortis Technologies Ltd, Cheshire, UK). Several sugar standards were used including D-glucose, D-sucrose, D-maltose, and D-galactose.

Using a Shimadzu HPLC system (Shimadzu Corp, Japan) with a diode array detector (DAD), the distribution and variety of polyphenolics were qualitatively analysed. Under ambient conditions, chromatographic separation was accomplished on an Xbridge Shield RP18 C18 column (5 µm, 100 x 4.6mm). The sample injection volume was maintained at 10 µl. Elution was performed in gradient conditions using 100% acetonitrile and 0.01M phosphoric acid as the mobile phase. The flow rate was kept constant at 0.5 millilitres per minute. The effluent's UV absorption was measured at 254 nm, 278 nm, and 320 nm.

3.2.2. Biochemical effects of sublethal Kilo Max herbicide on *Megamelus scutellaris*

3.2.2.1. Insect rearing and collection

Adult *M. scutellaris* insects were released on different *P. crassipes* treatments and allowed to feed for a week. The treatments included plants sprayed with different sublethal concentrations (0.2%, 0.4% and 0.6%) of Kilo Max herbicide and control plants that were only sprayed with distilled water, and the insects allowed to continue feeding for seven days. After seven days, the surviving adults were collected into an aerated plastic tub using an electric aspirator and brought to the laboratory. Twenty insects were placed in a 1.5 mL centrifuge tube and treated with liquid nitrogen. The samples were stored at −80℃ for preservation. Twenty insects were tested in each experiment, using three replicates for each treatment.

3.2.2.2. Investigation of insect bio compound profile after exposure to sublethal concentrations of herbicide

The extraction of carbohydrates from insects was performed according to Roe (1955). Twenty adult *M. scutellaris* insects (whole-body) from each herbicide treatment were homogenized in 100 µl of extraction buffer (20% sucrose, 50 mmol Tris-HCl pH 7.1, 0.5% Triton x-100) on ice, followed by centrifugation at 12,000 g for 10 min at 4 °C. Then, 30 µl of the supernatant was transferred to Eppendorf tubes and used to determine carbohydrate content as described in section 3.2.1.3.1. A standard curve was established with glucose, and the absorbance was read at wavelength 490 nm.

Similarly, protein extraction was conducted by homogenizing twenty *M. scutellaris* adults (whole-body) from each treatment in 100 µl of extraction buffer (20% sucrose, 50 mmol Tris-HCl pH 7.1, 0.5% Triton x-100) on ice, followed by centrifugation at 12,000 g for 10 min at 4 °C. Then, 30 µl of the supernatant was transferred to Eppendorf tubes and used to determine protein content as described in section 3.2.1.3.2. A standard curve was established with bovine serum albumin (BSA). The absorbance was read at 595 nm using a Hidex microplate reader (Bio-Tek Instruments Inc. Winooski, Vermont, USA) with KC Junior software®.

3.2.3. Statistical analysis

Data analysis was performed using R 4.2.3. and RStudio (R Development Core Team, 2023). Data were checked for normality and homogeneity using Shapiro-Wilk's and Levene's tests. Two-way repeated measures ANOVA compared the means of carbohydrate, protein, and phenolic contents between sprayed and unsprayed treatments at different time intervals. Significant differences between the means were determined with Tukey's HSD multiple pairwise comparison of means at a 5% significance level. Furthermore, metabolic compounds (carbohydrates, proteins, and phenolics) detected in *M. scutellaris* insects exposed to *P. crassipes* plants treated with different concentrations of the herbicide Kilo Max were analysed with one-way ANOVA. Tukey's HSD was used to perform pairwise comparisons of means at a 5% significance level.

3.3. Results

3.3.1. The effect of Kilo Max herbicide on *Pontederia crassipes* bio-compounds profile

The biochemical effect of sublethal herbicide application on carbohydrates, proteins and phenolic compound production of *P. crassipes* plants was measured by profiling the accumulation of bio-compounds on the sprayed and unsprayed plants.

3.3.1.1. Total carbohydrate content

The *P. crassipes* leaves were collected from the treatments on days 1, 4, 8, 16 until day 32. There were statistically significant interactions between treatments and time of exposure on the carbohydrate content of the plants ($F_{4,20} = 93.6$, $P < 0.001$). Interestingly, there was a spike in the amount of carbohydrate in the sprayed leaves on day 8, suggesting a short-lived stress response similar to an immune response. Generally, carbohydrate content between the treatments differed significantly, with unsprayed plants having greater carbohydrate content than the sprayed treatments ($F_{1,20} = 26.00$, $P < 0.001$), except on day 1 and day 8 (Figure 3.1).

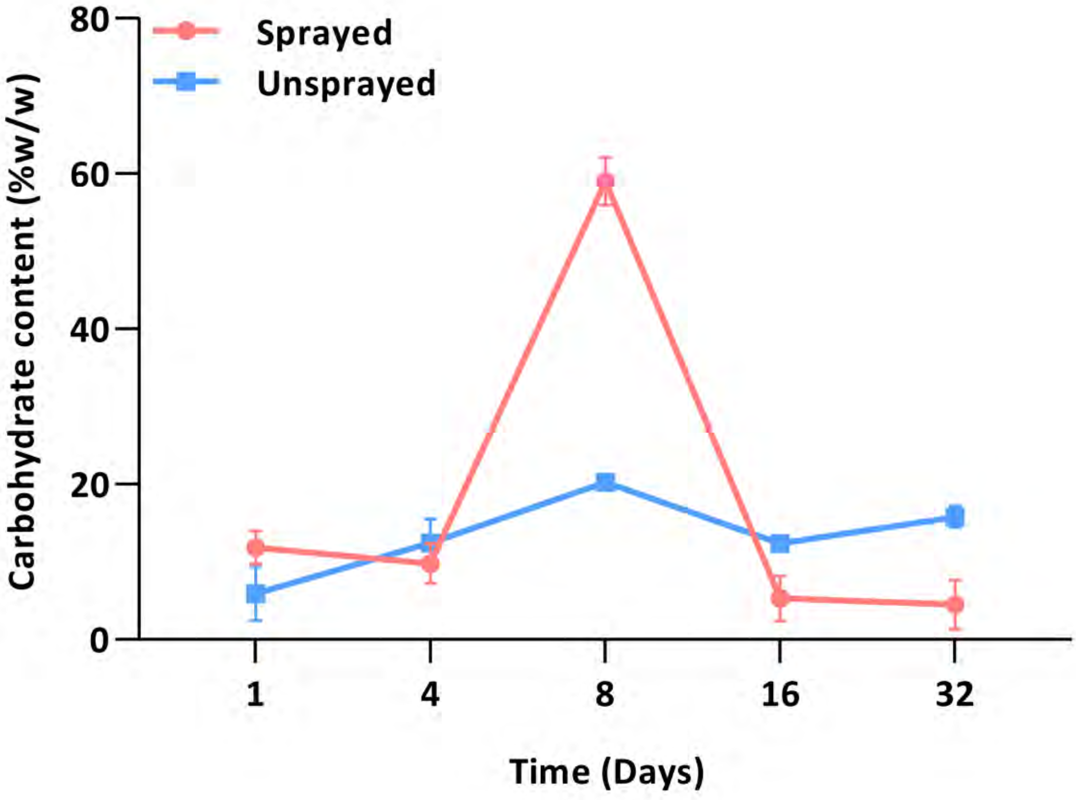

Figure 3.1: Carbohydrate content of *Pontederia crassipes* leaves collected 1, 4, 8, 16 and 32 days after application of sublethal concentration of glyphosate herbicide. Error bars indicate standard error of the means (n = 4).

3.3.1.2. Total protein content

The general trend of the results showed that there were significant differences in protein content between sprayed and unsprayed plants (Figure 3.2). There were significant interactions between the time of exposure (days) and the treatments ($F_{4,14} = 14.79$, $P < 0.001$). Unsprayed plants had significantly higher protein content than sprayed plants on day 1 and day 8 ($F_{1,14} = 8.33$, $P = 0.01$), while protein content of sprayed plants was higher than unsprayed plants on days 4, 16, and 32 (Figure 3.2). Furthermore, protein content increased significantly in the sprayed plants, over time.

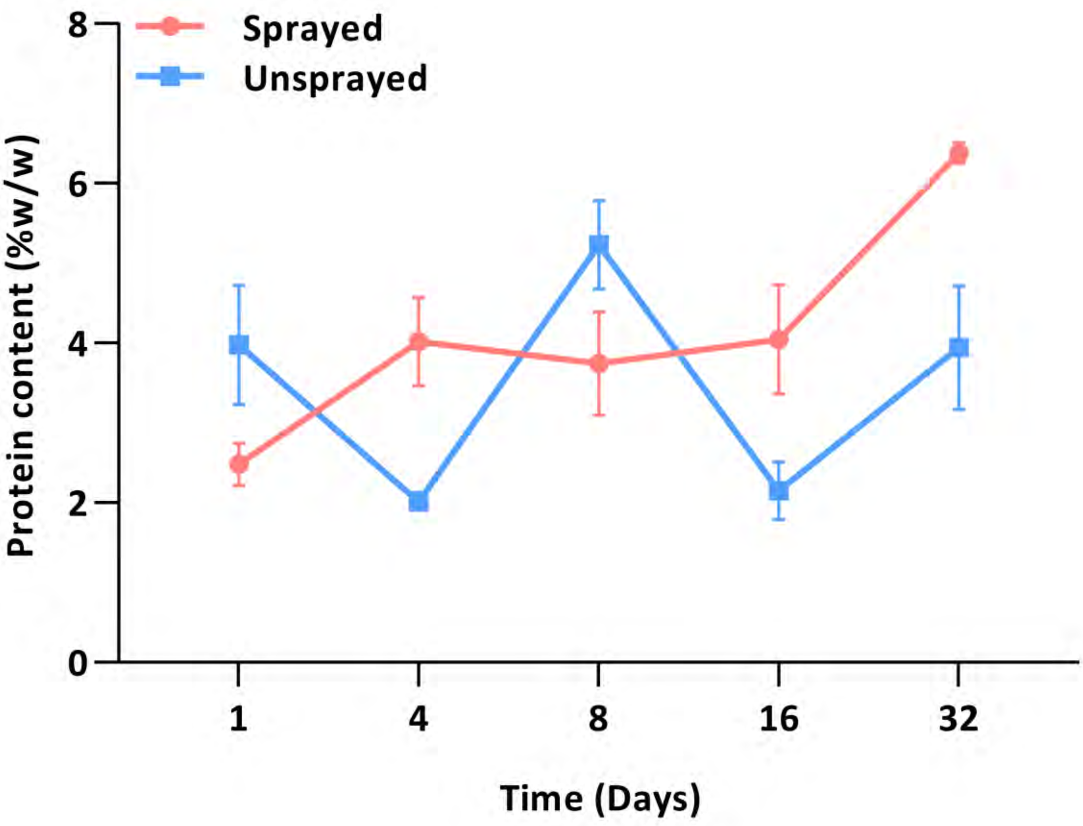

Figure 3.2: Protein content of *Pontederia crassipes* leaves collected 1, 4, 8, 16 and 32 days after application of sublethal concentration of glyphosate herbicide. Error bars indicate the standard error of means (n = 4).

3.3.1.3. Total phenolic content

The study demonstrated that the total polyphenolic content of *P. crassipes* leaves was unaffected by sublethal herbicide application. There were no significant interactions between the duration of exposure and the treatments ($F_{4,10}$ = 2.76, P = 0.08). Throughout the exposure time, there was no significant difference between the herbicide – sprayed and unsprayed control plants ($F_{1,10}$ = 2.32, P = 0.15) (Figure 3.3). This aspect was further investigated by HPLC.

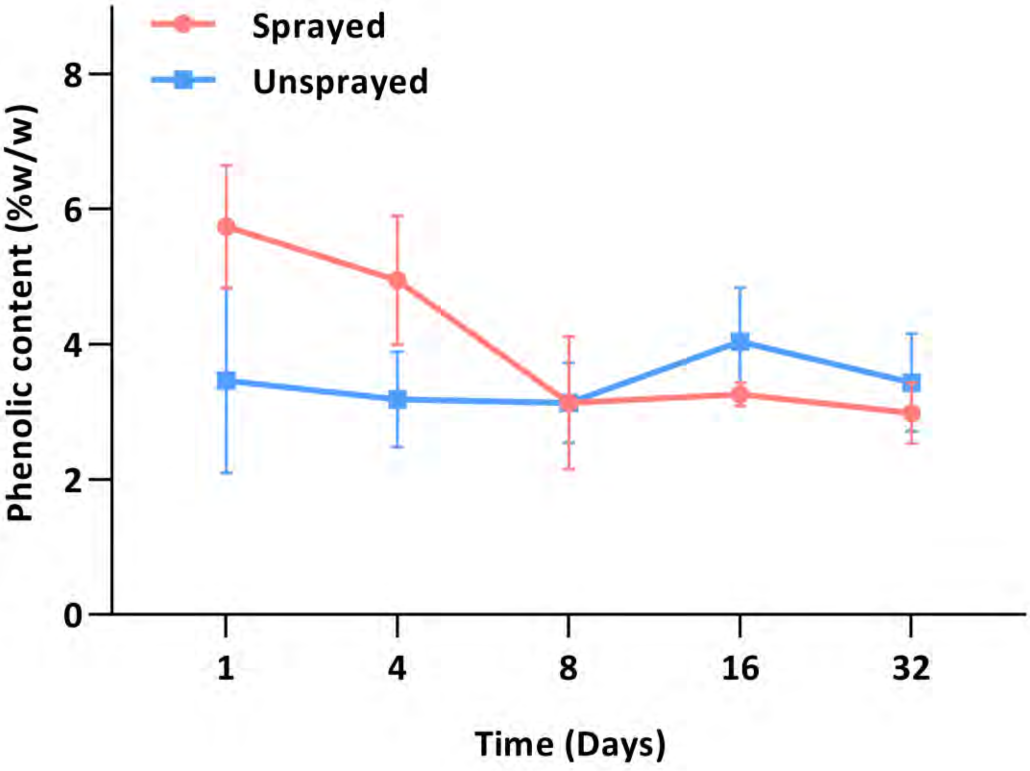

Figure 3.3: Phenolic content of *Pontederia crassipes* leaves collected 1, 4, 8, 16, and 32 days after application of sublethal concentration of glyphosate herbicide. Error bars indicate the standard error of means (n = 4).

3.3.1.4. Analysis of monosaccharide distribution within the *Pontederia crassipes* extracts

The profile of sugars within the *P. crassipes* leaf extracts was analysed by HPLC with a refractive index. The chromatographic results showed that the distribution of sugars was similar within the sprayed and unsprayed plants (Figure 3.4). The sugars were identified as fructose, glucose, maltose, and sucrose using the retention times of commercial standard sugars analysed parallel to the extracts. The intensity of maltose and sucrose peaks in both the sprayed and unsprayed plants was higher than glucose and fructose which could suggest abundance (Figure 3.4). Generally, sucrose content was more abundant on unsprayed plants than other sugars (Figure 3.4A).

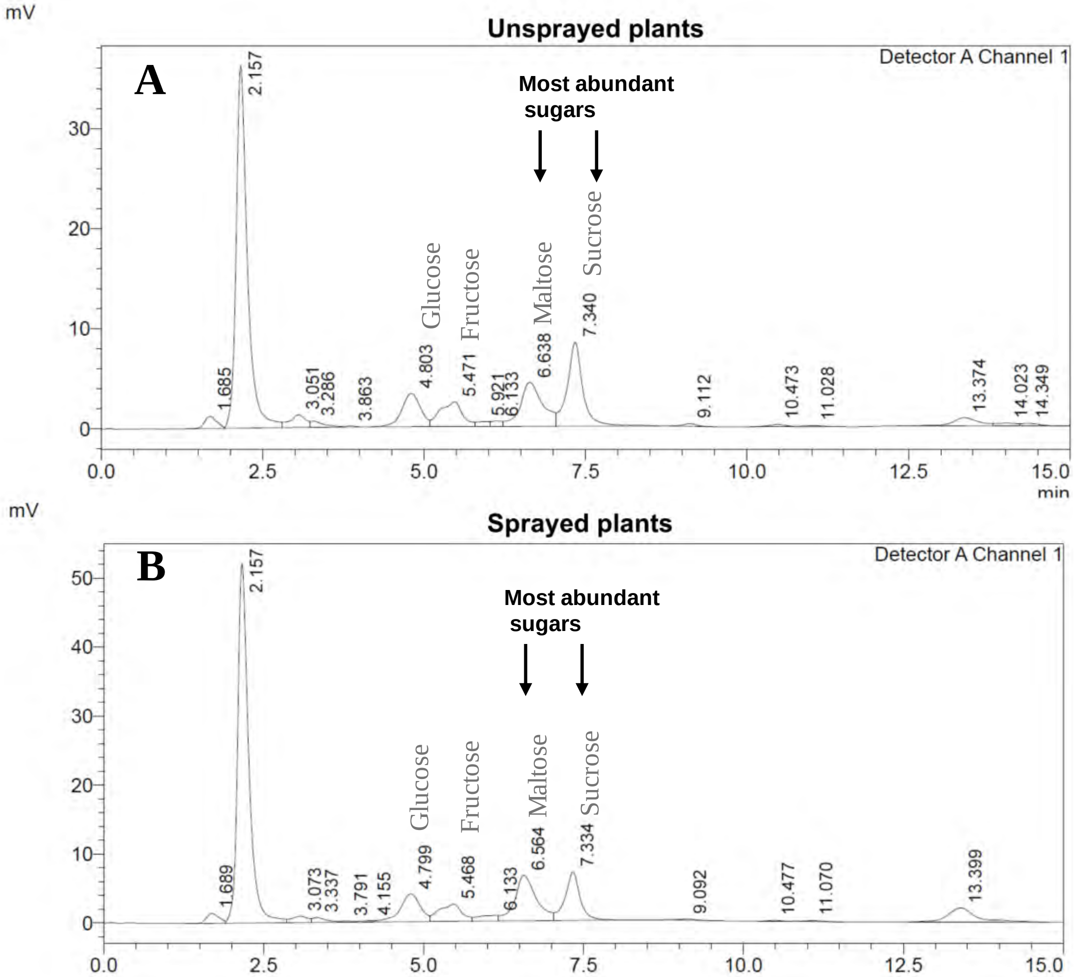

Figure 3.4: HPLC chromatogram of sugars detected in *Pontederia crassipes* plants A. unsprayed with herbicide and B. sprayed with herbicide.

3.3.1.5. Analysis of polyphenolics from *Pontederia crassipes* extracts

The HPLC results showed no difference in the phenolic compound profile between the herbicide-sprayed and unsprayed *P. crassipes* leaves. However, the individual phenolics indicated by the peaks at designated retention times consistently show higher signals (mAU), and peak areas within the sprayed plant extracts, clearly show an enhanced phenolic presence compared to the unsprayed plants (Figure 3.5). Available commercial standards could not identify any of the individual separated phenolic compounds.

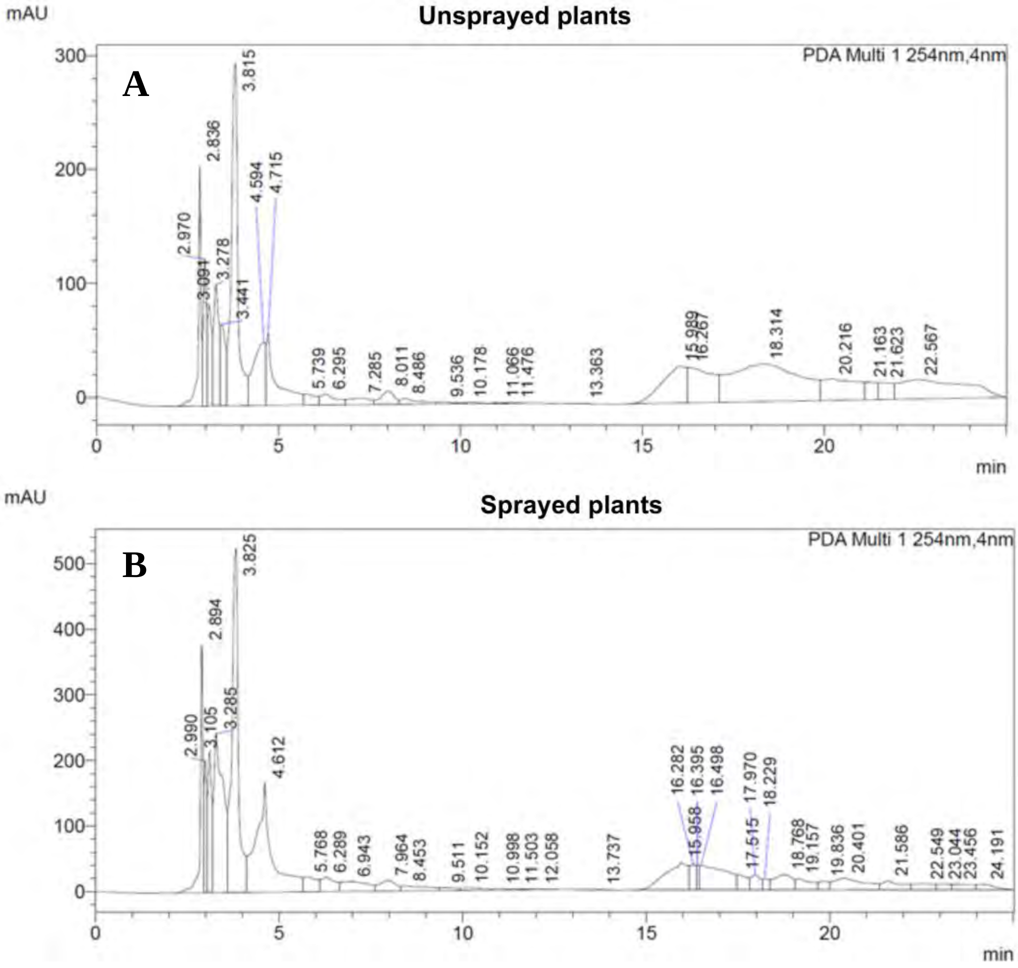

Figure 3.5: HPLC chromatogram of phenolic compounds detected in *Pontederia crassipes* plants, A. unsprayed and B. sprayed with herbicide.

3.3.2. Biochemical effects of Kilo Max herbicide on *Megamelus scutellaris*

Herbicides do not only show direct toxicity to insects but also influence population dynamics by affecting insect longevity, fecundity and physiological traits (Zhao et al. 2018, Bartling et al. 2024). The present study investigated the sublethal effects of Kilo Max on *M. scutellaris*.

Exposure to different concentrations of sublethal herbicides applied to *P. crassipes* leaves, did not impact the sugar content of *M. scutellaris*, ($F_{3,12} = 0.66$, $P < 0.59$) (Figure 3.6A). Even though not significant from other treatments, there was an observed reduction in carbohydrate content when insects were exposed to 0.6% and 0.2% concentrations.

Also, the sublethal Kilo Max concentrations had no significant influence on the protein content of *M. scutellaris* ($F_{3,12} = 0.28$, $P = 0.89$). However, the protein content was slightly higher in the control insects than in the insects exposed to 0.6%, 0.4%, and 0.2% (Figure 3.6B).

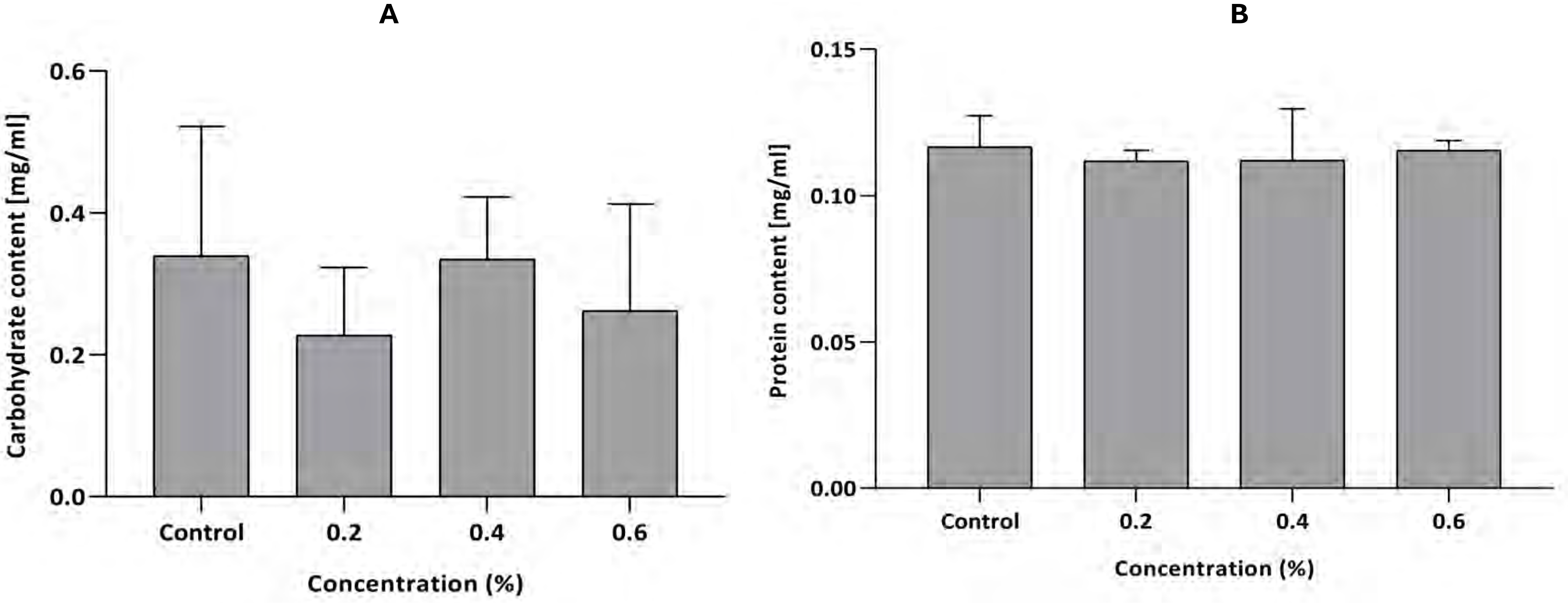

Figure 3.6: Total carbohydrate (A) and protein (B) contents in *Megamelus scutellaris* after exposure to sublethal concentration of Kilo Max. Error bars indicate standard error of means (n = 4).

3.4. Discussion

The application of 0.4% (w/v) of glyphosate-based herbicide, Kilo Max, was identified as a sublethal concentration in Chapter 2, as it slowed down the growth of *P. crassipes* plants without decreasing plant quality or causing rapid plant death. The current chapter evaluated the biochemical impacts of this sublethal herbicide concentration on *P. crassipes* leaves by measuring the accumulation of primary and secondary metabolites after treatment. Glyphosate herbicide has been reported to affect plant metabolism in various ways, including carbohydrate accumulation, protein synthesis, and phenolic formation (Duke et al. 1980, Carbonari et al. 2014, Zulet-Gonzalez et al. 2023); however, very few studies have reported the biochemical effects posed by exposure to sublethal herbicide concentrations.

The accumulation of carbohydrates in plants after treatment with herbicides has been previously reported (Zabalza et al. 2004, Fernández-Escalada et al. 2019). For example, Zabalza et al. (2004) reported an increased accumulation of soluble carbohydrates in the leaves of *Pisum sativum* L. (Fabaceae) after one and three days of applying lethal concentrations of Imazethapyr herbicide (IM) which is an acetolactate synthase (ALS) inhibitor. However, Scarponi et al. (2001) reported a decrease in the glucose of soybean and no effect on the total carbohydrate contents of maize treated with sublethal concentrations of IM herbicides. But Magné et al. (2006) showed that the leaf-soluble carbohydrate content of *V. vinifera* was significantly lower in the plants treated with recommended sublethal rates of flazasulfuron herbicide than that of unsprayed plants. These contradictory effects may be due to differences in concentrations of the herbicides used, with the latter results found at the sublethal levels. However, the current study showed that there was an accumulation of carbohydrates, including glucose, fructose, sucrose, and maltose, on plants treated with sublethal concentrations of glyphosate from day 1 to day 8. From day 8 to day 32, the carbohydrate content on the sprayed plants significantly diminished and was less than the content of the unsprayed plants. The accumulation of these carbohydrates confirmed that the primary metabolism is induced by the sublethal herbicide application on leaves. These results agree with Zulet-Gonzalez et al. (2023), who suggested that the primary metabolism of *Amaranthus palmeri* S. Wats. (Amaranthaceae), was more affected by sublethal glyphosate doses than by lethal ones. It is possible that the decline of carbohydrate content observed after day 8 is caused by the decline in carbon assimilation, making it difficult to maintain the same carbohydrate accumulation for the duration of the treatment period. This was previously reported by Scarponi et al. (1996) and Zabalza et al. (2004) under Acetolactate Synthase (ALS) inhibition.

Previous studies proposed that carbohydrate accumulation in the leaves of the plants treated with herbicide concentrations is due to a decrease in sink strength as the carbohydrates in the roots were not consumed (Zulet et al. 2013, Kumar et al. 2019, Zulet-Gonzalez et al. 2023) as plant growth is arrested. Growth inhibition caused by herbicides is explained by a possible carbon starvation in meristematic tissues caused by inhibition of assimilate translocation (Fan et al. 2013). In the previous chapter (Chapter 2), the low C:N ratio in the sprayed plants was evidence of carbon starvation. Impairment of carbon following the application of herbicide induces aerobic fermentation in roots (Zulet et al. 2013), thereby increasing the sugars in the roots and leaves. The increased sugars in the roots of herbicide-sprayed plants trigger a decrease in sink strength, which inhibits phloem transport and causes increased carbohydrate accumulation in the leaves (Zabalza et al. 2004, Orcaray et al. 2012, Zulet et al. 2013, Zulet-Gonzalez et al. 2023). The results of the present study were based on the excised leaves, and the root performance was not considered; therefore, the impact of sublethal concentrations of herbicides on sinks is yet to be determined to support this hypothesis. Additionally, studies have documented that carbohydrates play a key role in defense responses to abiotic factors such as herbicide exposure (Keunen et al. 2013, Jeandet et al. 2022). The carbohydrate accumulation on sprayed plants, particularly on day 8, could be upregulated due to herbicide-induced stress. Xu et al. (2022) reported similar results, where the sucrose content in the *Zea mays* L. (Poaceae) plants increased under nicosulfuron stress, compared to the control treatment. The accumulation of these carbohydrates is important for reducing the damage caused by herbicide stress in plants, as these soluble sugars act as osmoprotectants (Couée et al. 2006).

Inhibition of the enzyme EPSP–synthase by glyphosate herbicide may lead to decreased levels of aromatic amino acids, which subsequently inhibit protein synthesis (Cooley & Foy 1992, Martinez et al. 2018, Aldehoff et al. 2025). Some studies have reported that the biochemical effects of enzyme EPSPS inhibitors are an increase in the free amino acid pool and a decrease in the soluble protein content (Zulet et al. 2013, Zulet-Gonzalez et al. 2023). For example, Kumar et al. (2019) showed that the application of high concentrations of herbicides, propaquizafop, glyphosate, and clodinafop propargyl significantly reduced the protein content in *Pennisetum pedicellatum* Trin. (Poaceae) and *Indigofera hirsuta* L. (Fabaceae) at 5, 7 and 10 days after spraying. The key enzymes associated with the synthesis of amino acids and proteins are targeted by herbicides, which may be one of the possible reasons for decreased protein content under herbicide treatments (Singh et al. 2013). However, earlier studies by Patil and Kale (1975) reported that sublethal concentrations of herbicides 2,4–D and atrataf increased the protein content of wheat plants to the extent of 58% compared to the control. These studies have postulated that the effect of sublethal herbicide concentrations on protein content is due to the stimulatory effect of enzymes connected with nitrogen metabolism. The experiments in the previous chapter (Chapter 2) showed that the sublethal concentration of Kilo Max increased the nitrogen content and decreased the C:N ratio in the sprayed plants. According to these results, the increase in total protein on the sprayed plants was expected. Previous studies have reported that chemical alteration of the nitrogen metabolism leads to increases in protein content. The results of the current study show that the sublethal concentration of a glyphosate herbicide Kilo Max had affected the accumulation of proteins on the sprayed plants, indicating that the protein content in the sprayed plants could explained by an increase in the nitrogen content reported in the previous chapter of this thesis. This is evidenced by the slowed plant growth observed on plants treated with this sublethal herbicide concentration. From the results of this study, it may be inferred that this concentration of herbicide may be beneficial for the accumulation of essential primary metabolites which support the slow growth of the plants, thereby making the plants more susceptible to insect herbivory and palatable to insects.

Several chemical stimuli, including herbicide applications, influence the synthesis of polyphenols in plants. These secondary metabolites are involved in the protection machinery of plants owing to their antioxidant ability. Herbicide stress is associated with changes in the phenolic formation in plant tissues, with some herbicides increasing the level of these compounds in several plant species (Hoagland 1990, Scarponi et al. 1992, Alla & Younis 1995, Lang et al. 2023). Our research showed that even though the accumulation of phenolic compounds was observed in plants treated with herbicide, there were no significant differences from the untreated control treatment. A similar study by Becerril et al. (1989) reported that sublethal glyphosate treatments on plants did not have long-lasting effects on total phenolic acid content, so after a few weeks, the accumulation of such compounds recovered to almost control levels in leaves of velvetleaf plants, *Abutilon theophrasti* Medik. (Malvaceae). However, HPLC analysis here showed an increase in the phenolic compounds of the sprayed plants compared to unsprayed plants, although the individual compounds could not be identified. These studies coincide with the studies by Canal et al. (1987) who reported that sublethal glyphosate concentrations cause significant increases in the amount of soluble hydroxyphenol in yellow nutsedge (*Cyperus esculentus* L. Cyperaceae) leaf tissue. However, they disagree with Lee and Dumas (1983) who found that sublethal glyphosate concentrations caused marked decreases in the amount of hydroxyphenolic compounds per unit dry weight in tobacco *Nicotiana tabacum* L. (Solanaceae) callus. The results from this study suggest that sublethal concentrations of glyphosate herbicide elicit a general stress response in plants by upregulating individual phenolic compounds, indicating that glyphosate herbicide, even at low concentrations, is acting as an abiotic stressor to the plants.

Macronutrients, carbohydrates, and proteins are necessary chemical substances closely linked with several significant metabolic activities in an insect's body. Depending on the feeding conditions and growth stages, the amounts of these three chemicals may vary (Barragan-Fonseca et al. 2019, Ojha et al. 2021). Previous studies have indicated that exposure to glyphosate herbicides on the digestive microbiota of herbivorous insects significantly alters their metabolic functions, including the metabolism of essential macronutrients. Cullen et al. (2023) found that important physiological processes in the digestive tract of a pollinator, bumblebee *Bombus terrestris* (Hymenoptera: Apidae), were altered following exposure to sublethal glyphosate herbicides. These studies show that the proteins associated with oxidative stress regulation, cellular adhesion, TCA cycle and metabolism of carbohydrates, lipids and amino acids were significantly influenced by exposure to sublethal Roundup herbicide. Giglio et al. (2021) also showed that exposing ground beetles, *Pterostichus melas italicus* (Coleoptera: Carabidae), to pendimethalin-based herbicide had effects on the most predicted functional categories of gut microbiota related to metabolic functioning, including metabolism of carbohydrates, lipids and amino acids. The current study reported contradictory results, where total soluble carbohydrates and proteins in *M. scutellaris* adults were not affected by the exposure to different sublethal concentrations of glyphosate-based herbicide Kilo Max. These results suggest that exposure to this sublethal herbicide concentration of glyphosate did not affect the metabolic functioning of *M. scutellaris* insects.

3.5. Conclusion

Herbicide applications on weed plants significantly impact the physiological processes of primary and secondary metabolism. The current study reported that the Kilo Max sublethal herbicide concentration (0.4%) affected the accumulation of primary metabolism of plants by increasing the carbohydrate accumulation in sprayed plants. The increased carbohydrate content in the sprayed plants confirmed that sublethal herbicide application induces a primary metabolic response in plants. Moreover, the results presented in these studies indicate that this sublethal concentration did not have any toxic impacts on the biochemistry of *M. scutellaris*. The accumulation of metabolites in *P. crassipes* following sublethal herbicide sprays may affect biocontrol agent foraging and dispersal behaviour, thereby altering their population dynamics where integrated control is used as a weed management tool. Therefore, there is a need to optimise integrated management approaches for weeds that involve the combination of biocontrol agents and herbicides. The success of biocontrol insects largely relies on their ability to search and choose suitable host plants for oviposition and survival. The following chapter (Chapter 4) will determine the effect of sublethal herbicide concentrations of Kilo Max and Seismic on host choice preference, population dynamics and dispersal patterns of *M. scutellaris* for integrated control of *P. crassipes*.

CHAPTER 4: Investigating the effects of sublethal herbicide concentrations on populations and dispersal patterns of *Megamelus scutellaris*, a biological control agent for *Pontederia crassipes*

4.1. Introduction

Herbicide applications can alter host plant quality, potentially influencing insect survival by reducing nutritional value or increasing defensive secondary metabolites, affecting herbivore development and preferences. Some herbicides have been reported to cause mortality of biological control agents (Hill et al. 2012) or, at the very least, inhibit or shorten the feeding period of insects, thereby reducing their populations (Kraus & Stout 2019, Sánchez-Bayo 2021). Nevertheless, sublethal concentrations of herbicide may have benefits for biological control agent populations (Gettys et al. 2014, Tipping et al. 2017). For example, Chapter 2 of the current study showed that the sublethal concentrations of Kilo Max (0.4%) and Seismic (0.75%) reduced mortality and supported significant populations of *M. scutellaris* insects. Earlier studies by Stoyer & Kok (1986) suggested that after using reduced concentrations of the herbicide 2,4–D to weaken *Carduus* thistles, a high yield of its biocontrol agent adults, *Trichosirocalus horridus* Panzer (Coleoptera: Curculionidae), was achieved. Wright & Bourne (1990), also investigated changes in *P. crassipes* caused by sublethal 2,4–D treatment, where increased damage by larvae of the moth, *Niphograpta albiguttalis*, on treated plants was observed. This was because the 2,4–D herbicide decreased the petiole hardness and increased nitrogen levels, favouring the biological control agent. These studies suggest that applying sublethal concentrations could work synergistically with biological control insects to control invasive weeds sustainably.

The survival and success of biological control insects in herbicide-managed areas largely depend on their ability to locate suitable host plants. Host choice studies have been conducted in which insects are simultaneously exposed to herbicide-treated versus untreated plants (Olaya-Arenas et al. 2020, Piersanti et al. 2023). It would be expected that when presented with the choice, herbivorous insects would preferentially forage on herbicide-free plants (i.e., avoiding poisonous food should be highly adaptive). This expectation underlies the rationale that herbicides are toxic to plants, which in turn deters insects from feeding. However, insects use several cues in their host-finding process; in particular, volatile organic compounds (VOCs) emitted by plants are among the primary cues used by phytophagous insects to locate their host (Fernandez & Hilker 2007, Piersanti et al. 2020), and herbicide stress may affect the reliability of plant cues herbivores use to determine host quality. However, the previous chapter showed that a sublethal herbicide concentration of Kilo Max did not inhibit the accumulation of primary metabolites, which serve as crucial cues for insects in their foraging behaviour, enhancing the insects' ability to locate and accept the host plant. Primary metabolites, such as carbohydrates, play a significant role in attracting herbivorous insects, enhancing their ability to locate and accept the host plant.

Small-scale dispersal of insects may play an important role in the re-colonisation of herbicide-treated areas, as insects migrate from herbicide-treated plants to unsprayed plants (Goode et al. 2019). In *P. crassipes* control studies by Haag (1986a) and Haag et al. (1988), the plants were grown in tanks that were divided into herbicide-sprayed and unsprayed sections. The results reported that the *N. eichhorniae* adults were able to migrate to healthy plants in the untreated area when the treated plant quality declined. However, larvae died with the dying plants, because of their limited dispersal capacity. Haag (1986) showed that *Neochetina* weevils used to control *P. crassipes* moved from dying plants treated with standard concentration of 2,4 – D herbicide to nearby untreated ones, despite lacking the ability to fly. However, some studies have reported that some herbicides, mostly at sublethal concentrations, can attract biocontrol insects to the treated plants. A study by Perkins et al. (1978) showed that *P. crassipes* plants that were treated with sublethal concentrations of 2,4–D were attractive to *N. eichhorniae*. However, herbicide-treated plants become less attractive for insect feeding and oviposition as the plants degrade, so adult dispersal to untreated plants occurs, which increases oviposition and subsequent damage to the untreated areas (Ueckermann & Hill 2001, Sharma et al. 2018). Recently, Goode et al. (2021) found that the density of *M. scutellaris* was higher in unsprayed areas than in sprayed areas after spraying different herbicides at different concentrations, including 2,4–D, penoxsulam, and flumioxazin. However, *Neochetina* weevil densities did not differ between refuge areas and sprayed areas. Adult *Neochetina* weevils have reduced musculature and thus have limited dispersal capacity compared to *M. scutellaris*, and their sessile stages often sink with the sprayed mats as they feed inside the plant petioles (Grodowitz et al. 1997, Center & Dray, 2010).

Megamelus scutellaris has proved to be a successful biocontrol agent for *P. crassipes* in South Africa since its initial release in 2013 (Coetzee et al. 2022, Miller et al. 2023, Moffat et al. 2024). The success of this insect is attributed to its ability to disperse from unfavourable environments, allowing it to establish new populations in more favourable conditions. The dispersal of *M. scutellaris* is favoured by wing dimorphism, which includes fully winged macropterous forms capable of flight and a brachypterous form with reduced wings (Tipping et al. 2011, Fitzgerald & Tipping 2013). This wing dimorphism is presumably influenced by the host plant quality or the density of insects (Denno et al. 1991, Fitzgerald & Tipping 2013). Macropterous individuals of *M. scutellaris* and other wing-dimorphic delphacids can disperse over long distances. In contrast, brachypterous individuals have limited dispersal (Moran et al. 2016, Goode et al. 2019), and are capable of small-scale dispersal. A few studies have reported that compared to other biocontrol agents, *M. scutellaris* is less impacted by herbicidal treatments that are routinely used to control *P. crassipes* because all their stages (adults and nymphs) of development occur outside the plant petioles and the insect can leave when the plant deteriorates (Goode et al. 2020, 2022).

This study was developed to assess the impact of sublethal concentrations of glyphosate-based herbicides, Kilo Max (0.4%) and Seismic (0.75%), identified in Chapter 2, on *M. scutellaris* host choice selection, population dynamics on herbicide-sprayed plants and their dispersal patterns. The objectives of this chapter were to:

1. Determine the host-choice preference of *M. scutellaris* adults between *P. crassipes* plants treated with a sublethal concentration of glyphosate herbicide and unsprayed plants.

2. Assess the effect of sublethal herbicide concentrations on *M. scutellaris* densities in source mesocosms to observe its ability to persist following sublethal herbicide applications.
3. Monitor dispersal patterns of *M. scutellaris* by assessing insect populations in sprayed and unsprayed dispersal mesocosms.

4.2. Materials and Methods

Pontederia crassipes plants and *M. scutellaris* insects were collected from the cultures maintained at the Centre for Biological Control's Waainek Mass Rearing Facility, Rhodes University, Makhanda, South Africa. *Megamelus scutellaris* (ex. Argentina via USDA, Fort Lauderdale) adult insects used for these experiments were collected from cultures initiated in 2008 and maintained on *P. crassipes* plants. The insects were collected into a large, clean plastic container containing freshly cut *P. crassipes* leaves using an electric aspirator. The insects were collected and put into plastic tubs (65 mm diameter) lined with damp filter paper one day before the experiments and kept at 25 °C temperature with 65% relative humidity, and 12:12 (Light: Dark) photoperiod in a laboratory at the Department of Zoology and Entomology, Rhodes University. The insects collected were not divided according to sex, but it was assumed that the sex ratio was not significantly different from 1:1.

4.2.1. Host choice experiments

Initially the *P. crassipes* plants were grown in 25 L buckets fertilised with Culterra Multisol 'N' fertiliser and kept in the same tunnels. After acclimatising for a week, the plants were sprayed with sublethal concentrations of Kilo Max (0.4%) and Seismic (0.75%) and the control plants were treated with distilled water. The leaves from each treatment were collected 1, 4, and 8 days after the treatments and brought to the laboratory at the Department of Zoology and Entomology, Rhodes University for experiments.

To conduct paired choice test experiments, two *P. crassipes* leaves, one treated with a sublethal concentration of either Kilo Max or Seismic, and another unsprayed control leaves) were each put in separate Petri dishes and placed in a plastic container. A container with 20 *M. scutellaris* insects was placed in the centre between the Petri dishes, one containing sprayed leaves and one with control leaves, and the container was closed with an aerated lid. The insects were given a simultaneous binary choice to migrate to either sprayed or control leaves. The experiment was replicated three times. The movement of insects in each treatment was monitored and recorded by counting the number of insects found on the sprayed and unsprayed leaves every two hours on days 1, 8, and 16 after treatment.

Host choice tests were used to estimate the host preference of *M. scutellaris* between the sublethal herbicide-sprayed *P. crassipes* leaves and unsprayed control leaves. Further, *M. scutellaris* populations were monitored on indoor mesocosms after sublethal herbicide applications. Insect migrations from the source mesocosms to the dispersal mesocosms were also recorded to determine if they were attracted to the sprayed or unsprayed plants and if leaving refuges was effective for maintaining and increasing biocontrol populations.

4.2.2. Insect dispersal and population dynamics

Indoor mesocosm experiments were designed to monitor the population densities of *M. scutellaris* following sublethal herbicide applications, assess the combined impact of biocontrol and sublethal herbicide applications on *P. crassipes* populations and assess if leaving unsprayed plants for the biocontrol insects to migrate to during herbicide applications would affect insect populations. The experiments were conducted for 11 weeks at the Centre for Biological Control's Waainek Mass Rearing Facility, Rhodes University. Twenty-five experimental mesocosms (1.83 m length × 0.38 m diameter, 208 L polyethylene pools) were set up in a polyethylene greenhouse tunnel. Five medium-sized *P. crassipes* plants were placed in each mesocosm and allowed to grow to cover the mesocosm surface area. Mesocosms were filled with ~100 L of tap water and fertilised with Osmocote (Greenhouse Products© Pty Ltd, South Africa) (10 mg/L) slow release fertiliser. Iron chelate (0.9 g/L) (In Line Trading 112

Pty Ltd, South Africa) was applied at the beginning of the experiment to reduce chlorosis of the plants. The water level was maintained throughout the study, and plants were monitored weekly for growth for the duration of the experiment. Once the mats of *P. crassipes* covered 100% area of each mesocosm, *M. scutellaris* was released using two methods, first, by placing egg-infested plants in the centre of each pool, and second, by releasing adults (Goode et al. 2019). Insect populations were allowed to build up for four weeks before the herbicide application treatments started. The experiment was set up as a completely randomised design with five treatments, replicated five times (Figure 4.1).

After the plant coverage reached 100% and *M. scutellaris* had established, four plastic tubs (1.9 m × 0.8 m, 40 L) containing approximately three insect-free *P. crassipes* plants each were evenly placed around each source mesocosm to serve as dispersal tubs for the insects during herbicide application (Figure 4.1). Plants were fertilised with Culterra Multisol 'N' fertiliser (6N:1P:3K) (Culterra© Pty Ltd, South Africa) at a concentration of 10 mg N/L fertiliser, and iron chelate at 0.9 g/L (Miller et al. 2019) at the beginning of the experiment. Mesocosms containing insects were assigned as source plants, while the outside tubs served as dispersal areas for insects (Figure 4.1). The source plant treatments were either sprayed with herbicides or remained unsprayed; the sprayed treatments received either sublethal concentrations of the herbicides, Kilo Max (0.4%) or Seismic (0.75%) that were identified in Chapter 2. The treatment combinations were set up as: A. Kilo Max – source and two opposite dispersal treatments sprayed; B. Seismic – source and two opposite dispersal treatments sprayed, C. Kilo Max – source unsprayed and two opposite dispersal treatment sprayed, D. Seismic – source unsprayed and two opposite dispersal treatments sprayed, E. No herbicide sprayed (Figure 4.1). The herbicides were sprayed on the fifth week in all the treatments, on both source and dispersal treatments. In all the mesocosm treatments, the dispersal of *M. scutellaris* from the source to the dispersal areas was recorded by counting the number of insects on each plant in the dispersal tubs in each treatment. The source and dispersal mesocosms were spaced approximately 5 cm apart.

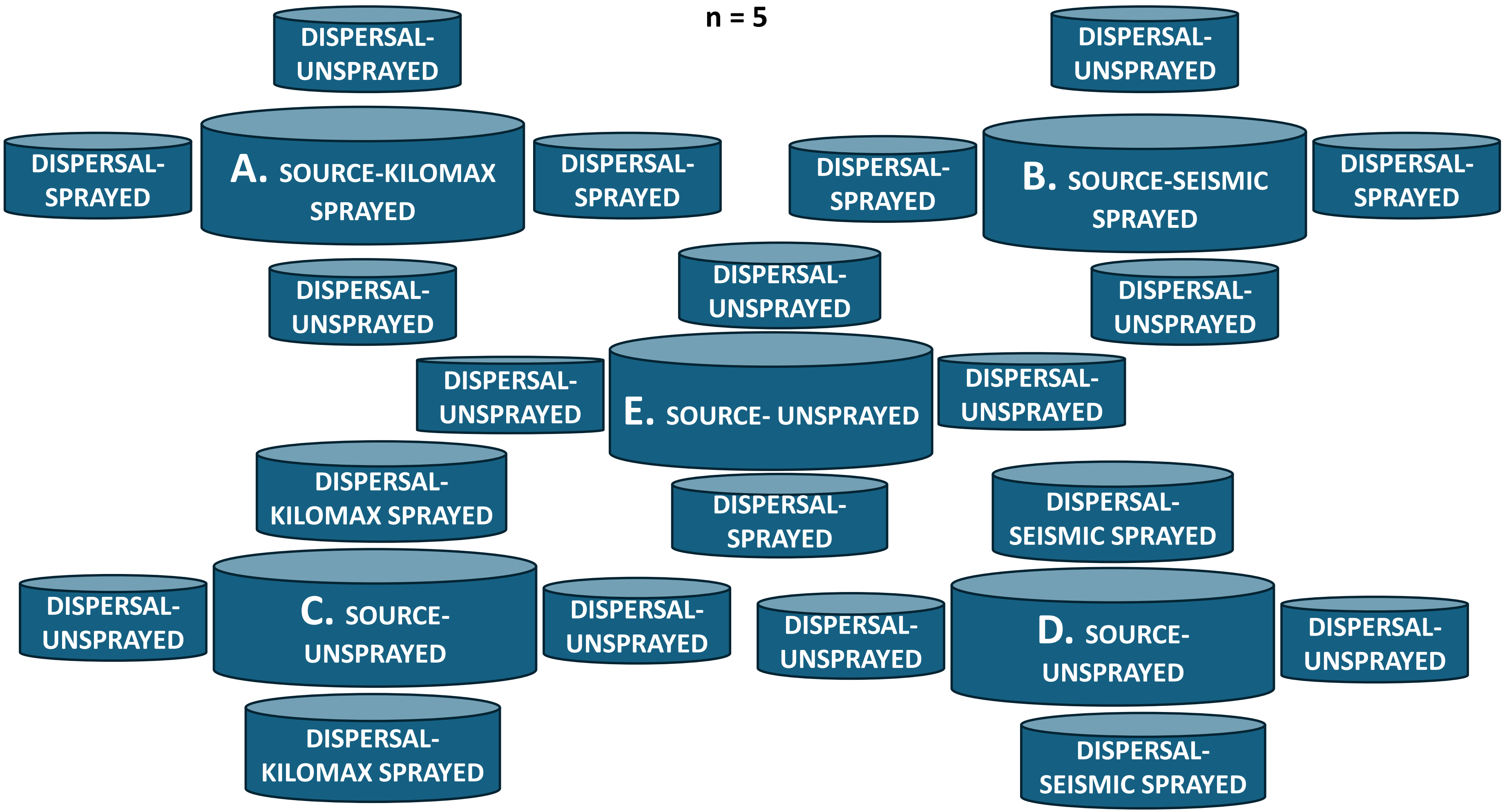

Figure 4.1: Schematic diagram of the indoor mesocosms experimental design. Treatments included A. Kilo Max – source and two opposite dispersal treatments sprayed, B. Seismic – source and two opposite dispersal treatments sprayed, C. Kilo Max – source unsprayed and two opposite dispersal treatment sprayed, D. Seismic – source unsprayed and two opposite dispersal treatments sprayed, E. No herbicide sprayed. The five replicated treatments were randomly arranged.

4.2.3. Source insect and plant populations

Insect populations from the source treatments were sampled from all mesocosms once a week throughout the experiment. The method for sampling *M. scutellaris* insects was adapted from Goode et al. (2021). *Megamelus scutellaris* populations were obtained using bucket samples, where a 25 L plastic bucket (39 × 26 × 26 cm) with the solid bottom removed and cross wires (26 × 26 cm) added was pressed down onto the plants. The wires submerged 0.07 m^2 of *P. crassipes* (~five plants), resulting in the insects climbing up the sides of the bucket, where they were easily identified and counted, after which the insects were released back into the pool by shaking the bucket. The initial and final plant densities were estimated by randomly placing a square PVC frame (0.5m ×0.5m) into the mesocosm, and then removing, counting and weighing the *P. crassipes* plants in the frame.

4.2.4. Statistical analysis

All data were analysed using R v4.2.3. and RStudio (R Development Core Team, 2023). Data were checked for normality and homogeneity using Shapiro-Wilk's and Levene's tests. To analyse the choice preference of *M. scutellaris* insects between the herbicide-treated and untreated *P. crassipes* leaves, a generalised linear mixed model (GLMM) with a Poisson distribution was used, and each container was used as a random effect. Dunnet's test was then used to compare the number of *M. scutellaris* insects recovered from plants treated with herbicide against the untreated control plants.

In the mesocosm studies, population densities of *M. scutellaris* from the source plants were compared using a generalised linear mixed model (glmmTMB package on R), and each pool was used as a random effect. To fit the model, backwards selection was used (drop1 function in R, lme4 package); this removes variables which contribute the least to the model until the lowest AIC is reached. A Poisson distribution with a logarithmic link function (family = poisson (link= "log")) was found to be the best-fit model. Multiple regression analyses were used to investigate relationships between insect densities and plant populations in all the treatments.

A generalised linear mixed model (glmmTMB package) was also used to test the effect of herbicide applications on *M. scutellaris* populations dispersal. Insect populations sizes were compared between treatments (sprayed and unsprayed) during exposure time (weeks). Following a significant main effect ($P < 0.05$), post hoc pairwise comparisons of means were conducted to identify specific group differences. Bonferroni correction was applied to adjust p-values for multiple comparisons.

4.3. Results

The capacity of an insect herbivore to select a host plant and feed on specific tissues generally improves its fitness.

4.3.1. Effect of sublethal herbicide concentrations on insect host preference

The attraction to plants and survival of insects on two treatments was quantified. Insect numbers changed with time and were significantly different between the sprayed and unsprayed control treatment for the Kilo Max herbicide over time ($\chi^2 = 6.96$, DF = 2, $P = 0.004$) during the choice test experiments. On day 1 and day 8, even though there was no significant difference, the insects selecting sprayed leaves were fewer than the insects on the unsprayed treatment (Figure 4.2). As the days after herbicide treatment progressed, fewer insects moved to the treated leaves, and there were significantly more insects recovered on the unsprayed plants on day 16.

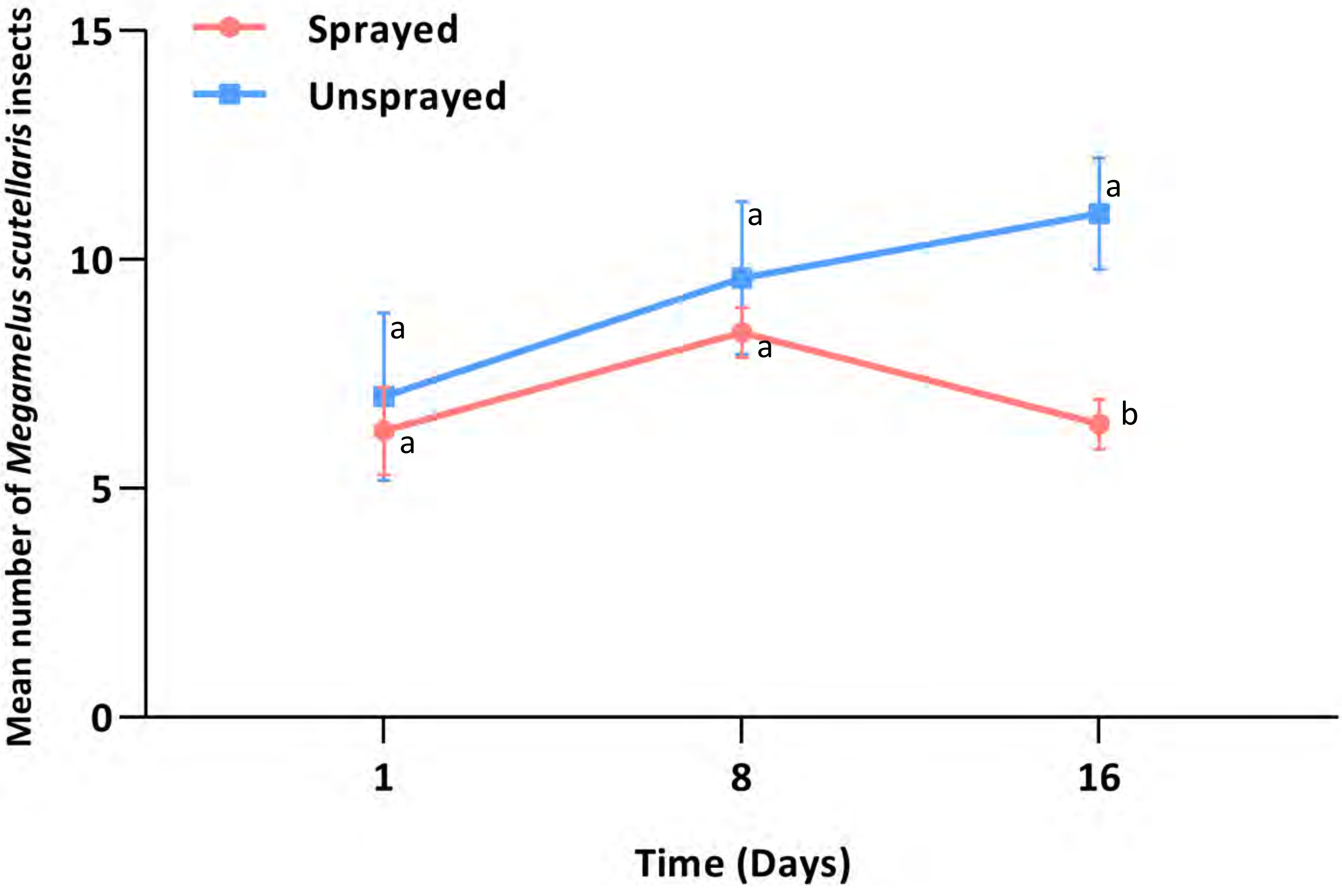

Figure 4.2: Mean number of *Megamelus scutellaris* recovered from *Pontederia crassipes* leaves sprayed with Kilo Max herbicide and unsprayed control leaves at 1, 8 and 16 days after treatment. Error bars represent standard error of means (n = 3). Means with different letters are significantly different from each other $P < 0.05$.

In the case of Seismic, there were significant interactions between treatments and the time that the insects were exposed to the treatments ($\chi^2 = 4.59$, DF = 2, $P = 0.02$). On day 1, there was no difference between sprayed and unsprayed treatments. As the time progressed, by day 8 and day 16, significantly more insects migrated to unsprayed leaves than to the sprayed leaves (Figure 4.3).

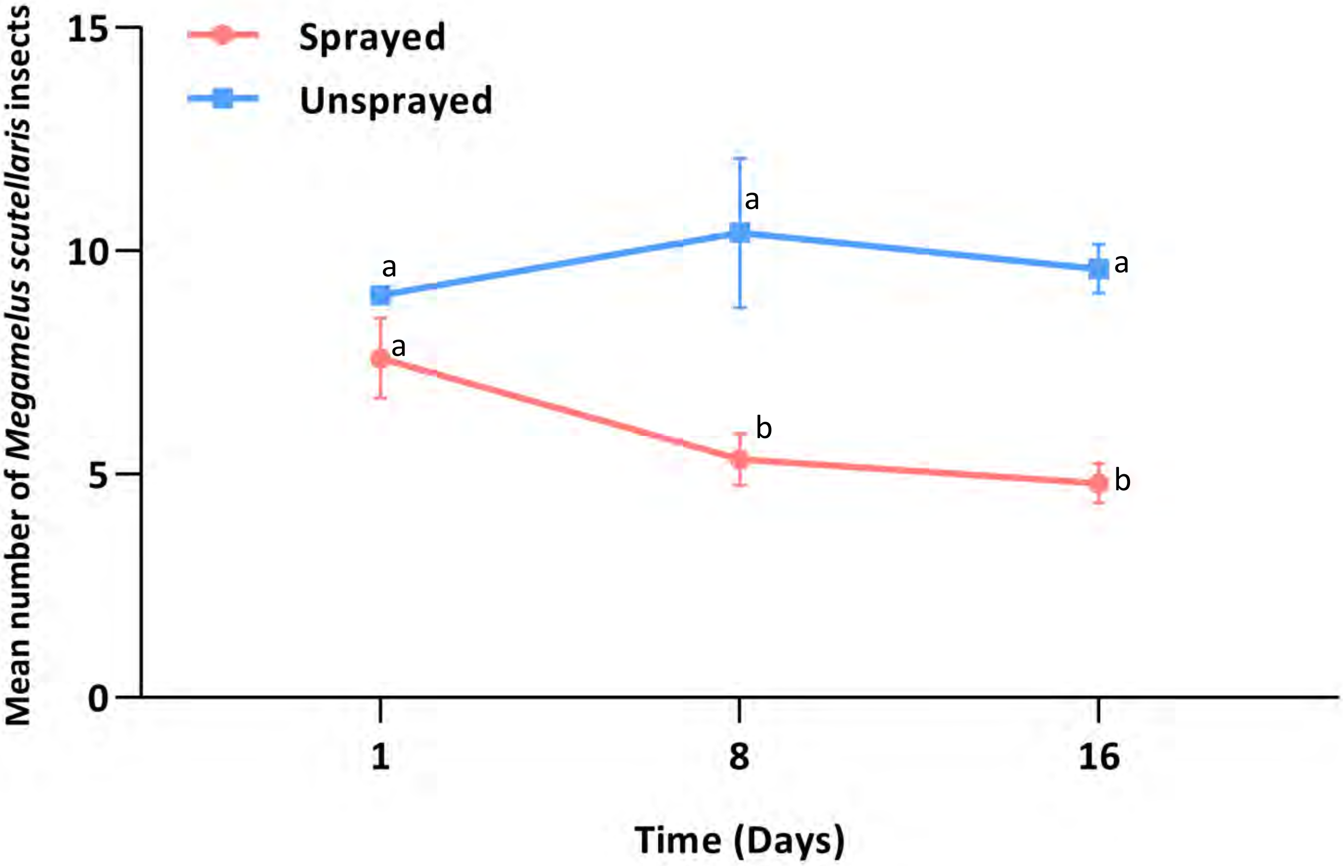

Figure 4. 3: Mean number of *Megamelus scutellaris* insects recovered from *Pontederia crassipes* leaves sprayed with Seismic herbicide and unsprayed control sprayed with distilled water at 1, 8 and 16 days after treatment. Error bars represent standard error of means (n = 3). Means with different letters are significantly different from each other *P* < 0.05.

4.3.2. Effect of sublethal herbicide concentrations on insect dispersal patterns between sprayed and unsprayed plants

Here I describe the insect and plant population patterns from the dispersal tubs placed around the source tubs. Dispersal of *M. scutellaris* insects from the source to the dispersal treatments was monitored for 11 weeks. Results indicated significant interactions between sprayed and unsprayed dispersal treatments, and the sampling time (χ^2 = 95.9, DF = 4, *P* < 0.001). For the first 3 weeks of the experiments, the insects were attracted to both the sprayed and unsprayed plants in the dispersal mesocosms. From week 5, insects started to disperse to the unsprayed dispersal treatments as source plants were beginning to deteriorate due to sublethal herbicide applications. Plants in the dispersal tubs that were treated with sublethal herbicides slowly wilted and died, thereby increasing the number of insects that were migrating to and ovipositing on the unsprayed plants. A significantly higher population density of *M. scutellaris* was found on the unsprayed dispersal treatments in all the treatments from week 7 to week 11 than the sprayed dispersal treatments (Figure 4.4, Table 4.1). Also, a significantly higher number of insects was recorded on the unsprayed dispersal treatments of Kilo Max – source and two dispersal sprayed, compared to other treatments (Table 4.1).

There were significant relationships in insect populations between the sprayed source and dispersal treatments on treatments Kilo Max – source and dispersal sprayed: $y = 6.38 + 0.364x$, $R^2 = 0.15$, $P < 0.034$ and Seismic – source and dispersal sprayed: $y = 1.13 + 0.255x$, $R^2 = 0.41$, $P < 0.001$ (Figure 4.5). However, the relationships between insect populations and unsprayed source treatments of treatments Kilo Max – source unsprayed and two dispersal sprayed: $y = 9.13 + 0.034x$, $R^2 = 0.04$, $P = 0.276$ and Seismic – source unsprayed and two dispersal sprayed: $y = 9.38 + 0.0358x$, $R^2 = 0.04$, $P = 0.280$, were weak and not significant (Figure 4.5). In all treatments, there was an increase in insect populations in dispersal treatments, as they decreased in source treatments, indicating a negative correlation between the source and dispersal treatments (Figure 4.5). This result suggests that as plant quality declined in the source mesocosms, insects dispersed to the neighbouring dispersal mesocosms. This was evidenced by the presence of macropterous *M. scutellaris* individuals in all treatments by week 5 ($F_{4,20} = 1.74$, $P = 0.18$) and week 7 ($F_{4,20} = 2.94$, $P = 0.05$), although they were not abundant. Overall, it was observed that as the plant populations decreased in the source mesocosms, the insect numbers decreased and subsequently increased in the dispersal treatments. It was clear that *M. scutellaris* insects dispersed and oviposited on the dispersal treatments as the host plant quality and quantity decreased on the source mesocosms.

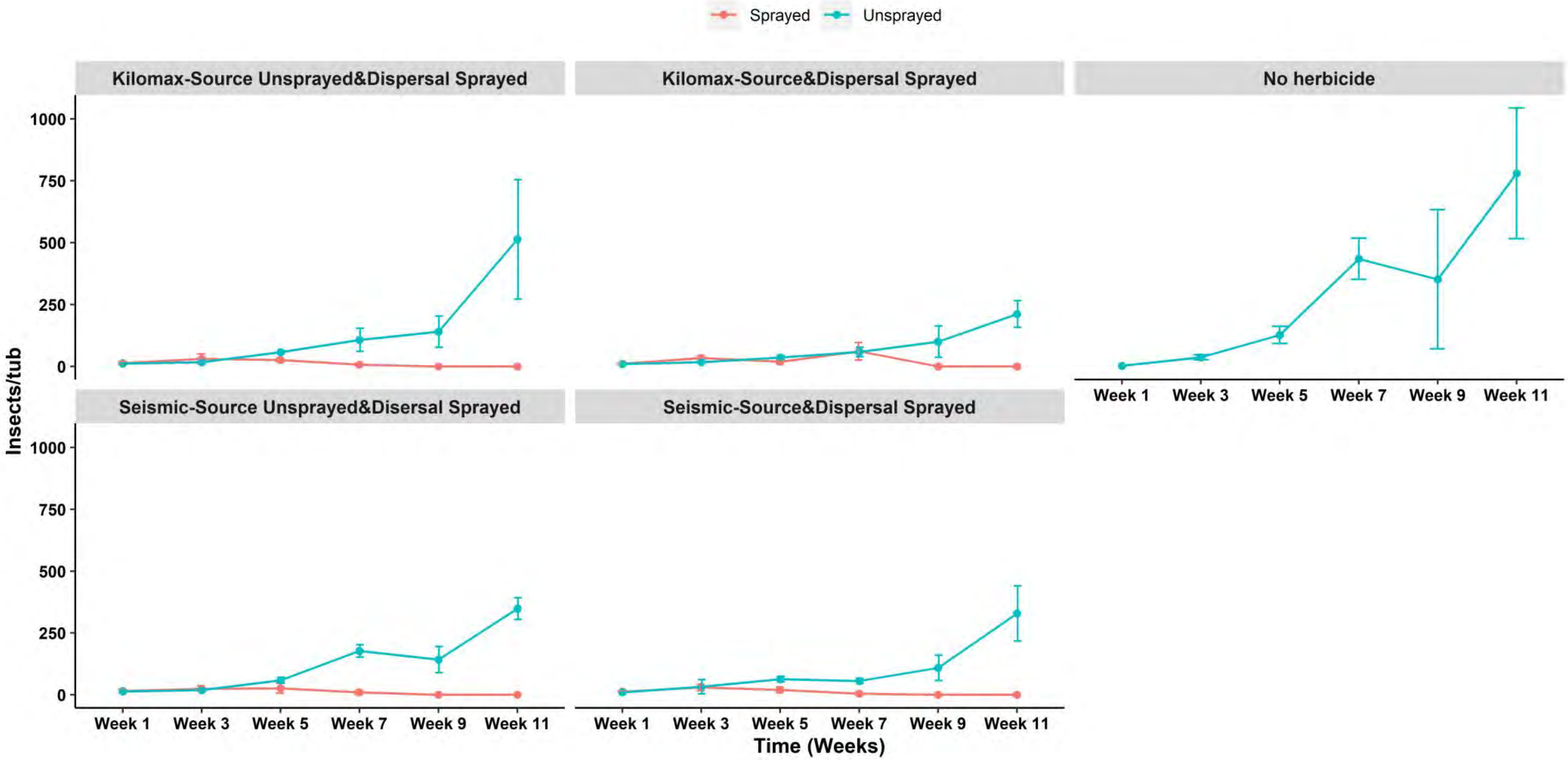

Figure 4.4: Population densities of *Megamelus scutellaris* on dispersal plants, from different treatments (1. Kilo Max – Source & two Dispersal sprayed, 2. Seismic – Source & two Dispersal Sprayed, 3. Kilo Max – Source Unsprayed & two Dispersal Sprayed, 4. Seismic – Source Unsprayed & two Dispersal Sprayed, 5. No herbicide), recorded biweekly for 11 weeks. Error bars represent standard error of means (n = 10).

Table 4.1: Interactions between source herbicide treatments (1. Kilo Max – Source & Dispersal sprayed, 2. Seismic – Source & Dispersal Sprayed, 3. Kilo Max – Source Unsprayed & Dispersal Sprayed, 4. Seismic – Source Unsprayed & Dispersal Sprayed, 5. No herbicide) and dispersal treatments (sprayed and unsprayed) on *Megamelus scutellaris* populations. Values presented in bold are significant.

Coefficients	**Estimate**	**Std. Error**	**z value**	***P***
Intercept	**2.559808**	**0.236862**	**10.807**	**< 2e-16**
Unsprayed	**2.388952**	**0.331461**	**7.207**	**5.71e-13**
Kilo Max Source & two Dispersal Sprayed	0.467100	0.333535	1.400	0.1614
Seismic Source & two Dispersal Sprayed	-0.002581	0.334983	0.008	0.9939
Seismic Source Unsprayed & two Dispersal Sprayed	-0.135005	0.335529	-0.402	0.6874
No Herbicide	**0.719165**	**0.327726**	**2.194**	**0.0282**
Unsprayed: Kilo Max Source & two Dispersal Sprayed	**-1.135497**	**0.467970**	**-2.426**	**0.0152**
Unsprayed: Seismic Source & Dispersal Sprayed	-0.103568	0.468792	-0.221	0.8252
Unsprayed: Seismic Source Unsprayed & two Dispersal Sprayed	-0.207918	0.469257	-0.443	0.6577

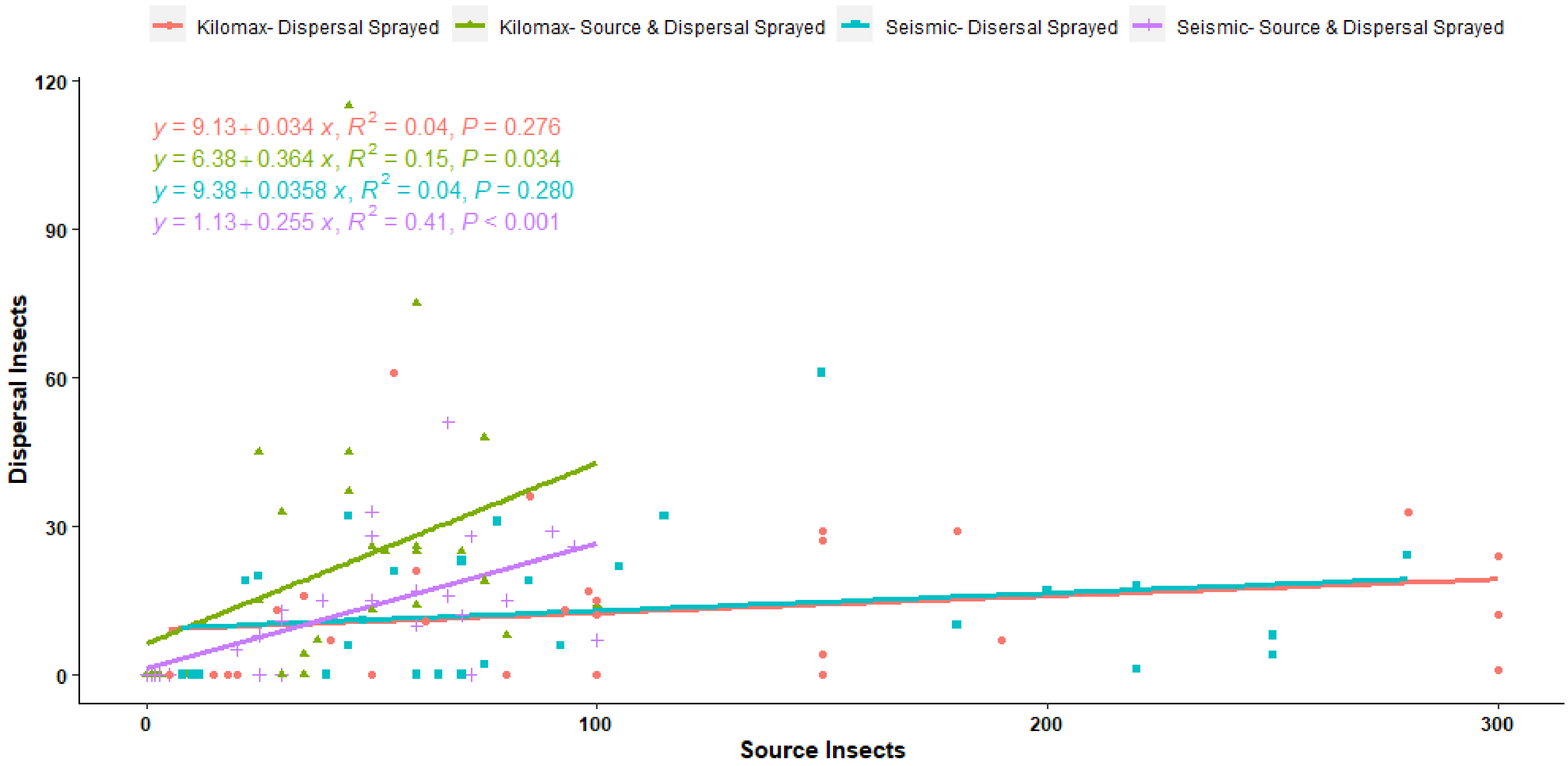

Figure 4.5: Relationship between dispersal and source treatments *Megamelus scutellaris* populations over different treatments.

4.3.3. Population dynamics of *Megamelus scutellaris* on the source plants

This section describes the plant and insect population patterns from the centrally placed source tubs. The insects established in all the source mesocosms for the first weeks of experiments. There were significant interactions between treatment and duration on the insect populations (χ^2 = 2.60, DF = 20, *P* = 0.0007). However, there were no significant differences in insect population size from the source treatments where the plants were not sprayed *viz*. Kilo Max – source unsprayed and two dispersal sprayed, Seismic – source unsprayed and two dispersal sprayed, and No herbicide – source and dispersal unsprayed and No herbicide treatment (χ^2 = 0.268, DF = 1, *P* = 0.60), throughout the duration of the study (Figure 4.6). The insect numbers in the Kilo Max – source unsprayed and two dispersal sprayed treatment showed a rapid increase from week 3 (96.2±18.7 *M. scutellaris*/m^2) to week 7 (208.0±39.8 *M. scutellaris*/m^2), after which they greatly decreased until week 11 (21.6±4.6 *M. scutellaris*/m^2). Although the population numbers were lower, the populations in Seismic – source unsprayed and two dispersal sprayed followed a similar trend. A rapid increase of insects was observed from week 3 (87.0±16.9 *M. scutellaris*/m^2) to week 7 (191.0±36.6 *M. scutellaris*/m^2), and quickly decreased from week 9 (63.0±12.4 *M. scutellaris*/m^2) until week 11 (20.2±4.3 *M. scutellaris*/m^2). The No herbicide treatment (source and dispersal unsprayed) routinely had higher insect populations than other treatments and showed a peak population on week 5 (mean ± SE = 225.0±43.0 *M. scutellaris*/m^2), but it was not different from the Seismic and Kilo Max unsprayed source mesocosms.

The insect numbers recorded on sprayed source treatments, Kilo Max – source sprayed and two dispersal sprayed, and Seismic – source sprayed and two dispersal sprayed were not significantly different from each other over the duration of the study (χ^2 = 1.35, DF = 2, *P* = 0.51). The insect populations in the Kilo Max – source and two dispersal sprayed treatment showed a gradual increase from week 1 (41.0±8.4 *M. scutellaris/*m^2), week 3 (53.0±10.45 *M. scutellaris*/pool) to week 5 (75.0±14.7 *M. scutellaris*/m^2). Although insects persisted on Kilo Max treated plants, a slow decline was observed from week 7 to week 9 (47.0±9.4 and 17±3.7

M. scutellaris/m^2) until the end of the study (1.0±0.5 *M. scutellaris*/m^2). The same pattern was observed for Seismic–source and two dispersal sprayed treatment, where *M. scutellaris* populations showed an increase from week 1 to week 3 (53±10.6 and 75.0±14.7 *M. scutellaris*/m^2) but declined from week 5 (68.0±13.4), until the end of the experiment (2.0±0.7 *M. scutellaris/*m^2).

Peak population densities were recorded in all the treatments from week 3 to week 7, after which the populations dramatically declined and remained low until the end of the experiment. By week 11, all treatments had almost no biological control insects, including unsprayed source mesocosms. This massive insect decline in unsprayed plants could be due to overcrowded insect densities, while the plant quality deterioration of *P. crassipes* could have contributed to the demise of *M. scutellaris* densities.

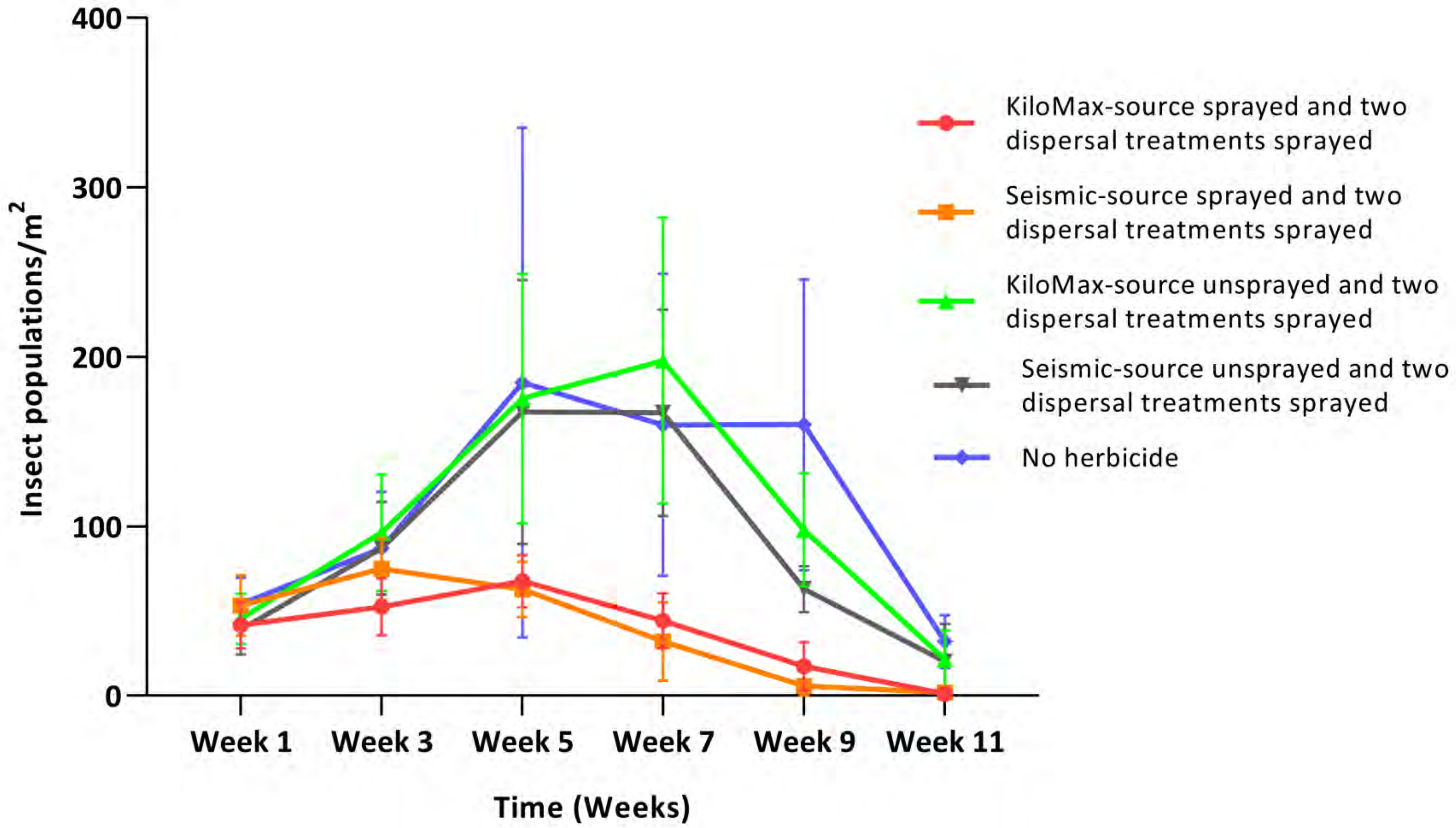

Figure 4.6: *Megamelus scutellaris* population densities (number of insects in the source mesocosms) in different treatments (1. Kilo Max – source and two dispersal sprayed, 2. Seismic – source and two dispersal sprayed, 3. Kilo Max – source unsprayed and two dispersal sprayed, 4. Seismic – source unsprayed and two dispersal sprayed, 5. No herbicide) over the 11 week sampling period. Error bars represent Standard error of means (n = 5).

At week 11, *P. crassipes* plant populations in the source treatments were significantly reduced by sublethal herbicide treatments, which resulted in a reduction in biological control insect populations. The results showed that plant populations influenced *M. scutellaris* population densities in all treatments ($F_{1,15}$ = 19.85, P = 0.0014). High densities of insects were recovered from the control (No herbicide) treatments (32±6.6 *M. scutellaris/*m^2) compared to other treatments, which also had high plant density (11±0.8 *P. crassipes*/m^2) by Week 11. The lowest insect population densities were recorded on treatments, Kilo Max - source and two dispersal sprayed (1.2± 0.5 *M. scutellaris*/m^2) and Seismic – source and two dispersal sprayed (2.0±0.7 *M. scutellaris*/m^2), at Week 11, which also yielded the lowest plant densities, 4±0.8 and 5.4±0.8 *P. crassipes*/m^2 respectively. The treatments, Kilo Max – source unsprayed and two dispersal sprayed and Seismic – source unsprayed and two dispersal sprayed, had higher plant densities (10±1.4 and 9±1.4 *P. crassipes*/m^2, respectively) than the other treatments, except the control. Consequently, there were relatively more insects recovered in these treatments (22±4.6 and 20±4.3 *M. scutellaris*/m^2 respectively) compared to other treatments. There was a positive and significant correlation between insects and *P. crassipes* populations in all the treatments (y = -14 + 3.6x, R^2 = 0.46, P = 0.00018) (Figure 4.7).

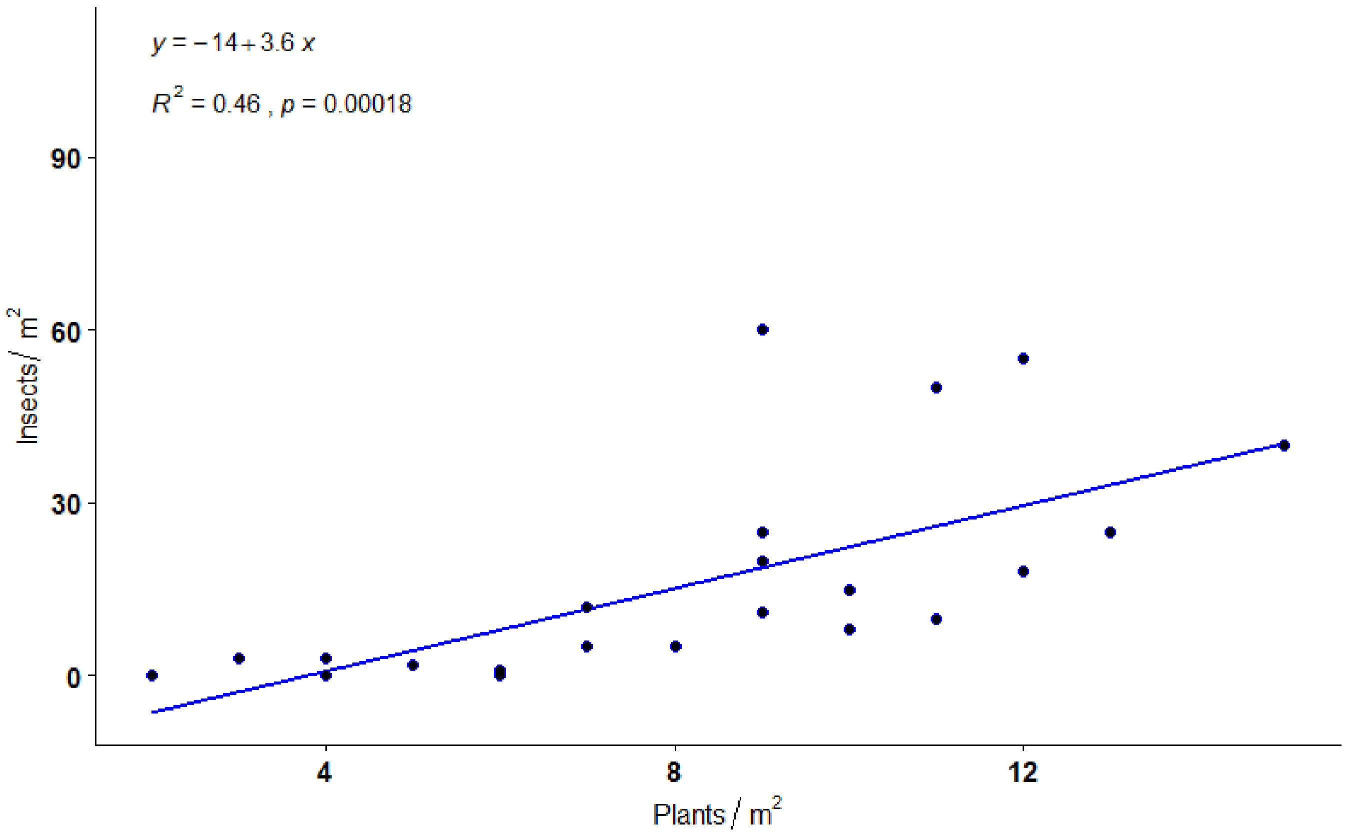

Figure 4.7: Relationship between *Pontederia crassipes* density and *Megamelus scutellaris* density in different herbicide treatments at week 11.

4.4. Discussion

The paired host choice tests in the current study revealed that *M. scutellaris* adults had a preference for unsprayed *P. crassipes* leaves over the unsprayed leaves, after a few days of exposure. This suggests that the herbicide-treated plants become less attractive for *M. scutellaris* feeding, and, to increase their reproductive output and survival chances, the insects are more attracted to the untreated leaves. A study by Del Fosse & Perkins (1977) reported that a natural *P. crassipes* allelochemical, kairomone acts as an attractant to the biocontrol agents, *N. eichhorniae* and *O. terebrantis*. It is possible that *M. scutellaris* uses contact or olfactory cues from the leaf surface to make a choice on preferred food and oviposition host. Anton & Cortesero (2022) reported that the balance of phagostimulants and antifeedants is crucial for herbivorous insects to accept their food host; however, a decision to feed on a plant after tasting it depends on a trade-off between the presence of toxic compounds and the nutritional value. Many studies have explored the effects of stress-induced changes in food quality on insect herbivore performance and population growth (Mattson & Haack 1987, Koricheva et al. 1998, Kansman et al. 2020). However, very little evidence has been provided on insect herbivore host selection due to herbicide stress. The results from the previous chapter suggest that the sublethal herbicide concentration raises the sugar levels on sprayed plants a few days following application, which could explain the initial attraction of insects to both the sprayed and unsprayed plants. Recently, Machado et al. (2021) also showed that sugar and benzoxazinoid production in maize acted as distinct and dynamically combined mediators of short-distance host finding and acceptance for western corn rootworm, *Diabrotica virgifera virgifera* LenConte 1868 (Coleoptera: Chrysomelidae). A similar study by Olaya-Arenas et al. (2020) reported that monarch butterflies (*Danaus Plexippus* L.) avoided oviposition on the plants that were treated with maximum field concentrations of herbicides, but did oviposit on plants treated with sublethal concentrations.

The dispersal treatments demonstrated that *M. scutellaris* dispersed from the source plants to dispersal tubs. However, initially, the insects migrated to both sprayed and unsprayed plants for the first three weeks in all the mesocosm experiments. These results suggested that even though there were no differences with the unsprayed plants, sublethal concentrations of glyphosate herbicides did not have detrimental effects to *M. scutellaris*. Previous studies have found that insects can establish populations on plants associated with stress (Larsson 1989, Teshome et al. 2020), possibly due to biochemical changes known to occur in stressed plants, and that such establishment may result in improved insect growth and performance (Durak et al. 2021, Rix & Cutler 2022, Hassan et al. 2023). It is possible that reduced herbicide stress stimulates growth of an insect herbivore population which then elicits stress signals in a host plant, resulting in the significant reduction of host plant communities. Some studies have investigated a concept of hormesis to understand the increasing insect density on plants treated with low herbicide concentration (Durak et al. 2021, Cutler et al. 2022). Calabrese and Blain (2009) introduced the concept of hormesis, demonstrating the considerable difference in the effects of high and low concentrations of herbicides, with a high concentration causing stress and inhibition and a low concentration being a stimulant of many processes, which favours the development of herbivorous insects. Durak et al. (2021) found that hemp aphids, *Phorodon cannabis* (Hemiptera: Aphididae), did not migrate from hemp plants treated with sublethal concentrations of graminicide herbicide; instead, the insect fecundity doubled. In the present study, it was evident that the loss of food sources drove *M. scutellaris* away from deteriorating habitats to search for new, healthy sources, as their densities increased in the unsprayed plants, and as they decreased in sprayed plants when the plants were dying. Haag (1986a) showed that *Neochetina* weevils used to control *P. crassipes* could move from dying plants treated with the standard concentration of 2.4 – D herbicide to nearby untreated ones. Furthermore, Goode et al. (2021) recently showed that *M. scutellaris* insects were able to disperse away from areas treated with different concentrations of herbicides to reach healthy mats of *P. crassipes*. Understanding concepts of dispersal patterns and population dynamics is important for the establishment of biological control agents in fluctuating environmental conditions created by frequent applications of herbicides to control *P. crassipes*.

Herbicides typically disrupt insect abundance by either increasing mortality or reducing their reproductive output (Sharma et al. 2018, Sánchez-Bayo 2021). Kraus and Stout (2019) reported that herbicides may alter the quality and quantity of weed populations. This, in turn, results in alterations in the structure of insect herbivore communities. A few studies have suggested that some herbicides, particularly at sublethal concentrations, can increase insect populations (Messersmith & Adkins 1995, Saska et al. 2016). The present study tested the effect of sublethal concentrations of two routinely used glyphosate-based herbicides (Kilo Max and Seismic) on *M. scutellaris* populations in semi-field conditions and showed that these herbicide concentrations affect the biocontrol insect populations. The results showed that *M. scutellaris* was able to persist on the sprayed plants and the populations were not different from the unsprayed source mesocosms for the first three weeks of the experiments. However, from week 5 to 11, the insect numbers decreased significantly on the herbicide-sprayed plants when compared to the unsprayed source mesocosms. According to these results, the sublethal herbicide concentrations did not reduce biocontrol agent populations, but the loss of their food sources as a result of herbicide spraying reduced insect populations in the treated plants. It was evident that *M. scutellaris* insect numbers decreased when the conditions of the host plant became unfavourable. Notably, the decrease of insects was also observed on unsprayed mesocosms after seven weeks of the experiments (Figure 4.4). This was not fully understood but was assumed to be related to increased insect density in untreated plants, which triggers natural insect dispersal and, therefore, a decrease of populations at release points. Dispersal may come at a cost of delayed reproduction, reduced fecundity and longevity, and increased risk of mortality while migrating (Denno et al. 1991, Fitzgerald & Tipping 2013).

4.5. Conclusion

The current study showed that sublethal concentrations of glyphosate-based herbicides supported the population growth of *M. scutellaris* insects, causing damage to the sprayed plants, thereby decreasing plant population size. From these observations, we can conclude that the deterioration of *P. crassipes* plants was due to combined insect herbivory and sublethal herbicide application. Despite the reduced insect abundance and performance resulting from herbicide application, the remaining insect populations were sufficient to cause observable damage when acting in conjunction with the herbicides. The insects dispersed to unsprayed plants when the condition of sprayed plants deteriorated, suggesting that plant quality rather than herbicide treatment is responsible for the dispersal of *M. scutellaris* insects. Biocontrol insect dispersal during herbicide applications is crucial for their population preservation as this influences their ability to navigate environments, find optimal habitats, and maintain fitness, thereby impacting overall weed management strategies. However, the results from this study are based on indoor mesocosm experiments, which present very different variables from field settings. Even though these were preliminary studies that aimed to mimic what happens in the field, the results suggest that the application of integrated chemical and biocontrol treatments of invasive plants should take into consideration the need to preserve biocontrol agents during herbicide applications. The influence of sublethal concentrations of herbicides on the populations of the insects, and therefore, the management of *P. crassipes* needs to be tested in outdoor field settings. The following chapter (Chapter 5) will examine the population dynamics of *M. scutellaris* and its dispersal patterns following sublethal herbicide applications on a large scale at a field site in Bronkhorstspruit, Gauteng, South Africa.

CHAPTER 5: Population dynamics and dispersal patterns of *Megamelus scutellaris* on *Pontederia crassipes* as influenced by sublethal concentration of glyphosate-based-herbicide applications in the field

5.1. Introduction

Understanding population dynamics and natural dispersal patterns of biological control agents from the release to target points has important implications for their establishment (Heimpel & Asplen 2011). The aim of biocontrol programmes is to rapidly build up biological control agent populations throughout the range of the weed and thereby reduce the weed's population. The rates of dispersal of agents are highly variable; for example, slowly dispersing agents may require numerous releases in relative proximity to the source, while agents that disperse rapidly may only require a few widely dispersed releases to achieve rapid population build-up (Paynter & Bellgard 2011). An ability to predict agents' rate of dispersal enables biocontrol practitioners to better plan and allocate resources towards optimising agent releases and establishment, to build up agent populations that are adequate for weed suppression.

The most efficacious agent, recently released for *P. crassipes* in South Africa, *M. scutellaris,* continues to disperse from release sites, causing significant damage, and it has successfully established across the country. However, the hypereutrophic status of South African aquatic ecosystems significantly enhances the rate of *P. crassipes* growth, thereby reducing the impact of the current suite of biological control agents (Coetzee & Hill 2012). Also, the interference from repeated herbicide sprays prevents the agents from achieving their maximum impact on *P. crassipes* because spraying rapidly reduces plant populations over a wide area, which then significantly reduces biological control agent populations (Center et al. 1999, Hill & Olckers 2001, El-Sayed 2005). Despite the repeated herbicide treatments that commonly occur, *P. crassipes* populations rebound quickly and can double in biomass in less than two weeks (Goode et al. 2021). In addition to vegetative growth, this species reproduces from seed, which can remain viable in the hydrosoil for up to twenty years (Barrett 1980, Sullivan & Wood 2012).

Previous studies have reported that neither herbicide application nor biological control alone can sustainably eradicate *P. crassipes* (Tipping et al. 2017). It has been suggested that active integration of biological control agents with herbicides could increase the efficacy of *P. crassipes* control. However, integrating these control strategies is difficult because of the coordination and patience necessary; biological control agents require time to build up their population size, during which plant populations grow rapidly with minimal suppression from herbivory. Dispersal of biological control agents plays an important role in the re-colonisation of herbicide-treated areas, as insects move from herbicide-treated mats of *P. crassipes* into the new expanding mats. Therefore, the objectives of this chapter were to monitor *M. scutellaris* population dynamics and dispersal patterns and to quantify its impact on *P. crassipes* populations in the field after an application of herbicide at the sublethal concentration identified in Chapter 2. I hypothesised that the biocontrol agents would disperse from the sprayed *P. crassipes* plants, increase their populations and cause damage to the adjacent unsprayed plants.

5.2. Materials and Methods

5.2.1. Study site

The population dynamics of *P. crassipes* and its biological control agents after sublethal herbicide applications were investigated at Bronkhorstspruit Dam (25° 53’ 15” S, 28° 43’ 15” E) (Figure 5.1), Gauteng Province, South Africa. Bronkhorstspruit is a human-made, concrete-arch dam that was established in the 1950s with the main purpose of supplying water for domestic supply and industry use. The dam also serves as a huge tourist attraction, for boating and fishing. *Pontederia crassipes* was first observed in Bronkhorstspruit Dam early in 2021 and is spreading rapidly, covering over 1.8 km of the Clover Hill Eastern shoreline, which makes it impossible to launch watercraft (Paddy Waller, Estate Manager, Pers. Comm. 2023). *Neochetina eichhorniae* and *N. bruchi*, which are both leaf-feeding weevils, and *M. scutellaris*, which is a sap-sucking plant hopper, were released on *P. crassipes* on Bronkhorstspruit Dam in 2021 (Paddy Waller, pers. comm., 2023). Recently, efforts to integrate biological control agents and sublethal concentrations of glyphosate herbicides have been trialled to manage *P. crassipes* proliferation in this system.

In Chapter 2, the sublethal concentrations of three glyphosate-based herbicides currently used in South Africa for the management of *P. crassipes* were identified. The identified sublethal concentration of Kilo Max (0.4%) increased insect populations in the sprayed plants and influenced their dispersal during the mesocosm-based studies. The effect of this sublethal herbicide concentration on population dynamics of *M. scutellaris* and their dispersal behaviour was assessed at Bronkhorstspruit Dam for sustainable integrated management of *P. crassipes*.

5.2.2. Study Design

Two insect release and herbicide spray sites were established and paired with two control (refuge) sites, which were located a meter from the sprayed sites, one at Clover Hill Western Shoreline and one at Summer Place Eastern Shoreline (Figure 5.1). *Neochetina* weevils and *M. scutellaris* insects were sourced from the Waainek Rearing Facility, Grahamstown. Approximately two thousand insects were released at both treatment sites every two weeks, prior to sublethal herbicide applications (Paddy Waller, pers. comm., 2023). Following herbicide applications with sublethal concentrations of Kilo Max herbicide (0.4%), the sites were monitored for three months, from March 2023 to May 2023. The sites were monitored for population fluctuations of both *M. scutellaris* and plant community persistence during *P. crassipes* control measures (herbicide treatments and water level changes), as well as local dispersal of *M. scutellaris* within existing mats of *P. crassipes* between the sprayed (treatment) and unsprayed (control) sites.

Figure 5.1: Bronkhorstspruit Dam, Gauteng Province. Sampling sites (Clover Hill and Summer Place) are represented by yellow pointers, the red dots represent the sprayed plots, while the blue dots represent the unsprayed plots.

5.2.3. Data collection at the release and refuge sites

Pontederia crassipes plant parameters from both sites (Clover Hill and Summer Place) in the sprayed and refuge areas were collected every month for the period of three months. A method by Coetzee & Hill (2012), where the longest leaf, leaf area, maximum root length, number of ramets (daughter plants), number of flowers, and number of leaves were recorded for ten randomly selected plants from each site was used for plant sampling. For biomass, three randomly selected quadrats (0.5 m^2) were thrown into the *P. crassipes* mat, and the plants were collected in each quadrat. The collected plants were divided into live emergent plant parts, live submerged plant parts, and dead plant material, and then the biomass was weighed and recorded (kg. m^2). The number of individual plants in each quadrat was also recorded.

The number of feeding scars caused by the weevils, *N. eichhorniae* and *N. bruchi*, was counted on leaf 2 (the second unfurled leaf) of ten randomly chosen plants, and the number of adult weevils on each of the ten plants was counted and recorded. Additionally, the total number of *M. scutellaris* per square metre from ten randomly selected quadrats at each site was determined using a specialised insect planthopper sampler. The sampler was created using "the Minteer Method" where an 80 L dustbin base was cut off, and a wire cross was attached 30 cm from the base (Minteer et al. 2016). In order to quantify *M. scutellaris* numbers, the sampler was used to totally submerge the plants in situ, forcing the insects to float to the water's surface and onto the sides of the bin, thereby allowing easy quantification. The number of insects floating on the sides of the bin was counted and recorded. The selection of sites was based on their accessibility, and monitoring was conducted by wading from the bank. Temperature data for the sites was obtained from South African Weather Services (SAWS) to determine if environmental conditions had an impact on insect populations.

5.2.4. Statistical analysis

All data were analysed using R v4.2.3. and RStudio (R Development Core Team, 2024). The normality and homogeneity of the data were checked using Shapiro-Wilk and Levene's tests. When the data failed normality tests, Kruskal-Wallis ANOVA followed by Dunn's tests were used. Repeated measures ANOVAs were used to determine the influences of sites (Summer Place and Clover Hill), treatments (Sprayed and Unsprayed) and months (March, April and May) on plant parameters and insect populations. Linear regression analysis was used to determine the relationships between the density of *M. scutellaris* and plant populations at both sites between the sprayed and unsprayed areas. The influence of treatments and environmental factors on insect populations was analysed using generalised linear mixed models (GLMM) fit by using Template Model Builder (glmmTMB package in R and lme4 package), and site was used as a random effect. The logarithmic link function, GLM (family = Poisson (link = "log")) was used. To fit the model, backwards selection was used (drop1 function in R, lme4 package); this removes variables which contribute the least to the model until the lowest AIC is reached.

5.3. Results

The influence of a sublethal concentration of glyphosate-based herbicide, Kilo Max, on the *P. crassipes* plant populations and its biocontrol agents, was recorded at two treatment and two refuge sites at Bronkhorstspruit Dam for three months.

5.3.1. *Pontederia crassipes* plant parameters

Throughout the sampling dates, significant interactions between the sprayed and unsprayed treatments in Clover Hill and Summer Place sites in leaf petiole length (Figure 5.2A) were observed ($F_{2,108} = 8.38$, $P < 0.05$). At the Clover Hill site, the tallest petioles were found on the untreated refuges rather than on the sprayed sites (Figure 5.2A). The plants from the sprayed area at Clover Hill decreased and wilted and sank by May, and therefore sampling was not possible. The same pattern for the longest petiole length was observed at Summer Place, although the plants persisted for the duration of the sampling period ($F_{1, 108} = 57.5$, $P < 0.05$). The plants sprayed with sublethal herbicides were shorter than the plants in unsprayed refuge areas in both sites (Figure 5.3A). Root lengths were significantly different across the sites on both sprayed and unsprayed areas throughout the sampling time ($F_{2,108} = 5.21$, $P < 0.05$). At Clover Hill, the roots were longer on sprayed plots in March and April, even though there were no differences compared to unsprayed plants (Figure 5.2B). The root lengths of plants in the sprayed area at Summer Place were also longer than the roots in the unsprayed refuge areas, but no differences between the sprayed and unsprayed treatments (Figure 5.3B). By May, the roots in the sprayed area were significantly longer than the roots in the unsprayed areas (Figure 5.3B).

There were significant interactions between treatments, site, and sampling dates on the number of leaves ($F_{2,108} = 4.19$, $P < 0.05$). More leaves were produced at the unsprayed refuge area at the Clover Hill site than at the sprayed area throughout the sampling period (Figure 5.2C). At Summer Place, the leaf production was not significantly different between the two treatments in March and April. However, in May, more leaves were evident at the unsprayed site than at the sprayed site (Figure 5.3C). Ramet production was not significantly influenced by treatments, site, and date of sampling ($F_{1,108}$ = 0.68, P = 0.50), although ramet production decreased over time at both Clover Hill and Summer Place sites, as the plants at the sprayed sites stopped producing ramets. However, there was no significant difference between sprayed and unsprayed treatments ($F_{1,108}$ = 2.36, P = 0.12) (Figure 5.3D).

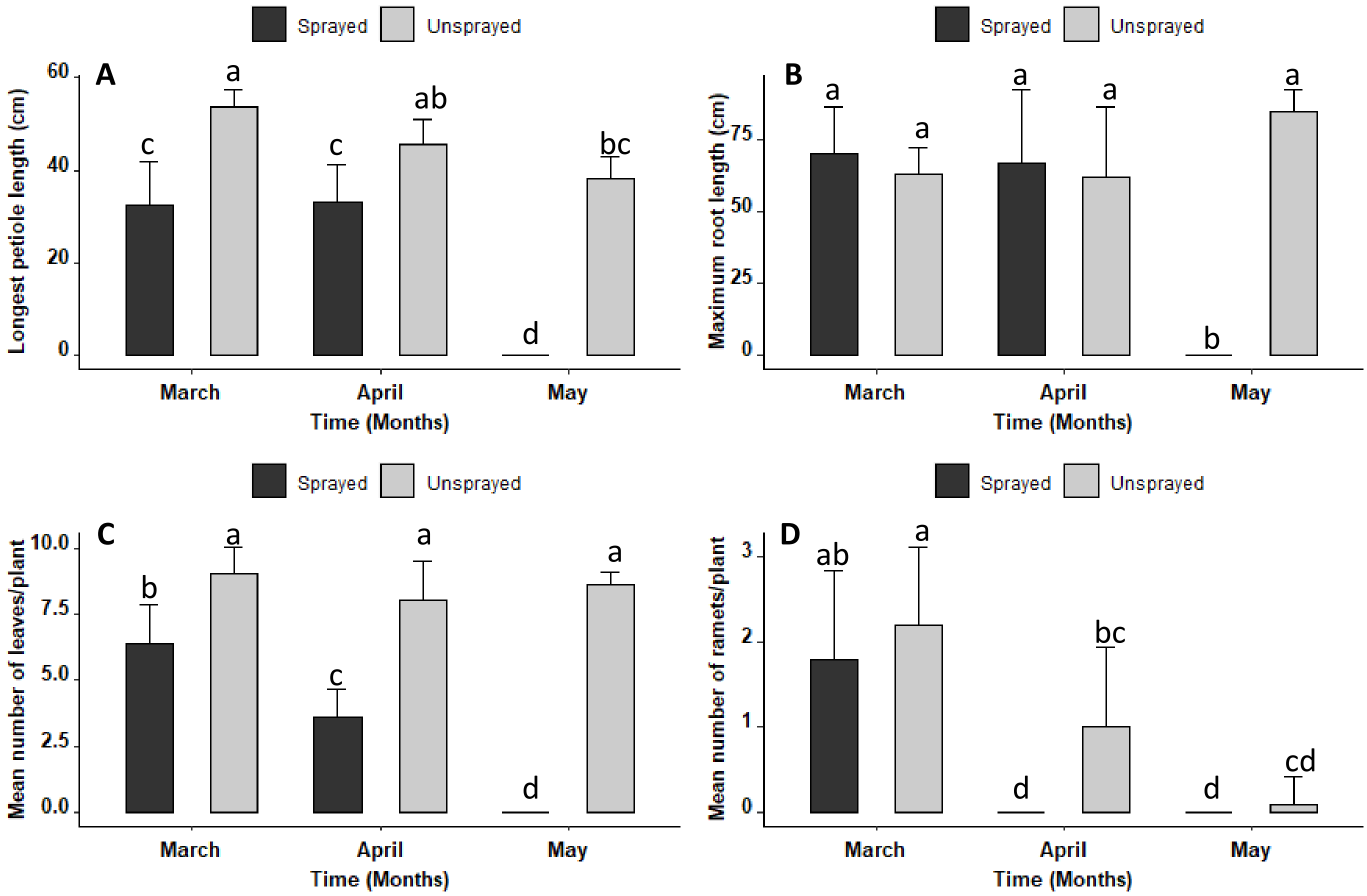

Figure 5.2: *Pontederia crassipes* plant parameters at Clover Hill (Bronkhorstspruit Dam) recorded over a three-month period (A- average long petiole length, B- average root length, C- average number of leaves per plant, D- average number of ramets per plant). Error bars represent Standard Error. Bars with the same letter are not significantly different from each other.

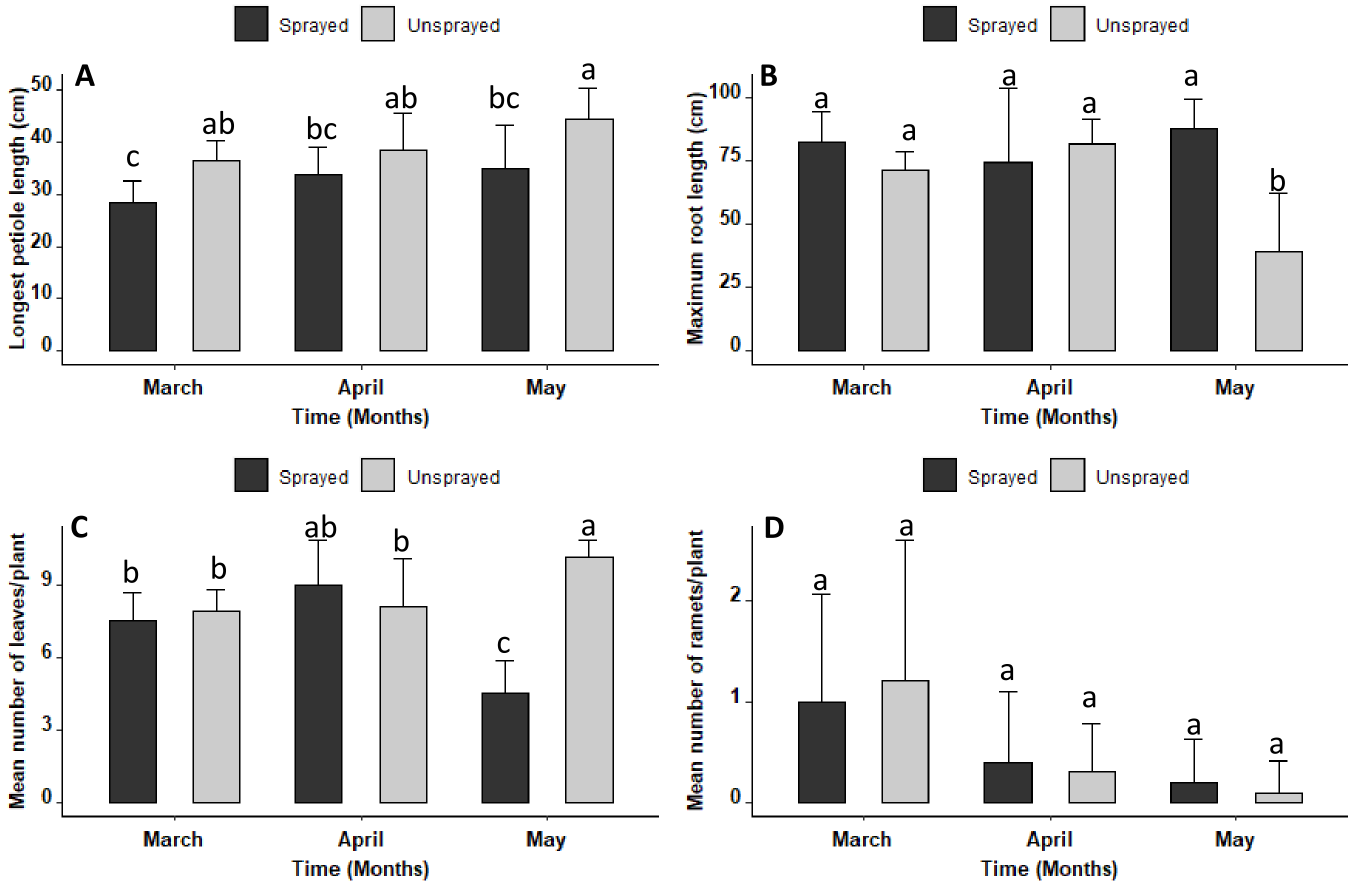

Figure 5.3: *Pontederia crassipes* plant parameters at Summer Place (Bronkhorstspruit Dam) recorded over a three-month period (A- average long petiole length, B- average root length, C- average number of leaves per plant, D- average number of ramets per plant). Error bars represent Standard Error. Bars with the same letter are not significantly different from each other.

Across the sampling dates, there were significant interactions between the sprayed and unsprayed treatments on the above-water plant biomass (fresh weight biomass) ($F_{2,24} = 16.77$, $P < 0.05$) (Figure 5.4A & 5.5A). However, there were no significant differences between the two sample sites ($F_{1,24} = 2.59$, $P = 0.21$). The below-water biomass (root biomass) was not significantly influenced by treatment, site, and sampling dates ($F_{2,24} = 1.86$, $P < 0.18$) (Figure 5.4B & 5.5B). Even though there was more root biomass in the unsprayed plants, there were no significant differences between the treatments. An exception with significantly more root biomass was recorded in the unsprayed treatment at Clover Hill during May ($F_{1,24} = 4.43$, $P <$ 0.05) (Figure 5.4B). Significant interactions for the accumulated dead material biomass ($F_{1,24}$

= 16.02, $P < 0.05$) were observed between treatments, sites and sampling dates (Figure 5.4C & 5.5C). *Pontederia crassipes* plant densities differed significantly between sites ($F_{1,24} = 13.96$, $P = 0.001$) (Figure 5.4D & 5.5D). However, no significant differences were observed between the treatments, even though the plant density of the sprayed plants was slightly greater than the plant density of the unsprayed plants ($F_{1,24} = 0.22$, $P = 0.64$).

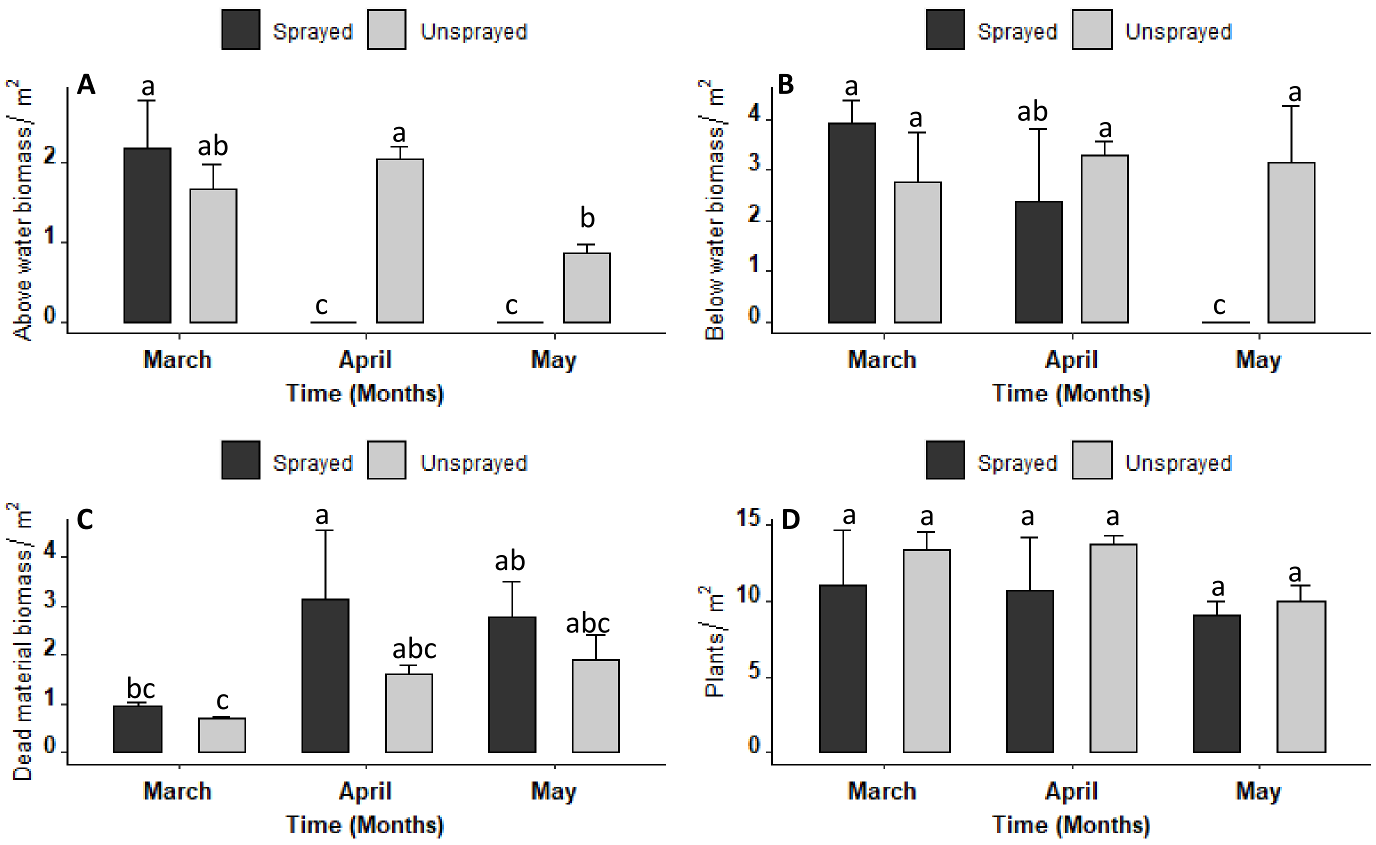

Figure 5.4: *Pontederia crassipes* plant biomass (A. Above water biomass, B. Below water biomass, C. Dead material biomass) and plant densities (D. Plants) in the sprayed and unsprayed refuge areas at Clover Hill. Error bars represent standard error. Means with the same letter are not significantly different from each other.

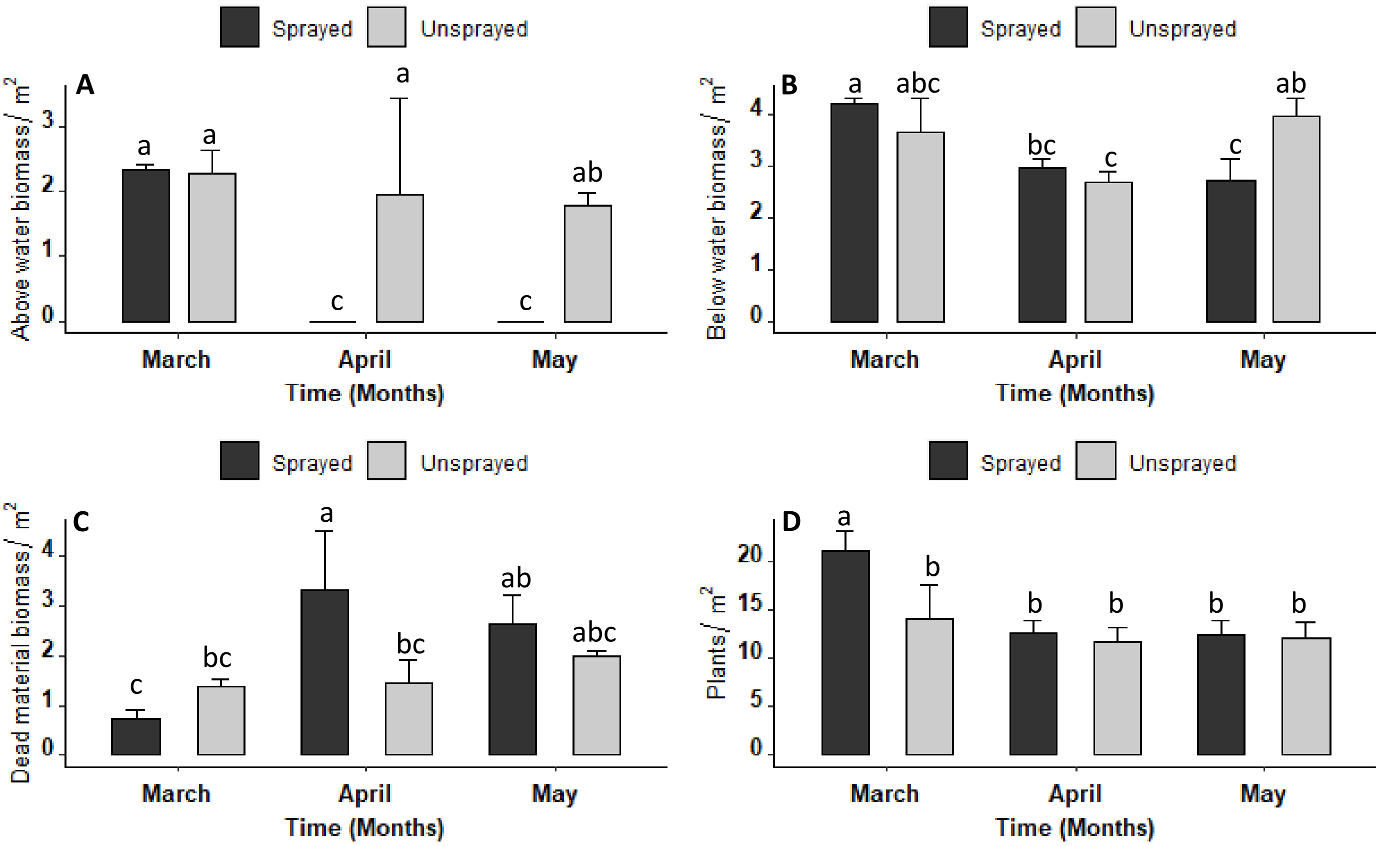

Figure 5.5: *Pontederia crassipes* plant biomass (A. Above water biomass, B. Below water biomass, C. Dead material biomass) and plant densities (D. Plants) on the sprayed and unsprayed refuge areas at Summer Place site. Error bars represent standard error. Means with the same letter are not significantly different from each other.

5.3.2. *Megamelus scutellaris* population parameters

Successful integration of biocontrol programmes with other management strategies for weed management requires a rapid increase in insect populations. There were significant interactions between treatments, sites and sampling dates on *M. scutellaris* population densities ($\chi^2 = 254.6$, DF = 2, $P < 0.001$). At Clover Hill, there was no significant difference in insect density between the sprayed plot (62.6±7.32 *M. scutellaris*/m^2) and the unsprayed plot (40.9±7.32 *M. scutellaris*/ m^2) in the first month of sampling (Figure 5.6). However, in the second and third months, the insect densities decreased significantly in the sprayed plants and increased in the unsprayed plants (54.9±7.32 *M. scutellaris*/m^2 in April and 40.6±7.32 *M. scutellaris*/ m^2 in May). At Clover Hill, the decrease in plant populations in sprayed plots caused a decrease in insect populations and an increase in unsprayed plots with high plant populations, thereby showing a significant positive correlation ($y = -39 + 6.2x$, $R^2 = 0.32$, $P =$

0.0014) (Figure 5.7). At Summer Place, the insect density on the sprayed plants increased with time (Figure 5.9). In March, the insect density from the sprayed area was significantly lower than the densities from the unsprayed plants. In the second and third months, however, there were no differences in insect densities between the sprayed plants (86.8±10.6 *M. scutellaris*/m^2 in April and 68.6±10.6 *M. scutellaris*/m^2 in May) and the unsprayed plants (75.6±10.6 *M. scutellaris*/m^2 in Apil and 59.5±10.6 *M. scutellaris*/m^2 in May). The insects decreased where there were high plant populations. There was a significant negative relationship between the plant and insect populations in Summer Place (y = 150 – 5.5x, R^2 = 3.1, P = 0.017) (Figure 5.9).

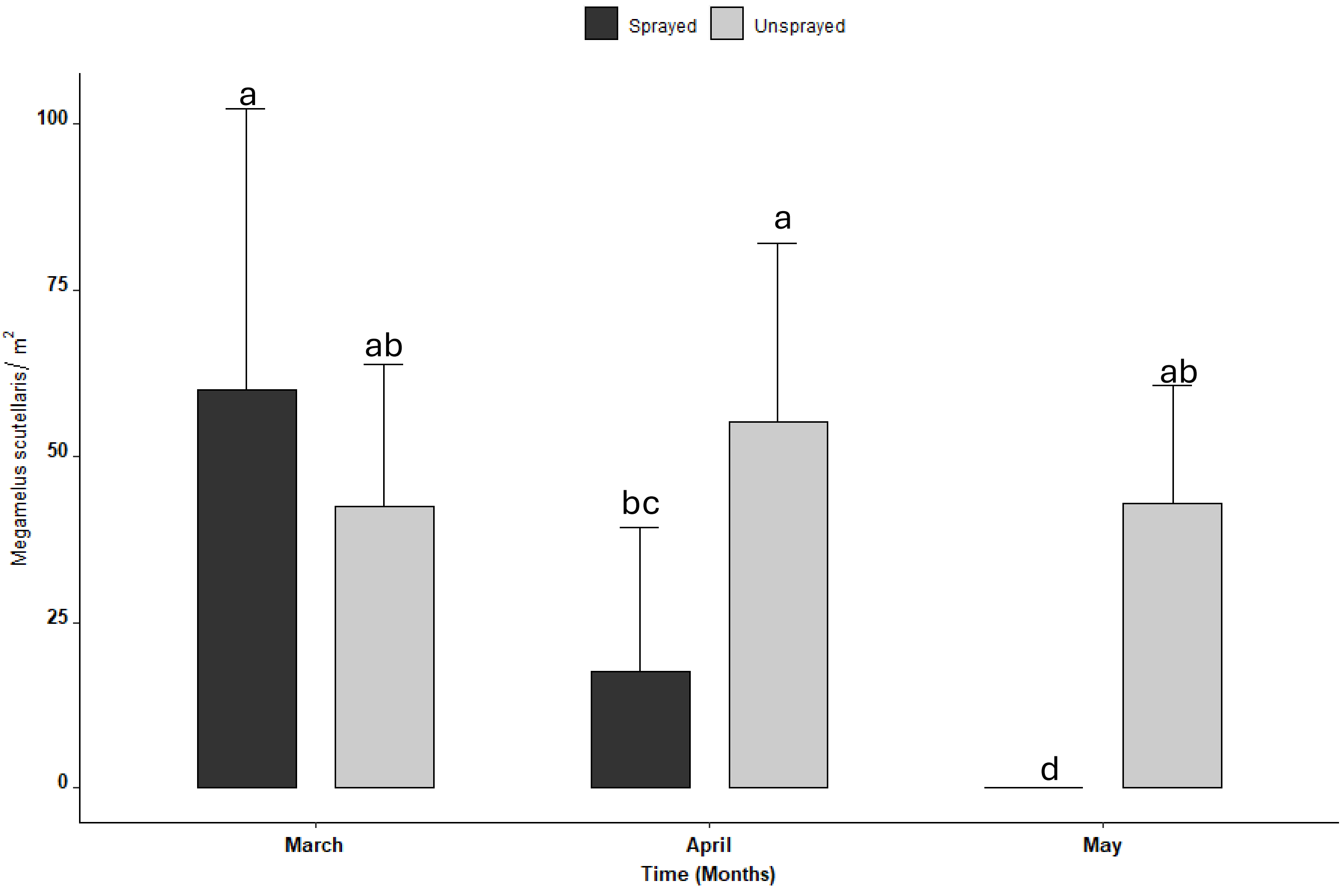

Figure 5.6: *Megamelus scutellaris* densities recorded in Clover Hill at the herbicide-sprayed and refuge areas for three months (March, April, May). Means with the same letters are not significantly different from each other; error bars represent standard error.

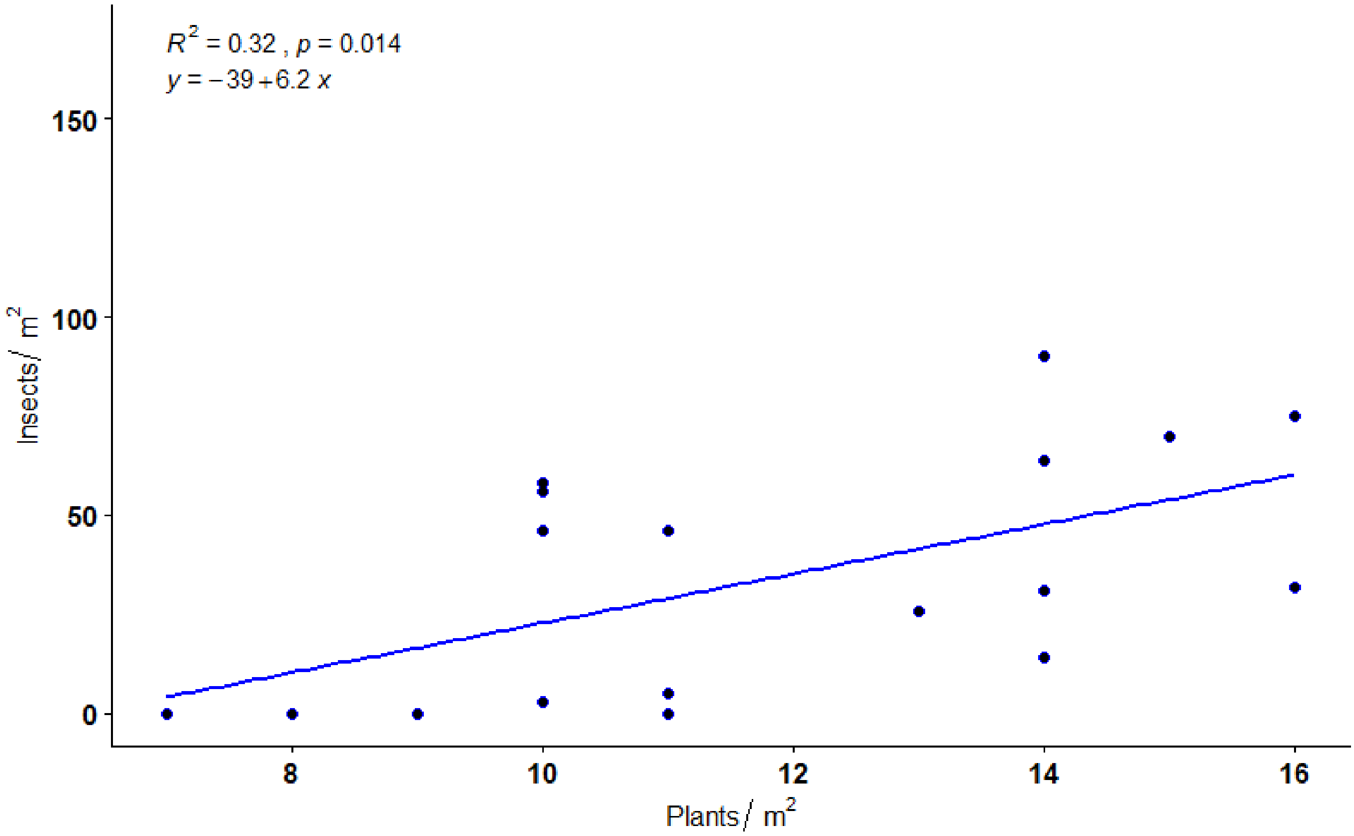

Figure 5.7: Relationship between *Megamelus scutellaris* insects and *Pontederia crassipes* plant populations in Clover Hill.

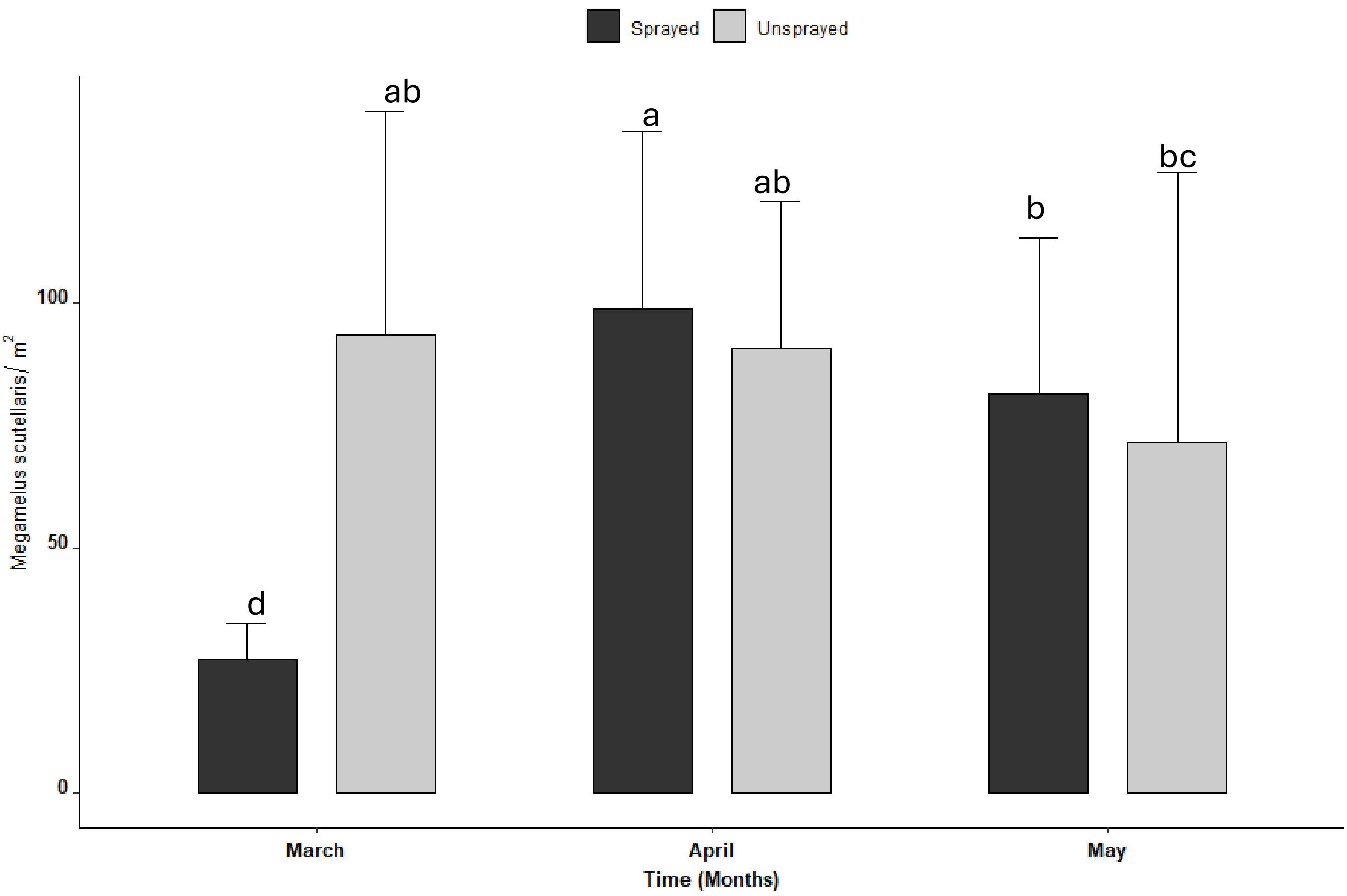

Figure 5.8: *Megamelus scutellaris* densities recorded at Summer Place from the herbicide-sprayed and refuge areas for three months (March, April, and May). Means with the same letters are not significantly different from each other; error bars represent standard error.

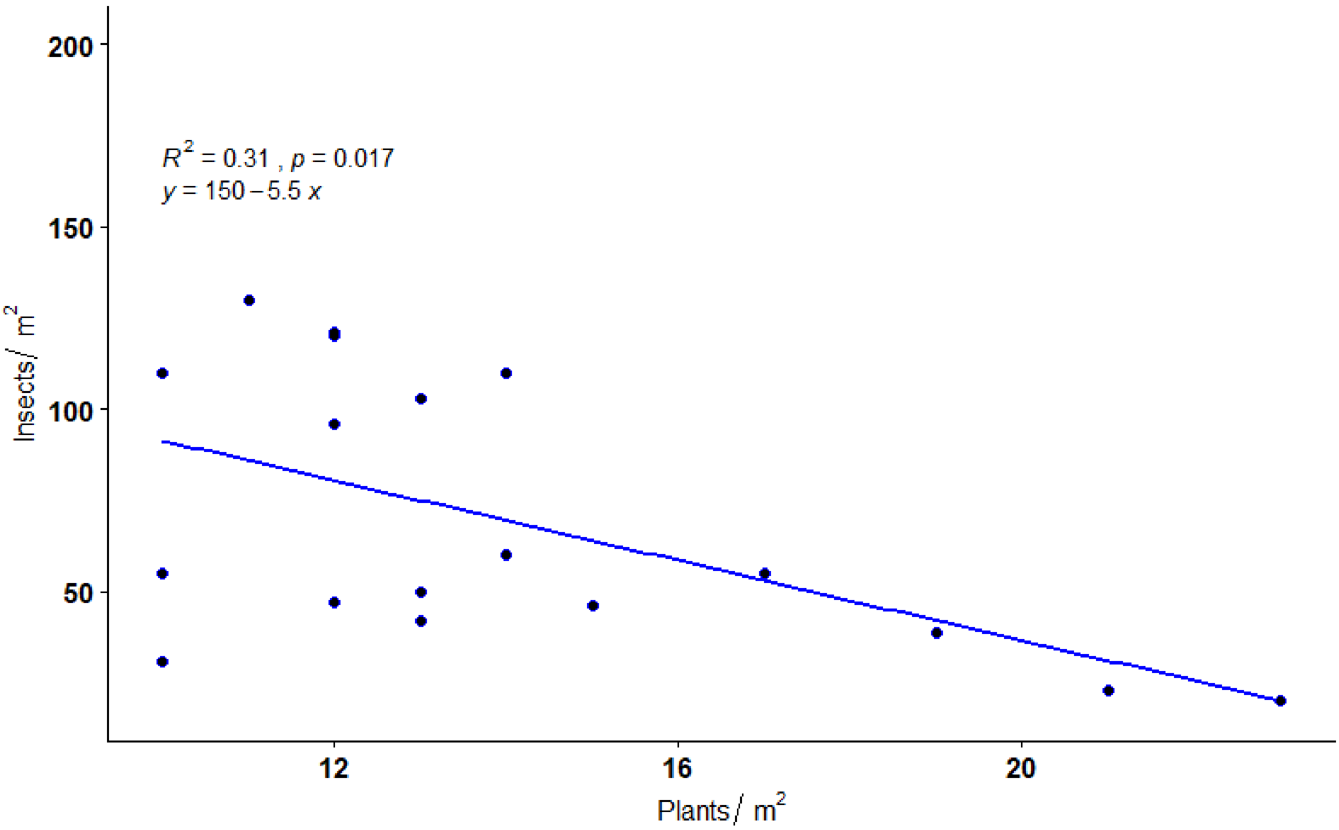

Figure 5.9: Relationship between *Megamelus scutellaris* insects and *Pontederia crassipes* plant populations in Summer Place.

The effect of sampling dates and environmental conditions on insect densities was tested with a generalised linear model. The treatment (χ^2 = 50.02, DF = 1, $P < 0.001$), site (χ^2 = 82.5, DF = 1, $P < 0.001$), and minimum temperature (χ^2 = 55.9, DF = 1, $P < 0.001$) significantly influenced the insect numbers. As plants became unsuitable, insects decreased in sprayed treatments and dispersed to the unsprayed treatments. The insects decreased with time (Figures 5.6 & Figure 5.8) at both sites; it is possible that the minimum temperatures experienced in May (which is the beginning of the winter season) caused a reduction in insect development.

5.3.3. Feeding damage by *Neochetina* weevils

The feeding damage caused by weevils differed significantly throughout the sampling period at the sprayed and unsprayed treatments at Clover Hill and Summer Place (χ^2 = 91.8, DF = 2, $P < 0.001$). *Neochetina* weevils persisted and continued to damage the plants, even in the sprayed areas. At Clover Hill, the damage was significantly higher in the refuge areas than in the sprayed plants (Figure 5.10). The feeding scars increased on the unsprayed plants in the second month of sampling (9.52±1.32 scars/leaf 2). However, a significant decline was observed in the third month (4.05±0.93 scars/leaf 2). At Summer Place, plants in the untreated areas had more damage (20.27±4.2 scars/leaf 2 and 15.17±3.7 scars/leaf 2), than those in the sprayed areas (12.73±2.8 scars/leaf 2 and 8.63±1.9 scars/leaf 2) in March and April, respectively. However, in May, there were more weevil feeding scars on the sprayed plants (10.17±2.5 scars/leaf 2) than on the unsprayed plants (5.63±1.2 scars/leaf 2) (Figure 5.11).

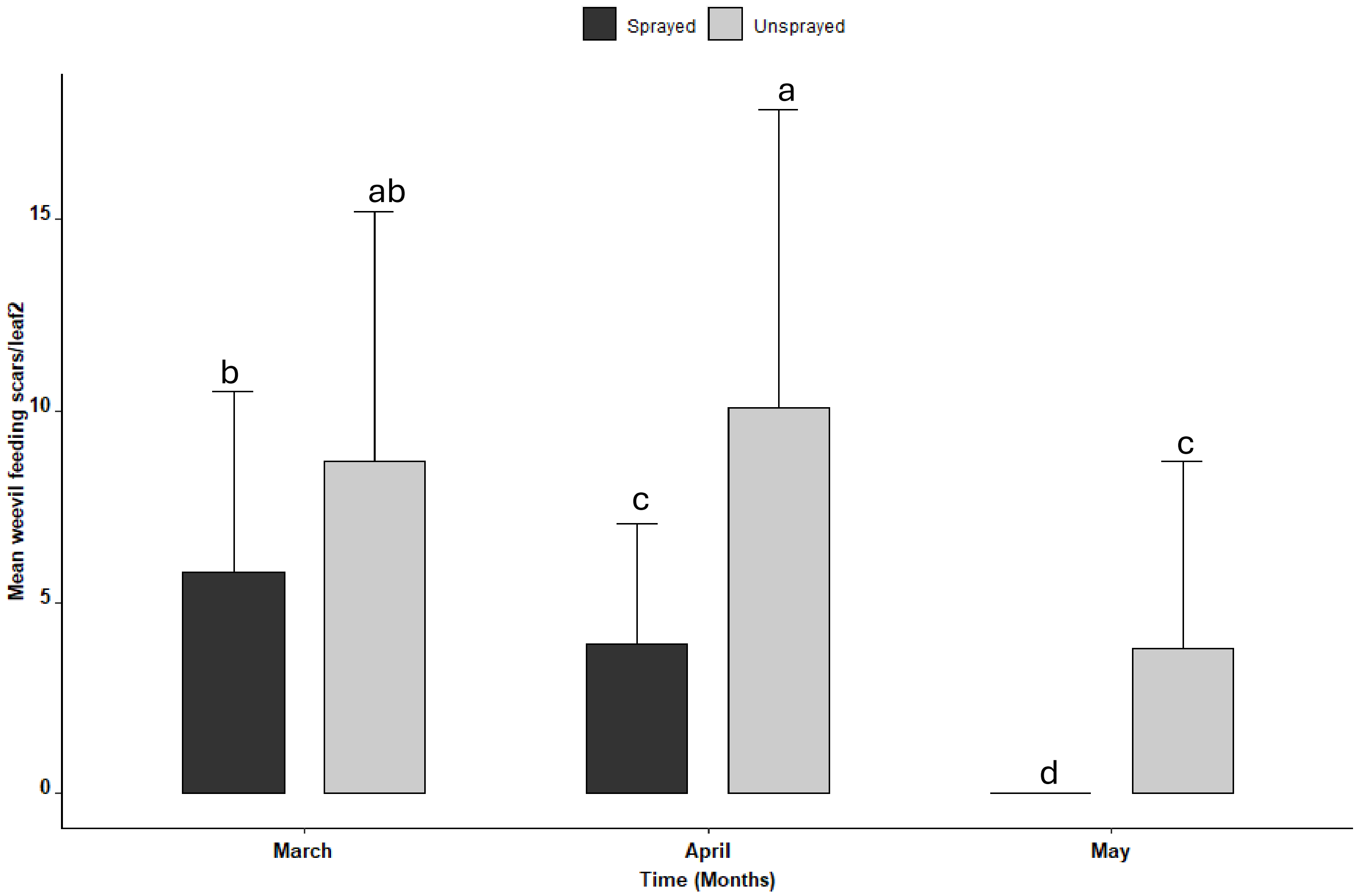

Figure 5.10: *Neochetina* weevils feeding damage on leaf 2 over a 3-month period at Clover Hill. Means with the same letters are not significantly different from each other; error bars represent standard error.

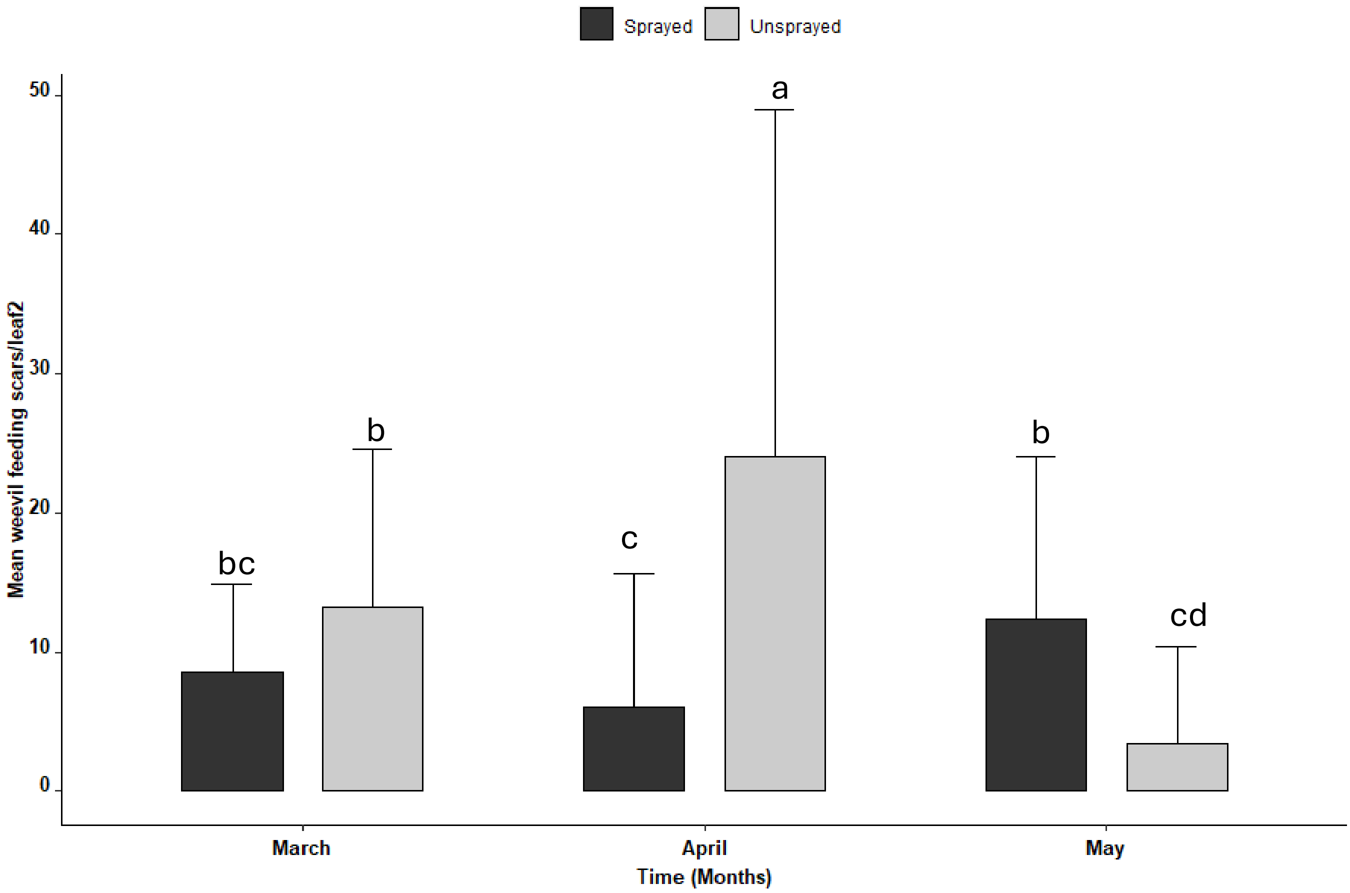

Figure 5.11: *Neochetina* weevils feeding damage on leaf 2 over a 3-month period at Summer Place. Means with the same letters are not significantly different from each other; error bars represent standard error.

5.4. Discussion

The results of the field studies were similar to those reported for indoor mesocosms in the previous chapter (Chapter 4). At Clover Hill, the plant parameters (longest petiole, root length, leaves, ramets) were significantly reduced in the sprayed plots compared to the control unsprayed area. Moreover, the unsprayed plants had more living biomass and higher plant density than the sprayed plants. Similarly, at Summer Place, even though there were no significant differences, the plant parameters were reduced in the sprayed site compared to the unsprayed refuges. By May, the plant quality and plant densities of the sprayed plants had significantly declined at both sites. It is possible that this decrease in plant growth and plant density in the sprayed sites was due to applications of sublethal glyphosate herbicide combined with herbivory by released biocontrol agents. Studies have reported that the sublethal herbicide-induced hindrance of vegetative growth of the *P. crassipes* plants results in substantial levels of damage by the biological control agents and further suppression of the weed through continued herbivory (Gettys et al. 2014, Goode et al. 2021).

The reduction in plant populations led to a decrease in insect populations in the sprayed plants at the Clover Hill site. The *M. scutellaris* densities and *Neochetina* weevil feeding damage increased in the unsprayed area as the plant density in the sprayed area declined. In this study, it was clear that the biocontrol agents dispersed from the sprayed plants and increased their populations in the unsprayed areas. Similar results were reported from the indoor mesocosm in Chapter 4, where *M. scutellaris* insects survived on the sublethal herbicide-treated plants and dispersed to the unsprayed healthy mats when plant quality and density reduced. However, at the Summer Place site, *M. scutellaris* populations persisted and feeding damage by *Neochetina* weevils increased in the sprayed plants compared to unsprayed plants, even though the population numbers were relatively low. During field herbicide applications, it seems feasible to spray weed mats selectively, where portions of the weed mats could be left unsprayed, to preserve the populations of biocontrol insects. Studies by Haag et al. (1988) highlighted that *Neochetina* weevil density increased on unsprayed plants following herbicide application at a recommended rate of 6.7 kg/ha on Alachua Co. Ponds, Florida, USA. Recently, Tipping et al. (2017) reported that when combining a reduced rate of herbicide and biological control treatment, the insects dispersed from the sprayed dying plants and became highly concentrated on the few surviving *P. crassipes* plants that were missed during spraying. However, some studies have shown that herbicides, particularly at sublethal concentrations, can positively influence insect populations (Rohr & Crumrine 2005, Sharma et al. 2018). This was observed at Summer Place site where the insect populations were higher in the sprayed plants compared to unsprayed plants, even though the numbers were relatively low. There is a widely accepted theory based on many observations, that when herbivorous insects are under stress, they tend to reach outbreak populations (Durak et al. 2021). This hypothesis is based on two arguments: First, there are many observations that herbivorous insects frequently reach explosive densities on plants associated with environmental stress (Larsson & Tenow 1984, Subedi et al. 2023); Second, certain biochemical changes known to occur in stressed plants have been suggested as stimulating herbivorous insect performance (White 1974, Galway et al. 2004). Lipok (2009) reported that the use of sublethal concentrations of phosphate herbicides decreased the plant resistance and increased populations of aphids on the treated plants. However, this was not the case during the current studies, as the insects did not reach explosive numbers in the sprayed areas. The dispersal of *M. scutellaris* populations in the current research may be attributed to disruptions of physiological processes that impact nutrient composition in plants after sublethal glyphosate herbicide applications. The role of plant quality in the population dynamics of herbivorous insects has been much debated in recent years (Larsson 1989, Larsson et al. 2000, Kozlov et al. 2022) and was discussed in the previous chapter of this study (Chapter 3).

The dispersal behaviour of biological control insects is an important aspect of their lifecycle that has obvious implications for where populations will be exerting pressure on weed populations (Parry 2022, Ramya & Singh 2022) During the degradation process caused by herbicide application, treated plants may release chemicals which can be sensed by insects, subsequently causing an alteration in the insect's searching behaviour (Collin & Marshall 2003). Another possibility for dispersal is the insect's ability to maximise the offspring's survival through a non-random selection of oviposition sites, which is referred to as the 'preference-performance hypothesis' or 'mother knows best hypothesis' (Jaenike 1978, Thompson 1988, Clark et al. 2011, Salgado et al. 2020). This implies that the female insects prefer to lay their eggs on suitable host plants which possess qualitative traits that appear to favour offspring survival and performance. Even though there were high densities in unsprayed plants, the populations declined in winter at both sites. Minimum temperatures during this study had a significant influence on insect densities. Cold temperatures offer significant metabolic challenges to insects, causing reduced survival, fecundity and feeding frequency (Kingsolver 1989, Miller et al. 2020). Foley et al. (2016) reported that the adult lifespan and fecundity of *M. scutellaris* change with temperature. In South Africa, the populations of *M. scutellaris* are affected by cold winter temperatures. The lower developmental threshold (t_0) for *M. scutellaris* is 11.458 ℃, and insect development below this threshold ceases (May & Coetzee 2013). Indeed, Miller et al. (2020) reported a decline in

M. scutellaris populations at the onset of winter at the Kubusi Dam, South Africa. During our field study, the mean daily minimum temperatures in May went as low as –3°C, which is very low for *M. scutellaris* development and survival.

Overall, the results from the current study showed that a sublethal concentration of glyphosate-based herbicides was not toxic to the *M. scutellaris* insects; however, the diminished plant populations and changes in season contributed to the decreased insect populations, thereby potentially impacting their population dynamics and dispersal patterns and the overall control of the plants. During this study, it was difficult to assess the efficacy of biocontrol agents on plant damage on unsprayed areas, as *M. scutellaris* population numbers were low at both field sites. It is possible that the number of insects released at the dam resulted in populations that were unable to sustain themselves due to disturbances of plant populations by environmental conditions. Goode et al. (2019) reported that low release numbers of biological control agents is linked to failure to establish damaging populations in the field. Coetzee et al. (2022) showed that multiple releases of *M. scutellaris* in large numbers to inundate *P. crassipes* is most likely to lead to successful control of the plants; however, this was not the case for the current research study. Bronkhorstspruit Dam may benefit from this multipronged approach where inundative releases (i.e., large insect numbers released at multiple life stages) of biocontrol agents are made frequently, particularly when sublethal herbicide applications are also being done. This approach may enhance the establishment of sustainable damaging biocontrol populations, that will provide a quick knockdown of the *P. crassipes* plants that are skipped during herbicide applications.

5.5. Conclusion

The local populations of biological control insects in natural environments are primarily influenced by the abundance and suitability of food, shelter, and population density. This study showed biological control agents persisted in areas sprayed with sublethal herbicide concentration and caused damage to the plants. Fresh weight biomass and plant density were decreased in sprayed compared to unsprayed areas at both sites, suggesting that sublethal herbicide concentrations combined with biocontrol by *M. scutellaris* improved *P. crassipes* control. From these findings, it can be concluded that the combined effect of *M. scutellaris* herbivory and sublethal herbicide stress reduces the vigour of *P. crassipes* plants, and thus, this integrated approach may reduce the reliance on herbicide use. It is recommended to combine inundated insect herbivory with sublethal herbicide applications as part of an integrated weed management approach to improve the sustainability and efficacy of weed control initiatives. Insect feeding might further reduce plant vigour and possibly increase herbicide uptake, whereas sublethal herbicide concentrations can weaken target weeds, leaving them more vulnerable to harm from insect herbivores. This strategy can provide longer-term weed population control and lessen the need for lethal herbicide dosages. However, considerable consideration must be given to the timing, intensity, and environmental factors impacting both herbicide efficacy and herbivore activity.

CHAPTER 6: General discussion

6.1. Introduction

Biological control of *P. crassipes* in South Africa has been considered slow-acting and in some cases unsatisfactory by land users (Hill & Coetzee 2008, Katembo et al. 2013). Insects for weed biological control often take years to establish effective population densities and may be only marginally effective, therefore a combination of control methods may offer faster control, that is sustainable in the long term. Integrated control, combining herbicide applications and biological control agents has been considered a more successful approach for *P. crassipes* management (Van Wyk & Van Wilgen 2002, Xu et al. 2024). Studies have reported that this method provides the most satisfactory control by reducing weed density more quickly than a biocontrol insect alone, or by increasing success with biological control insects where they would be marginally effective alone (Byrne et al. 2010, Hill et al. 2012, Katembo et al. 2013). Scientists in the past usually assumed that classical biocontrol and chemical weed control techniques were incompatible (Messersmith & Adkins 1995, Hill et al. 2012) but now recognise that their integration may result in improved weed control programmes. However, the use of herbicides in a traditional manner (i.e., lethal concentrations) interferes with the establishment of biological control agents and adds toxic chemicals to the environment (Hill et al. 2012).

To counteract or reduce the negative impacts induced by herbicides on biocontrol agents, and to reduce the amount of herbicide used in chemical control programmes, the use of sublethal concentrations of herbicides has been suggested. Sublethal herbicide concentrations reduce plant growth rates, and if tested correctly, have no harmful effects on biological control agents. Moreover, some studies recommend leaving unsprayed areas to serve as a refuge for biocontrol agents to increase the effectiveness of this method (Ainsworth 2003, Tipping et al. 2017, Goode et al. 2019). The current study demonstrated that applying sublethal concentrations of glyphosate-based herbicide reduces *P. crassipes* proliferation by retarding its growth and decreasing its biomass. Moreover, it was determined that the sublethal herbicide concentrations influenced the overall primary metabolism of *P. crassipes*, which lowered the accumulation of its high–energy compounds (i.e., proteins and carbohydrates). This study also highlighted that integrating biological control with sublethal herbicide treatments enhances insect dispersal patterns to unsprayed areas, aiding the preservation of biocontrol agent populations during herbicide application.

6.2. Identifying sublethal concentrations of herbicides and their effects to biocontrol insects

The use of sublethal herbicide concentrations in combination with biocontrol agents for weed control has been previously reported. Herbicides applied at label-recommended rates can be toxic to biocontrol agents, either by killing them directly or by rapidly eliminating their host plants (Hill et al. 2012, Kraus & Stout 2019). However, sublethal concentrations can preserve habitat for biocontrol agents, allowing them to build up their populations. The current study emphasised the importance of identifying sublethal herbicide concentrations during integrated management of *P. crassipes*. The sublethal concentrations of herbicides maximise the impact of both biological and herbicidal control without sacrificing the efficacy of weed control.

The current study also showed that applying sublethal concentrations of glyphosate-based herbicides slowed the vegetative growth of *P. crassipes* plants and reduced its biomass accumulation (Chapter 2). The reduced vigour in *P. crassipes* growth has been previously reported where plants treated with sublethal concentrations of glyphosate-based herbicide produced significantly fewer ramets and leaves (Jadhav et al. 2008; Katembo et al. 2013). These herbicide concentrations were not toxic to *M. scutellaris*, as was illustrated by reduced insect mortality when they were exposed to sublethal herbicide concentrations. *Megamelus scutellaris* survived and oviposited on plants treated with Kilo Max (0.4%) and Seismic (0.75%). There was also an increased number of insects in the *P. crassipes* plants sprayed with the sublethal herbicide concentration of Kilo Max compared to unsprayed control plants (Chapter 2). These findings support the hypothesis that sublethal concentrations preserve habitat for biocontrol agents and enhance their control efficacy and agree with several other studies reported in the literature. A study by Ahn et al. (2001) showed that there were no direct harmful effects caused by the sublethal glufosinate–ammonium herbicide to the coccinellid *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) insects. Adult insects of *Galerucella calmariensis* L. (Coleopetera: Chrysomelidae), a biological control agent for *Lythrum salicaria* L. (Lythraceae), were alive and robust after exposure to a sublethal concentration of glyphosate herbicide. The possibility of implementing sublethal herbicide concentrations on spray regimes during integrated control management relies on their compatibility with biological control agents. Even though the application of sublethal herbicide concentrations reduces their toxic effects in several plant species and are non–toxic to insects, they still disrupt their biochemical pathways, which are essential for producing nutrients that are beneficial to insect herbivores.

6.3. Biochemical effects of sublethal herbicide concentrations on *Pontederia crassipes* plants

The biochemical responses evoked in plants treated with the sublethal concentrations of glyphosate-based herbicide, Kilo Max (0.4%), indicated metabolite accumulation was induced in the treated leaves. The current study showed that there was a significant accumulation of carbohydrates for the first few days of treatment, which declined significantly after more than a week. A similar study by Xu et al. (2022) showed increased sucrose content in maize plants treated with low concentrations of nicosulfuron herbicide. It is possible that plants exposed to herbicides might increase sugar storage to ensure they have sufficient energy to survive the stress, which could lead to an accumulation of sugars in plant tissues (Dos Santos et al. 2022, Jeandet et al. 2022). Some studies have reported that herbicide exposure inhibits phloem transport and causes increased carbohydrate accumulation in treated leaves (Zulet et al. 2013, Zulet-Gonzalez et al. 2013).

The increased accumulation of proteins was also observed after exposure to sublethal concentration of Kilo Max herbicide application. This indicated that primary metabolism was affected by sublethal concentrations of herbicide. Previous studies have reported that EPSPS inhibitors target the enzymes that are associated with the synthesis of proteins. For example, Kumar et al. (2019) suggested that high concentrations of glyphosate-based herbicide significantly reduced protein content in treated plants compared to untreated plants. However, the sublethal concentration used in the current study did not inhibit protein synthesis in the treated plants. This concentration of herbicide proved to be beneficial for the accumulation of essential primary metabolites that support slow growth of plants, while making them more palatable to insect herbivores.

It is well understood that insect dispersal is strongly driven by the nutritional quality and accumulation of metabolites in the host plants. The success of biocontrol insects largely relies on their ability to search and choose suitable host plants for oviposition and survival. Previous research has exhibited that insects use primary metabolites as cues to identify nutritious hosts and tissues (Chapman 2003, Machado et al. 2021). A study by Wang et al. (2020) showed that soluble carbohydrate and protein content in plants were positively associated with tuberworm [*Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae)] host preference and survival. Dispersal of insects to suitable host plants with higher nutritional contents will offer them the optimal nutrient balance required to maintain metabolic normalcy, thereby increasing their chances of survival and consequently, their populations.

6.4. Investigating the effects of sublethal herbicide concentrations on biocontrol host preference, population dynamics and dispersal patterns.

The second chapter (Chapter 2) of the current research showed that sublethal herbicide treatments can support the development of biological control agent populations, as they do not rapidly reduce plant abundance. However, it is possible that after a few days, the sublethal herbicide treatment will reduce the nutritional quality of the plants by producing metabolites which are not favoured by the insects. The survival and success of biological control insects in herbicide-managed areas depend largely on their ability to locate suitable host plants. This study revealed that after a few days of sublethal herbicide applications, *M. scutellaris* adults preferred feeding on unsprayed plants than sprayed *P. crassipes* leaves. *Megamelus scutellaris* use their contact or olfactory cues from leaf surfaces to choose a suitable food source. A natural allelochemical, kairomone, produced by healthy *P. crassipes* plants, acts as an attractant to biocontrol agents. It is possible that a sublethal herbicide spray disrupts the production of chemicals that attract insects after a few days of application, which could explain why the insects are more attracted to the unsprayed plants.

A few studies have suggested that some herbicides, particularly at sublethal concentrations, can increase the populations of insects in the sprayed areas. Our results showed that for the first three weeks of treatment, *M. scutellaris* populations on the sprayed mesocosms were not different from those on the unsprayed mesocosms. After three weeks, however, the plant populations in the sprayed mesocosms decreased, leading to a significant reduction in insect populations, explaining a positive significant relationship between *P. crassipes* and insect populations. This decrease in plant population was attributed to the combination of insect herbivory and sublethal concentrations of herbicide. It is possible that a sublethal herbicide concentration stimulates growth of an insect herbivore populations which elicits stress signals in a host plant, resulting in significant reduction in host plant communities. Biocontrol agents have been shown to reduce both biomass and reproduction in *P. crassipes*, while rendering the weakened plants more susceptible to herbicides (Tipping et al. 2017). Consequently, the loss of habitat in the source mesocosms due to the combined effects of sublethal herbicide applications and insect herbivory initially triggered insect dispersal to the neighbouring plants.

The increase in *M. scutellaris* density in unsprayed dispersal mesocosms in our study was due to the insects dispersing from sprayed dying plants. This study showed that the insects increased in the dispersal mesocosms as they decreased in the source mesocosms, indicating a significant negative correlation. Similar results have been reported during biocontrol and herbicidal control integration for weed management. Recently, Goode et al. (2021) found that when plants are treated with different concentrations of herbicides and begin to degrade, adult *M. scutellaris* insects will disperse to the new mats, while nymphs migrate to healthy untreated plants within the same mat of plants and increase their local densities. Earlier studies by Haag et al. (1986a) also showed that *Neochetina* weevils dispersed away from dying *P. crassipes* mats treated with a standard recommended concentration of 2, 4–D herbicide to nearby unsprayed plants. Even though the present study used sublethal concentrations, they presented similar results.

The results of this study indicated that a change in plant quality after herbicide application triggered *M. scutellaris* dispersal to unsprayed plants. The capacity to disperse can preserve critical densities of biological control agents, subsequently improving the efficacy of *P. crassipes* biological control. Our experiments were conducted in indoor mesocosms, intended to mimic field conditions, and therefore, we implemented this concept in larger–scale field environments (Chapter 5). Although our experiments were in controlled environments, which may under- or overestimate the field conditions, they provided a basis for active adaptive management of *P. crassipe*s in the field.

6.5. Effects of sublethal concentrations of herbicides on *Megamelus scutellaris* population dynamics and dispersal behaviour for *Pontederia crassipes* control in the field.

In natural habitats, there is strong evolutionary selection pressure on herbivorous insects to avoid danger and choose suitable host plants. Similar selection pressures may drive dispersal and choices of oviposition hosts by herbivorous insects used as biological control agents in areas where herbicides are often used for weed management. In our study, *P. crassipes* plant biomass and plant populations were significantly reduced in sprayed areas as a consequence of integration of *M. scutellaris* herbivory and sublethal herbicide applications to the plants. The decrease in plant populations in sprayed areas subsequently led to a decrease in biological control insects in both sites that were sampled. These results were not different from those reported in the indoor mesocosms. The insects increased in unsprayed plants as they decreased on the sprayed plants, probably due to insects dispersing from sprayed dying plants to healthy unsprayed plants. Our study was similar to a study by Goode et al. (2021) who reported that in a few sites in Florida, where different herbicide concentrations were used, *M. scutellaris* adults were found moving 15 – 20 m, weeks after following a herbicide application at a site. These confirm that when plants are treated with herbicides and their quality decreases, adults will disperse to new mats, and their densities in unsprayed mats will increase. The unsprayed *P. crassipes* mats provided a stable habitat for biocontrol agents. The higher densities within the unsprayed plots indicated that sublethal herbicide sprays facilitated the dispersal of biocontrol insects away from the sprayed areas in search of high-quality plants. This has previously proved to help maintain biological control agent populations in locations where herbicide treatments occur regularly (Haag 1986a, Haag & Habeck 1991, Goode et al. 2019).

The increase of insect populations in unsprayed areas was expected to enhance the level of herbivory damage, which would subsequently cause the death and collapse of the unsprayed *P. crassipes* mats. However, similar to the previous chapter (Chapter 4), insect herbivory in this study did not cause any significant damage to unsprayed *P. crassipes* plants. *Megamelus scutellaris* populations could not increase at rates comparable to the *P. crassipes* mats, therefore their level of control for the weed was less effective. This study agrees with previous studies conducted by Haag et al. (1988) and Haag (1986b) in Alachua County Ponds, Florida who reported that after sprayed plants sank, *P. crassipes* populations in the unsprayed ponds expanded to fill open water and developed larger mats of *P. crassipes*, thereby reducing the intensity of *Neochetina* weevil feeding damage. This was owing to weevil populations increasing more slowly than *P. crassipes* plants. To achieve successful integrated control of *P. crassipes*, it is important to have high levels of biocontrol agent populations in the unsprayed plots, which will increase the feeding damage, when the plant growth rate increases. Studies indicate that releasing multiple life stages of *M. scutellaris* increases propagule pressure where there is increased environmental variability (Sinclair & Arnott 2016). Coetzee et al. (2022) suggested that an augmentative approach to *P. crassipes* control, whereby biocontrol agents, particularly *M. scutellaris*, are released often and in high numbers to inundate and overwhelm the *P. crassipes* mats, is most likely to lead to success. Furthermore, Goode et al. (2021) reported that as more *M. scutellaris* populations are released at sites, more insects disperse to surrounding untreated areas. This approach may help buffer the dynamism of *P.*

crassipes populations, especially in herbicide-treated areas, thereby establishing persistent insect populations. Our studies gave an insight into the necessity of leaving unsprayed areas for biocontrol insects when integrating herbicides for the management of *P. crassipes* while suggesting the additional releases of the control agents. The benefits of using this approach include the maintenance of critical and sustainable biological control agent populations.

6.6. Sublethal herbicide application and the threat of resistance

While herbicides play a key role in weed management, the risk of resistance developing through sublethal exposure presents significant challenges. Previous studies reported that exposure to sublethal herbicide rates could be detrimental to long term weed management as several weed species have evolved resistance after recurrent exposure to sublethal herbicide rates (Busi et al. 2012, Ashworth et al. 2016). The current research suggested that sublethal concentrations of glyphosate-based herbicides show potential in controlling P. crassipes proliferation. However, the phenotypic response of the weeds, such as herbicide resistance induced by recurrent sublethal exposure to herbicides, was not investigated. *Pontederia crassipes* plants at Bronkhorstspruit Dam in the present study showed susceptibility to sublethal herbicide applications in the dam, as the growth of the plants in the sprayed areas was reduced following herbicide exposure. However, in the Inanda Dam, Kwazulu–Natal, South Africa, *P. crassipes* growth and mat coverage did not reduce after herbicide spray, possibly due to herbicide–induced resistance to sublethal concentrations of glyphosate herbicide. Weed populations are known to display high levels of genetic diversity and phenotypic plasticity, capable of resisting low herbicide rates and control measures. For example, sublethal dose recurrent selection leading to herbicide resistance has been reported for glyphosate and dicamba in *A. palmeri*, acetyl CoA carboxylase inhibitors in *Lolium rigidum* Gaudin (Poaceae), and 2,4–D in *Raphanus raphanistrum* L. (Brassicaceae) (Neve & Powles 2005a, b, Norsworthy 2012, Ashworth et al. 2016). These studies demonstrated the potential for reduced rates of herbicides to rapidly select for resistance in weeds that are susceptible to herbicides. These studies provided important implications that inform effective management practices of weeds and help with long-term resistance management. However, studies addressing the glyphosate resistance levels on *P. crassipes* selected by sublethal glyphosate concentrations are scarce, warranting for further research. Future studies involving applications of sublethal herbicides to *P. crassipes* should address whether resistance is induced due to recurrent exposure of sublethal herbicides and whether applying low concentrations of different herbicides with different modes of actions will yield the same results. Further, additional studies elucidating whether synergistic interactions of biocontrol agents and sublethal herbicides will manage resistance induced by the herbicide applications could be crucial for enhanced integrated management of the weed.

6.7. Conclusion and recommendations

As herbicides are one of the most frequently applied pesticides worldwide, and their use is likely to increase as climate change promotes the expansion of invasive weeds (including aquatic weeds), it was crucial to further our understanding of their impacts on biological control agents used as natural enemies. The results for the current research showed that sublethal concentration of glyphosate herbicide (0.4%) combined with *M. scutellaris* insects provided substantial control of *P. crassipes*, more so in controlled environments, where significant numbers of biocontrol agents were achieved in plants sprayed with herbicides. Based on these results sublethal herbicide applications combined with a biocontrol agent, *M. scutellaris* offer a great potential for better integrated control of *P. crassipes* and should be implemented in more systems for sustainable control.

Sublethal herbicide concentrations used in this study are compatible with biocontrol agents and they reduced *P. crassipes* proliferation by slowing its growth rate (Chapter 2). However, this approach may take time to reach full *P. crassipes* control, particularly in field conditions. For example, under field conditions, the control of *P. crassipes* was not achieved through integration of a sublethal herbicide spray and release of biocontrol agents at Bronkhorstspruit Dam. This was attributed to the low numbers of biocontrol agents present in the dam, which were not sufficient to cause adequate damage to *P. crassipes* and decrease coverage. Similarly, in April 2024, a sublethal application of KiloMax was applied to a water hyacinth infestation at Inanda Dam, according to the conditions of the General Authorisation awarded to the managers by DFFE. This spray also yielded inadequate *P. crassipes* control because the abundance of *M. scutellaris* was considered too low to effect integrated control (Julie Coetzee Pers. Comm. 2024). Since then, managers opted for a lethal application of Kilomax at the dam because the immediate control of water hyacinth was required. Even though applying lethal herbicide concentrations of herbicides provides rapid knockdown of *P. crassipes* and is approved by the herbicide's label, they will cause high mortality to biocontrol agents and cause damage to non-target species (Hill et al. 2012). Furthermore, lethal herbicide sprays often require frequent reapplication because the weed quickly rebounds from plants not sprayed, and from seedling germination, and this could be more expensive than beneficial.

This study provided important insights on integration of sublethal herbicide applications and biocontrol agents. Although the sublethal herbicide concentration is compatible with *M. scutellaris*, the insect populations remained low in the field. This suggests that sublethal herbicide applications could be augmented with multiple additional releases of biocontrol agents. The approach of integrating sublethal herbicide sprays with inundative releases of biocontrol agents should be implemented by land users and implementing agents as it promises rapid knockdown of the weed, while using less herbicide. This is a promising and reliable strategy that could further limit the need to use herbicides in our vulnerable aquatic systems. This method is safe, cost-effective, environmentally friendly, and sustainable, and should be implemented in more systems, even without the application of sublethal herbicides. Further studies on herbivore impacts on the susceptibility of *P. crassipes* to sublethal herbicides and the speed at which plant populations rebound following herbicide treatment may reveal additional benefits and guide a sturdier integration of herbicide and biological control.

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