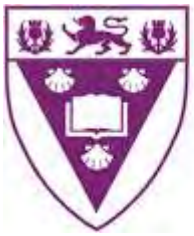

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Biology, ecology and management of the Keurboom moth, *Leto venus* Cramer and the leafhopper *Molopopterus* sp. Jacobi in cultivated Honeybush (*Cyclopia* spp.)

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ABSTRACT

Honeybush, *Cyclopia* spp. Vent (Fabaceae), farmers have raised pest concerns following commercial cultivation. The Keurboom moth *Leto venus* Cramer (Lepidoptera: Hepialidae) and the leafhopper *Molopopterus* sp. Jacobi (Hemiptera: Cicadellidae), are two of the major pests identified in cultivated Honeybush. Laboratory and field studies were conducted to gain an understanding of the biology of these two pests to inform future pest management solutions. Additionally, entomopathogenic fungi were isolated from Honeybush farms and screened for virulence against *Molopopterus* sp. as a possible management strategy. This study showed that the *L. venus* infestation on Honeybush was a product of four fixed effects; stem diameter, species of *Cyclopia*, Farm location and age of the plants. *Cyclopia subternata,* had the highest likelihood of infestation. Increase in age of the plants resulted in an increase in the stem diameter and therefore a higher probability of infestation. Stem diameter was also shown to be a significant predictor of infestation likelihood. Infestation severity, determined by the number of larvae per plant, was shown to be influenced by three fixed effects; stem diameter, plant species and Farm location. The results also showed that *L. venus* prefers to initiate penetration at, or just aboveground level.

Laboratory studies showed that the leafhopper *Molopopterus* sp. undergoes five nymphal instars with an average egg incubation time of 20 days, development time from 1st instar to adult of 26 days and average generation time of 47 days. Laboratory experiments revealed variations in host preference by the leafhopper over a period of 15 days. *Cyclopia longifolia* was identified to be the most preferred species for feeding compared to the two other commonly cultivated species, *C. subternata* and *C. maculata*. The results were consistent with those obtained from the field survey which showed that leafhopper density was influenced by four fixed effects; plant species, age of the plant, Farm location and harvesting practices. There were significant differences in leafhopper density in different species with *C. longifolia* having the highest number of leafhoppers per plant. There were differences in leafhopper density in different farms as 57% of the sampled farms had leafhopper infestations, of these farms, Lodestone and Kurland had the highest leafhopper densities. Harvested plants were shown to have significantly higher leafhopper density than non-harvested plants. Age was also shown to influence leafhopper density, which reduced with an increase in the age of the plants. A total of 20 fungal isolates were recovered from 98 soil samples of which 70% were from Honeybush

fields and 30% were from surrounding refugia. *Fusarium oxysporum* isolates comprised 20% of the recovered isolates, with *Metarhizium anisopliae* isolates making up the remainder. Laboratory bioassays against adults and nymphs of the leafhopper, *Molopopterus* sp., showed that *F. oxysporum* isolates induced 10 – 45% mortality and *M. anisopliae* isolates induce 30 – 80% mortality. *Metarhizium anisopliae* isolates J S1, KF S3, KF S11, KF S13, LS1 and LS2 were the most virulent and induced over 60% mortality in both *Molopopterus* sp. nymphs and adults. The results of this study showed pest preference towards different *Cyclopia* species. As such, they should be managed differently. Furthermore, *L. venus* was observed to occur in low densities, hence, it cannot be considered a major pest. However, *Molopopterus* sp. recorded high population densities making it a major pest in Honeybush production. Positive results indicated that some of the isolated fungal isolates have potential for control, an avenue worth investigating further.

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
ARC	Agriculture Research Council
AICc	Akaike Information Criterion correction
cm	Centimeters
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
DAFF	Department of Agriculture Forestry and Fisheries
DHARMA	Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models
EIL	Economic Injury Level
ETL	Economic Threshold level
FAO	Food Agriculture Organisation
GLM	General Linear Model
GTR	General Time Reversible
GAP	Good Agricultural Practices
g	gram
IPM	Integrated Pest Management
ITS	Internal Transcribed Spacer
IUCN	International Union for Conversation of Nature
km	Kilometre
L	Litre
LSD	Least Significant Difference

µl	Microlitre
mg	Milligram
ml	Millilitre
MuMin	Multi-Model Inference
PCR	Polymerase chain reaction
rpm	revolutions per minute
SDA	Sabouraud Dextrose Agar
SAHPA	South African Honeybush Producers Association
SAHTA	South African Honeybush Tea Association
WHO	World Health Organisation
w_i	Akaike Weights
ZAR	South African Rand

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CHAPTER 1

General Introduction

1.1 Background of the Tea Industry

1.1.1 Overview of the black tea industry

Tea is one of the most popular consumed beverages (Mukhopadhyay and Mondal, 2017). It is famous for its health benefits including antioxidant, anti-inflammatory and weight loss properties and comprises several vitamins with trace amounts of potassium, magnesium, manganese, calcium minerals and different amino acids such as L-theanine (Mitra and Khandelwal, 2016). These health benefits have been a major driving force in global tea demand as consumers are becoming increasingly health conscious, moving away from carbonated drinks in particular (Adam, 2015; Food Agriculture Organisation of the United (FAO), 2018). According to the FAO (2018), the demand for tea has also been driven by the ongoing retail revolution and growing investment into tea. Furthermore, changes in consumer preferences, the rise in café culture, and growth in disposable incomes, and more importantly infusions of additional healthy ingredients in traditional teas, are some of the market forces that have driven the growth of the tea market (Authur, 2018; Sinha, 2017).

Primarily tea can be categorised by degree of fermentation into black tea and green tea. Black tea is produced from withering, rolling and fermentation of *Camellia sinensis* L. (Kuntze) (Ericales: Theacea) plant leaves (Sinha, 2017). Black tea has its origins in China where it was known as red tea before being cultivated globally, most of which is produced in South East Asia, China and Kenya (Department of Agriculture, Fisheries and Forestry (DAFF, 2011)). Green tea is produced from *C. sinensis* leaves that have not undergone oxidation processes as in black tea. The global market further categorises tea based on type, geographical location, packaging and distribution channel (Sinha, 2017). Of interest is the differentiation by type where the market is divided according to production practices into organic and non-organic tea (Ruan et al., 2020).

In South Africa, tea was first cultivated in 1870 on an estimated 200 ha in KwaZulu-Natal, but failed to gain traction due to preference given to sugar production. However, it has since grown

substantially with an average of between four and seven million kilograms annual production (DAFF, 2011). In general, the tea industry plays a very important role in the economy of South Africa as it is a US dollar traded commodity (DAFF, 2016) and thus generates foreign currency. Although black tea has dominated the South African tea industry, it is declining due to several factors. According to Mahomedy (2018) the value of tea has plummeted from ZAR 30 million in 2008 to just over ZAR 5 million in 2017. This fall has been attributed to poor harvests as a result of drought in the recent years and rising production costs due to inflationary pressures (Mihalis and Adeyeye, 2015; Khumalo et al., 2016). South African black tea is also facing intensified competition from Asian markets.

1.1.2 The South African herbal tea industry

Herbal teas differ from traditional teas as they are not made from *C. sinensis*, but rather from infusions of fruit, leaves or other parts of other plants. Some of the common herbal teas include Rooibos, *Aspalathus linearis* (Burm. f.) R. Dahlgren (Fabaceae) and Honeybush, *Cyclopia* spp. Vent (Fabaceae) (Mihalis and Adeyeye, 2015). Globally, the herbal tea market is expected to experience substantial growth opportunities in the near future. Medical practitioners have reported that high caffeine levels in the body cause prolonged ailments, thus discouraging the widespread usage of black and green teas. In contrast, herbal teas are caffeine free and thus recommended by health professionals (North et al., 2017). Change in consumer lifestyle with a shift towards health-beneficial diets has amplified the importance of herbal tea. Segmentation of the herbal tea market is done on the basis of product type, raw materials, flavour and region (Sinha, 2017).

The herbal tea industry offers respite to the South African tea industry as it has a unique competitive and sustainable advantage. The semi-arid and temperate climate of the South African Cape fynbos region favours the production of two very important herbal teas: Rooibos and Honeybush (Marsh, 2009). Both Rooibos and Honeybush are endemic to the fynbos region of South Africa (du Toit et al., 2008). Rooibos is characterised by needle-like leaves and is polymorphic with various wild forms having been described differing both in geographic location and distribution. Rooibos occurs naturally in the Cederberg area in the Western Cape Province of South Africa. The Rooibos industry has a rich history which dates to as early as 1904 when commercial activity was initiated by a South African businessman who later became the pioneer of commercialisation of Rooibos tea, Benjamin Ginsberg. Early during

production, the industry relied on wild harvesting, experiencing a boom in demand during the Second World War due to shortage of oriental teas leading to the commercialisation of the industry (Marsh, 2009; du Toit et al., 2008).

The economic importance of the herbal tea industry cannot be overlooked as it is labour intensive, hence important in terms of job creation. Rooibos alone employs approximately 8 000 Farm labourers with more employment created directly and indirectly in downstream activities (Mahomedy, 2018). Furthermore, it is a major player in terms of export earnings, with over 6 000 tons in exports as of 2016 (Swart, 2018). As such, the Rooibos industry has a very significant role in the economy of the country generating millions of foreign currencies each year from foreign export. Although the Rooibos industry is on a growth trajectory, it is under threat from the global phenomenon of climate change and the resultant instability of market forces coupled with high production costs. The combination of these factors question the sustainability of the industry (Pretorius, 2008).

1.1.3 Enter Honeybush tea

Honeybush is an alternative crop whose aim is to aid the South African economy as it has high export value (Rampedi and Olivier, 2008). Honeybush tea is not "new" to South Africa, rather a rediscovered indigenous herbal tea (Toit et al., 2008). Honeybush tea has gained global recognition due to its pleasant flavour and like other herbal teas, it does not contain caffeine (McGregor, 2017). The industry is moving from a small and informally organised industry (cottage industry) to international markets, hence, the adoption of cultivation practices which are more sustainable than wild harvesting (Schutte-vlok and Herbarium, 1998). According to the South African Honeybush Tea Association (SAHTA, 2018) statistics, the Honeybush tea industry employs over 1 258 people ranging from primary to tertiary level of production and research. The Honeybush tea industry is still a fledgling industry. As of 2016, 30 000 ha were being harvested in wild areas and only 200 ha under cultivation (McGregor, 2017). These figures show that Honeybush is currently being produced on a limited commercial basis producing approximately 221 tons. Of this, 52 tons were packaged for local consumption generating ZAR 7.6 million and a further ZAR 4.4 million bulk loose tea value generated from exports (DAFF, 2014). Currently, consumer demand for Honeybush products far outweighs the supply. This growing demand for Honeybush tea, both locally and globally, calls for further commercialisation of the industry and a shift from wild harvesting (Slabbert, 2016).

Honeybush has several uses. Chief among these is human consumption where leaves and stems are used to make herbal tea commonly referred to as Honeybush tea. Furthermore, extracts from Honeybush have potential to be used as flavourings in ready-to-drink beverages such as fruit juice blends and iced tea (DAFF, 2014). The most recognised and acknowledged benefit of Honeybush tea is lack of caffeine making it suitable for night consumption. It also has a low tannin content, thus a less acidic taste as compared to other teas even when steeped for a long time. Traditionally, it has been used to treat cough because it contains pintol, a modified sugar, which is an expectorant (substance which promotes secretion of sputum by the air passages). According to Hesbert (2015), Honeybush tea also contains isoflavines and coumestans which are classified as phyto-estrogens, used in the treatment of menopausal symptoms, an application for which Honeybush has been greatly promoted. The flowers of Honeybush also contain luteolin the primary yellow pigment which has been used historically as a dye (Bates, 2000). Honeybush tea also plays a significant role in improving the immune system as it is a natural source of many antioxidants, including major phenolic compounds. Phenolic compounds shield cells from oxidative stress and help modulate the immune system. Evidence also exists that phenolic compounds in Honeybush tea help protect from inflammatory diseases and prevent development of chronic inflammatory disease (Hesbert, 2015). In women, the tea is thought to help regulate menstruation and reduce the risk of osteoporosis. It is also good for gastrointestinal health as it is drunk to alleviate heartburn, nausea and constipation. In some instances Honeybush tea is used to treat abdominal cramps and colic pain in infants (Visagie et al., 2015).

1.2 Target Plant: *Cyclopia* spp.

1.2.1 Description of Honeybush

Honeybush, *Cyclopia* spp. Vent (Fabaceae), is a papilionaceous genus endemic to South Africa, used to produce a unique herbal tea with a pleasant honey like taste and flavour (McGregor, 2017; Schutte, 1995). The genus *Cyclopia* comprises 23 species with subtle differences. Species are differentiated by their bracts, calyxes, leaves and fire survival strategies (Joubert et al., 2011). Of the 23 species, only six species are of economic importance: *C. genistoides* L (Vent), *C. intermedia* E.Mey, *C. subternata* Hofmeyr (E.Phillips), *C. maculata* Andrews (Kies), *C. longifolia* Vogel and *C. sessilifora* Eckl (Zeyh). *Cyclopia* occurs on coastal

plains and mountainous areas of the Eastern and Western Cape provinces of South Africa in the Fynbos biome (Figure 1.1) (Joubert et al., 2011; Miller and van Emden, 1993; Walt, 2000). The fynbos biome is one of the five biomes within the Greater Cape floristic region that is unique and species rich (Mucina and Rutherford, 2006).

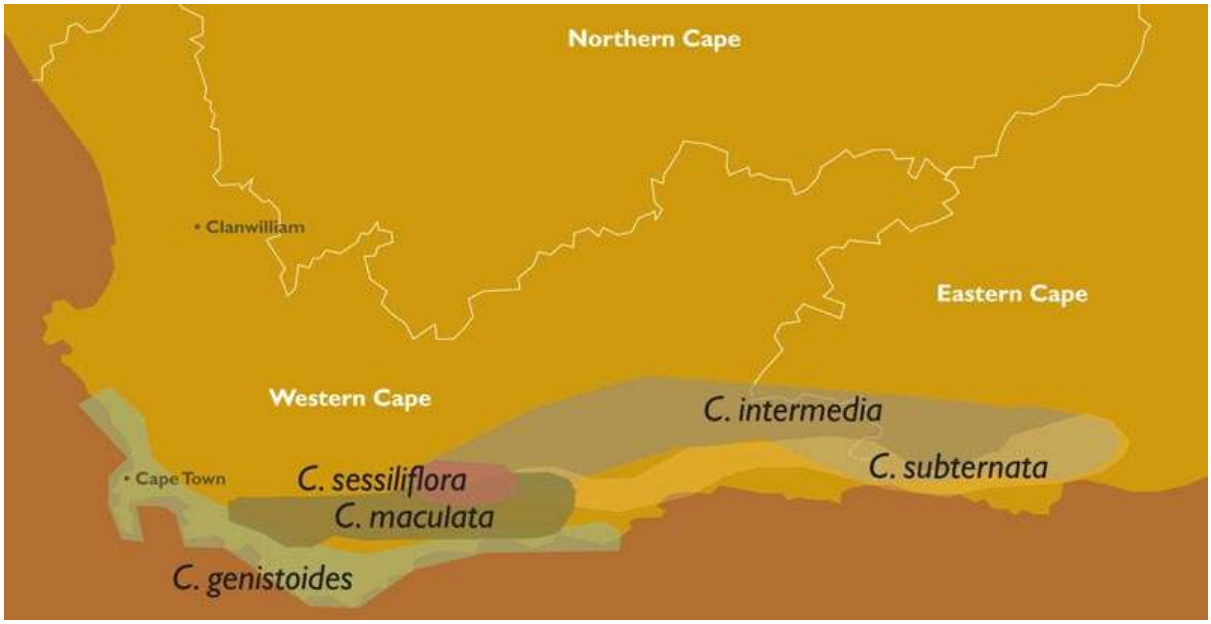

Figure 1.1: Distribution of *Cyclopia* species of economic importance (SAHTA, 2017).

Fynbos is a vegetation that is characterised primarily by woody plants with small leathery leaves. *Cyclopia* spp. are characterised by trifoliate leaves, single flowered inflorescences and sweet scented, bright yellow flowers with prominent grooves on the standard petal. It has a thrust in calyx base and two bracts fused at the base around the pedicel (Figure 1.2) (Schutte, 1995; Stepanova and Kotina, 2012).

Figure 1.2: The bright yellow Honeybush flower with grooves on the petal (adapted from (SAHTA, 2018).

Honeybush are long lived perennial plants whose growth is dependent on climate and terrain and can reach 3 m in height (Joubert et al., 2011). Honeybush favours well drained sandy loam soils with low pH, phosphorous and nematode count. Constantly faced with wildfires, *Cyclopia* spp. have evolved strategies to survive fire as either resprouters or reseeders. Resprouters are identified by the woody rootstock which reproduce new coppice after a fire (Schutte, 1995). Reseeders produce seed with a hard coat which requires scarification as a fire survival strategy (Schutte et al., 1997). Fynbos vegetation thrives on fire, making it an essential component of the fynbos ecosystem (Stepanova and Kotina, 2012). The role of fire is to assist in the rejuvenation of local plant populations. Most species of Honeybush flower in spring (September – October) with a few flowering in winter (May – June) e.g. *C. sessiflora* (Joubert et al., 2011).

The most common species in the wild, *C. intermedia*, a resprouter often referred to as "bergtree", has proven difficult to cultivate as it can only be harvested every second season. This is because it does not accumulate sufficient energy reserves to enable it to reach maturity on an annual basis making it uneconomic for agricultural production. However, *C. intermedia* is by far the most important species, making up 85% of the wild crop. Due to its abundance, it is the most recommended species for wild harvesting (McGregor, 2018). Bergtree is found on the south facing slopes of the Cape Fold Mountains. Its bright yellow flowers bloom in spring with buds forming as early as April (Figure 1.3).

Figure 1.3: *Cyclopia intermedia* plant growing in the wild (McGregor, 2018).

Only a few species have managed to be commercially exploited under cultivation (DAFF, 2014). *Cyclopia subternata*, *C. genistoides* and recently *C. longifolia* are the three most cultivated species (SAHTA, 2017). *Cyclopia subternata*, a reseeder, occurs in relatively high stands along drainage lines in kloofs and around freshwater seeps. Unlike *C. intermedia*, this plant species has been exploited under cultivation because they can produce flowers in the first year of growth. An adult plant produces many flowers hence many seeds to ensure perpetuity of the plant (Figure 1.4) (McGregor, 2018).

Figure 1.4: Cultivated *Cyclopia subternata* at Hidden Woods Farm in Harkerville (Photo credit: Tapiwa Mushore).

During harvesting, the shoots, which comprise twigs and leaves of the plant, are gathered in bunches for further processing. At the processing plant, they are chopped into smaller pieces in preparation for fermentation. The product is dried, sieved, graded according to size and flavour before being packaged for the market. The fermentation process is omitted in making green Honeybush, with green Honeybush being more popular amongst consumers due to its high antioxidant properties. Each species of Honeybush has a different flavour profile and to ensure a more consistent product, processors often blend two or more species together (Muller et al., 2012; SAHTA, 2017).

1.2.2 Trends in Honeybush tea production: past, potential and future perspectives

The European history of Honeybush dates back as far as the 18th century. In 1772, the Swedish botanist, Thunberg, first documented Honeybush tea when he reported that he found “honig tee” during one of his excursions in the Cape. It was since used by settlers as a restorative medicinal drink in 1830 with the earliest scientific research documenting Honeybush as a caffeine-free herbal drink in 1881.Wild harvesting of Honeybush for sale started in 1930 with

the price increasing during the Second World War in the 1940s. Half a century later, in the 1990s, scientific research in cultivation and propagation of Honeybush began and still is the key focus of research. Harvesting of Honeybush from commercially cultivated stands began in 1996 with small scale farmers being brought on board in 1998. This marked the commencement of intensive research on the cultivation of Honeybush. Officially, the commercial Honeybush tea industry came into existence in 1999 when the South African Honeybush Producers Association (SAHPA) was formed, later renamed South African Honeybush Tea Association (McGregor, 2017; North et al., 2017; SAHTA, 2018).

Before gaining economic importance, Honeybush was wild-harvested mainly for home consumption until the early to mid-1990s (North et al., 2017). Honeybush tea has remained in the shadow of Rooibos which gained commercial status as far back as early 1940. This unique herbal tea has gained economic importance due to increased demand both locally and globally. Demand has been a result of emerging markets, products and changes in consumer preferences. Of late, consumers have started exerting pressure in need for natural products and increasing awareness of health issues (McGregor, 2017). This has led to the formalisation of the industry and a steady increase in prices of the product on the market. Honeybush harvesting is still dependent on wild harvesting, an unsustainable practice both environmentally and commercially (Joubert et al., 2011). Harvesting is done during the flowering period as bushes are easily visible in fields. However, due to increasing market demand and pressure by processors, harvesting is often done during early to late summer. Early and unsustainable harvesting practices are placing wild Honeybush populations under threat and resulting in negative impacts on the livelihoods of local harvesters (SAHTA, 2017). Poor harvest management practices have led to *C. plicata* Kies, one of the species of Honeybush, to be listed on the International Union for Conversation of Nature (IUCN) Red list for threatened species. If these harvest practices are left unchecked, this can lead to more species of *Cyclopia* becoming extinct (McGregor, 2017).

Honeybush is also under threat from other factors such as growth of agriculture and urban settlements. Being an indigenous plant, it is also under threat from alien plant invasion (a common phenomenon in the fynbos) such as pine and black wattle (McGregor, 2018). As aforementioned, the current production trends do not meet the demand, posing questions as to what needs to be done. Commercialisation of Honeybush entails moving from the traditional

cottage industry to cultivation on a large scale. The greatest challenge is the complexity of multiple factors that need to be considered for a successful transition. One of the challenges of commercialising Honeybush was the price of raw Honeybush per kilogram which is based on the zero maintenance of wild Honeybush. The price does not justify or does not meet the cost and financial risk involved in cultivation (Platt, 2014).

For successful commercialisation of Honeybush as an indigenous product, conventional product development paths need to be transformed. Recognition and promotion of indigenous resources goes a long way in repositioning indigenous products in a much broader spectrum beyond just communities, but globally as well (Rampedi and Olivier, 2008). Very few South African indigenous plants have been commercialised, with Rooibos tea being one of the few success stories. Honeybush has been a subject of intensive research over the years with scientists striving to develop Good Agricultural Practices (GAP) to facilitate cultivation and propagation. Several baseline questions have thus been raised to better understand this crop. Being a fairly new crop, very little is known with respect to cultivation and propagation (Mbangcolo, 2008; Slabbert, 2016).

Successful cultivation of Honeybush faces both biotic and abiotic challenges with pests being a major biotic challenge. Insect pests are an integral part of the ecosystem and are usually found coexisting with their hosts (Brasier, 2008). They are one of the main sources of biotic stress on crops and as such, they have a major influence on agriculture production (Bonsignore and Vacante, 2018). Generally, pests comprise of organisms that compete for resources with humans including disease vectors (van Emden and Service, 2004). Several approaches are used in determining the pest status of an organism. In most cases, however, pest status is defined in terms of economics and an organism is considered a pest when it is economic to control it (Hill, 1990; Moore, 1967). Exceptions do occur where the “pests” are controlled in response to rational and irrational responses e.g. nuisance organisms, hence pest definitions often reflect human value (Hill, 1990). Human disturbance through domestication often lead to sporadic pest outbreaks and their devastating effects become more pronounced (Gilbert, 2002). Pests are one of the constraints that can hinder successful commercialisation of any indigenous plant (Wright, 1995). Insect pests, if uncontrolled, may cause significant yield losses which ultimately translate to economic losses (Bonsignore and Vacante, 2018). Honeybush tea is a perennial crop that is cultivated under monoculture practice; hence it provides favourable

conditions for pest establishment. Limited information exists on the impact of pests in Honeybush and how they shape the ecosystem (Slabbert, 2016). Given the current emphasis in shifting from wild harvesting to commercial cultivation, if it is to be achieved, insect pests associated with Honeybush need to be understood.

1.3 Role of Pests in Tea Production

Tea is an intensively managed monoculture crop with all parts of the plant (leaves, stems and roots) being fed upon by at least one species (Ruan et al., 2020; Xiao, 2018). Tea plantations are extensively managed monoculture stands which resemble a "single species forest" and insects co-exist by way of inter-tree distribution of well-defined ecological niches (Harries and Douglass, 1994). Most tea pests occur throughout the year, and are residential as they complete their lifecycles in tea plantations (Mamun and Ahmed, 2011). In tea production, pests that attack new shoots have low Economic Injury Levels (EIL) since their injury affects both yield and quality (Chen and Chen, 1989). Meanwhile, pests that infest other parts of the plant have higher EIL because their damage affects growth of new shoots of the next crop, with very little impact on current yield and quality (Chen and Chen, 1989; Kawai, 1997; Roy et al., 2015).

1.3.1 Classification of tea pests

Tea pests are classified according to their degree of impact, i.e. major pests and minor pests (Hazarika et al., 2009). The most common classification, however, is based on site of attack; root pests such as nematodes and cockchafer grubs, stem pests such as shot hole borer, red borer and sapling borer and leaf pests such as mosquito bug, caterpillars and aphids (Rahaman et al., 2016). The mosquito bug, *Helopeltis theivora* Waterhouse (Heteroptera: Miridae), is a major tea pest in most tea producing countries (Roy et al., 2015). It sucks sap from tender leaves and new shoots resulting in heavy crop losses of 40 – 60% (Mamun and Ahmed, 2011; Roy et al., 2015). The shot hole borer, *Euwallacea fornicatus* Eichhoff (Coleoptera: Scolytidae), is a stem borer which damages tea plants by making short round holes in primary branches. This pest results in dieback symptoms and in severe cases mortality of infested branches (Hazarika, 2018).

Due to the semi-arid climatic conditions in South Africa, the most common tea pests are mites. The red spider mite, *Oligonychus coffeae* Nietner (Acari: Tetranychidae) attacks foliage in hot

dry summers and is difficult to control (DAFF, 2016). Rooibos, the only other commercially cultivated endemic herbal tea in South Africa, has its fair share of pests. The clearwing moth, *Monopetalotaxis candescens* Felder (Lepidoptera: Sessidae) is one of the most pestiferous pests in Rooibos which is especially damaging due to its larval feeding habits (Hatting, 2017; Hatting et al., 2013). As a root borer, it causes extensive tissue damage thereby limiting translocation capacity of nutrients and water uptake. A chemical based approach with minimal pesticide residues was developed as a control strategy. The pesticide is placed at the base of the plant using a custom designed applicator to avoid foliage contact. Furthermore, two entomopathogenic fungi and an entomopathogenic nematode, along with other natural enemies, have been recorded to be associated with this moth (Hatting et al., 2018). The leafhopper *Molopopterus theae* Theron (Hemiptera: Cicadellidae) is another pest associated with Rooibos. The pest causes symptomatic yellow colouration of the leaves under high leafhopper populations. As a result, there is impaired plant growth and, in extreme cases, mortality, which is exacerbated by moisture stress (Hatting, 2017).

1.3.2 Management of pests in tea production

Over the years, the concept of pest control has undergone several changes with a strong emphasis now being placed on integrated pest management (IPM). IPM can be defined as an effective, modern approach that is environmentally sensitive to pest management, due to its encouragement towards the combined use of common sense practices (Sullivan, 1999). Alternatively, IPM can be defined as a cohesive system for the selection, implementation and integration of pest control methods based on socio-economic levels (Mamun and Ahmed, 2011). IPM is centered around preventative measures and comprehensive information on pest life cycles which is used to formulate or choose appropriate control actions. For successful implementation of IPM in Honeybush, one of the basic requirements is the economic threshold level (ETL) at which the control measure is justified. The economic injury level (EIL), the lowest population which will cause economic damage, is another basic component required in IPM (Pedigo, 1996).

Several tactics of IPM are employed in the control of both major and minor tea pests. One of the most common strategies employed, is the use of synthetic pesticides to complement natural enemies. Pesticides are commonly applied as a pest remedy; however, their application is a burden to both farmers and the environment. Injudicious use of pesticides often results in

secondary and primary pest resurgences, resistance development, environment contamination and unwanted residues on tea. The demand for contaminant free tea and the need to sustain productivity and quality maintenance pioneered organic tea cultivation, which began in Sri Lanka in the early 80's (Hazarika et al., 2009). In Rooibos cultivation,pesticide residue concerns and suspected resistance development, have promoted research on biological insecticides and other benign pest management practices (Hatting, 2017).

Cultural control methods, common in tea, are preventative measures which are integrated in tea production systems and are compatible with natural processes. It involves intelligent manipulation of crop management practices such as pruning and weed management. Several cultural control methods including sanitation, trap crops along with pruning help keep pests within tolerable limits (Shelton and Badness-Perez, 2006). Mechanical control is employed in small tea plantations or plantations with large labour force. Collection and destruction of pests has been proven to be successful in control of chrysalids of *Buzzura suppressia* Guen (Lepidoptera: Geometridae) (Hazarika et al., 2009). There are significant morphological and genetic differences among tea cultivars to which pests react differently. This has been exploited to create resistant cultivars. The most common host plant resistance approach is the leaf aspect and structure which is known to inhibit insect feeding (antibiosis) (Banerjee, 1987; Chen et al., 1996).

Mating disruption using pheromone trapping is a direct management technique for pests that can provide excellent suppression of lepidopteran pests at low pest densities; hence, it is often augmented with other control options. Pheromones are chemicals secreted by insects as a means of communication (mostly to attract potential mates) (Welter et al., 2010). Trapping and monitoring adult stem borers are another option for the control of tea pests, and this is normally started early in the season continuing throughout. This is achieved by sticky traps baited with pherolures. For example, the use of sticky delta traps has been used with considerable success for monitoring the population of *Adoxophyes* sp. Meyrick (Lepidopera: Tortricidae), thereby enabling timeous intervention (Hazarika, 2018; Hiyori et al., 1986).

Biological control uses natural enemy complexes to suppress the population of pests. Tea plantations are regarded as highly supportive for biological control due to cultivation patterns (monoculture), duration of the crop and scale of planting (Namasivayam et al., 2015). Delayed response, low efficacy and challenges in its implementation have been reported as limitations

to successful use of biological control (Pedigo, 1996). Natural enemies may include predators, parasites and pathogens. Predators such as hemipteran bugs, coccinellid beetles, chrysopid lacewings and syrphid larvae, tend to be polyphagous and have the potential to persist in the tea agro-ecosystem as they will not be affected by pesticides as they do not feed directly on the plant material (Mailafiya et al., 2011). *Macrocentrus homonae* Nixon (Hymenoptera: Braconidae) was successfully introduced from other tea producing countries, into Sri Lanka and managed to suppress *O. coffearia* populations (Hazarika, 2018). Meanwhile, *Bacillus thuringiensis* is the most successfully utilised microbial insecticide in Tea against lepidopterans and *Agromyza theae* (Bigot) Meij. (Diptera: Agromyzidae).

1.4 Insects Associated with Honeybush

Research on cultivation and field management practices of Honeybush has largely focused on propagation and mass production. The Honeybush tea ecosystem is especially ideal for pests as it provides a permanent environment with uninterrupted food supply coupled with an ideal habitat. Currently, the role of insects and related arthropods on Honeybush regarding their (pollination, pests status and natural enemies) remains unknown (Slabbert, 2016). In addition, no research has been conducted to promote biological control of pests as a way of complementing the organic industry to ensure its success. Knipe and Rosenberg (2008), conducted the first ever insect survey to determine and quantify insects associated with cultivated Honeybush in the Western Cape. Insects were classified into three categories namely harmful, beneficial and incidental. Scale insects (Hemiptera: Diaspididae), *Aonidiella aurantii* Maskell, *Lindingaspis rossi* Maskell and *Hemiberlesia lataniae* Signoret were observed to cause the most noticeable damage on Honeybush followed by caterpillars of the leaf roller moth *Epichoristodes acerbella* Walker (Lepidoptera: Torticidae) which feeds on the flowers and leaves. Several beneficial parasitoids and generalist predators were also recorded. For example, larvae of the leafminer were reported to be parasitised by five different hymenopteran parasitoids namely *Apanteles* spp., *Chlorocytus* spp., *Pediobius* spp., *Elachertus* spp. and *Cotesia* spp. indicating strong presence of natural enemies. Following this survey, various insects have been identified in cultivated Honeybush such as the monkey beetle, *Scelophysa trimeni* Peringuey (Coleoptera: Scarabaeidae) which was found to be attracted to the sweet smelling flowers at the tip of branches and responsible for pollination (Slabbert, 2016). A more recent survey on cultivated Honeybush by Slabbert (2016) revealed approximately 130 insect

families with a peak in phytophagous arthropod abundance during the flowering stage of Honeybush. No attempts were made to identify insects beyond family level as this was beyond the scope of the study.

There have been reports by farmers of infestation by a damaging larva, which has potential to develop quickly and disrupt Honeybush plantations. In their insect survey, Knipe and Rosenberg (2008) observed burrowing larvae that caused severe dieback in Honeybush but could not identify the causative agent. Metcalf et al. (2018), did an initial assessment to better understand this pest, which was identified as larvae of the Keurboom moth, *Leto venus* Cramer (Lepidoptera: Hepialidae). The moth was not reported to cause substantial damage to Honeybush until after cultivation efforts. In their assessment, Metcalf et al. (2018), concluded that the Keurboom moth has potential to develop quickly and disrupt Honeybush plantations. As a result, some farmers have begun opting for other cash crops that are less prone to attrition at the expense of Honeybush. The threat posed by this pest has far reaching consequences and does irreparable damage to the Honeybush industry as there are no protocols in place to manage the infestation effectively (Metcalf et al., 2018).

Metcalf et al. (2018), also identified another pest that was causing significant damage, a leafhopper, which they referred to as the blue winged leafhopper. The pest was later identified to genus level by the Agriculture Research Council (ARC) *Molopopterus* sp., Jacobi. Though only one Farm, Kurland, the damage was significant and a cause for concern.. Since then, this pest has been recorded in other farms as well. Streak lines were observed on infested leaves of *C. subternata* and *C. longifolia* coupled with stunted growth. Leafhoppers cause severe damage as both adults and nymphs and are destructive especially during early stages of growth (Backus et al., 2005).

At the early stages of production, in the 1990s, a decision was made to apply organic production principles as the cost for registration of chemicals to control pests, diseases or weeds in plantations would be prohibitive for such a small industry (Joubert et al., 2011). A shift in cropping pattern, from wild to commercial cultivation, as in the case of Honeybush, causes ecological imbalances in pest populations (Andow, 1983). The Honeybush tea industry is a certified organic industry (DAFF, 2014), and has zero tolerance towards the use of synthetic pesticides, hence the need to explore alternative pest control options to eliminate residue concerns. Increasing consumer awareness is exerting pressure in reaching minimum pesticide

residues in tea (FAO, 2009). Furthermore, the use of synthetic pesticides against *L. venus*, a target pest of interest in this study, is undesirable since larvae are no longer vulnerable to contact pesticides and are not often detected until after they have caused considerable damage (Moolman et al., 2014). Cultivation of Honeybush means elimination of certain plants which natural enemies may rely on and thus, may lead to increased pest populations (Sullivan, 1999). The taxonomy, morphology and ecology of *L. venus* and *Molopopterus* sp., are going to be discussed in Chapter 2 and 3 respectively.

Many entomopathogenic fungi are used for management of tea pests alone or in combination with synthetic insecticides (Hazarika and Puzari, 2001). Amidst natural epizootics, entomopathogenic fungi can cause rapid population decline of their arthropod hosts. Due to their application, many entomopathogens have been studied and exploited for commercial use (Batta, 2016; Sultana et al., 2017). Entomopathogenic fungi have gained significant interest in research and use as mycoinsecticides. This is due to their ease in application as they can be administered similarly to synthetic pesticides (Zimmermann, 2007). Entomopathogenic fungi are considered advantageous in the management of tea pests as they are cost effective, increase yield as some have endophytic relationship with plants, do not have significant side effects on natural enemies and leave no chemical residues (Hussain et al., 2014; Wang et al., 2010, 2018).

1.5 Objectives of the Study

The development of pest management strategies for control of the two pests in cultivated Honeybush necessitates knowledge of their biology and ecology. Since very little is known about their life cycle, expectancy and behaviour at different stages of growth, the overall objective of this study was to improve current knowledge regarding the biology and ecology of the Keurboom moth, *L. venus* and the leafhopper, *Molopopterus* sp. information. Therefore, the aim of this study was to (1; Chapter 2) gain an understanding of the pests' biologies with respect to host preference and overall impact of the insect pest and confirm the presence, location and, if possible, the developmental rate of the suspected pre-boring life stages of *L. venus*. Control aimed at this life stage would prevent or reduce the entry of larvae into the stems of the plants and hence economic damage, (2; Chapter 3) gain an understanding of *Molopopterus* sp., bionomics with regards to life history traits, host preference and population density drivers, (3; Chapter 4) isolate entomopathogenic fungi naturally occurring in the soil

of cultivated Honeybush fields, which may be used for later control strategies against *L. venus, Molopopterus* sp. and other associated pests and, (4; Chapter 4) conduct a pathogenicity test of the isolated fungi on nymphal and adult stages of *Molopopterus* sp..

CHAPTER 2

Biology and Ecology of *Leto venus* in Cultivated Honeybush

2.1 Introduction

The Silver Spotted Ghost moth commonly known as the Keurboom moth, *Leto venus* Cramer (Lepidoptera: Hepialidae) is an endemic stem borer found in the Southern Cape region of South Africa (Grehan and Ralston, 2018; Grehan, 2012). The family Hepialidae is the largest family in the suborder Exoporia the sister group of Heteroneura, which includes most Lepidoptera species (Grehan, 1989). Exoporia is one of the most primitive lineages of the extant Lepidoptera (Nielsen et al., 2000). Traditionally, this family was identified morphologically by the absence of tibia spurs and comprises 80 genera with over 500 species described. The suborder Exoporia is a geographically circumscribed and conservative group with 68 genera and 616 valid species. The suborder is distinguished by the absence of a common cloaca or separate copulatory orifice, a characteristic feature of all other Lepidoptera (Peretti and Aisenberg, 2015; Sánchez et al., 2011). Sperm transfer is achieved via an external seminal gutter between the ovipore and the ostium with a few modifications to this pattern occurring in other genera (Kristensen and Nielsen, 1994). It has two superfamilies, Mnesarchaeoidea and Hepialoidea. The superfamily Hepialoidea contains the families Hepialidae, Anomosetidae, Neotheoridae, Prototheoridae and Palaeosetidae, though their inter-relationships are not clear (Nielsen, et al., 2000). Of the five families, Hepialidae is the most common with 56 genera and 537 valid species occurring in Asia, South America, Australia and Africa. Hepialids are commonly referred to as ghost moths as they are large and spectacular insects leading to their elevated status among their relatives (Scoble, 1992).

Due to its size and brightly contrasting colours, adult *L. venus* moths have been a favourite for collectors and recently, has captured the attention of nature photographers (Figure 2.1) (Grehan et al., 2018). *Leto venus* adults are thought to live only for a few days as they lack functional mouthparts for feeding (Metcalf, et al., 2018). Another phenomenon common to ghost moths is that they prefer to fly during wet foggy nights (Nielsen et al., 2000). Metcalf et al. (2018), also observed that the adults of *L. venus* often emerge under similar conditions. Hepialid adult males are fairly mobile with females being clumsy at flying due to their heavy bodies which

are laden with eggs (Janse, 1945). Several authors speculate, based on studies conducted on similar species that this enigmatic pest lays its eggs at the base of its host where they mature over a one to two week period (Grehan and Ralston, 2018; Grehan et al., 2018; Janse, 1945). It is highly suggestive, though inconclusive, that early instars of *L. venus* have a ground dwelling stage where they feed on litter before transferring to the host. According to Paukku and Kotiaho (2008), the availability of resources to the offspring is influenced by egg laying decisions by the females that strongly affects offspring survival. The moth is reported to emerge in late summer (February to March) and the silver spots begin to show through the skin when the chrysalis is reached (Grehan and Ralston, 2018). *Leto venus* pupae are morphologically identified by a distinct suture, a characteristic feature synonymous with Hepialids. Furthermore, the wings are entirely encased but the wing venation is clearly visible (Figure 2.1) (Janse, 1940).

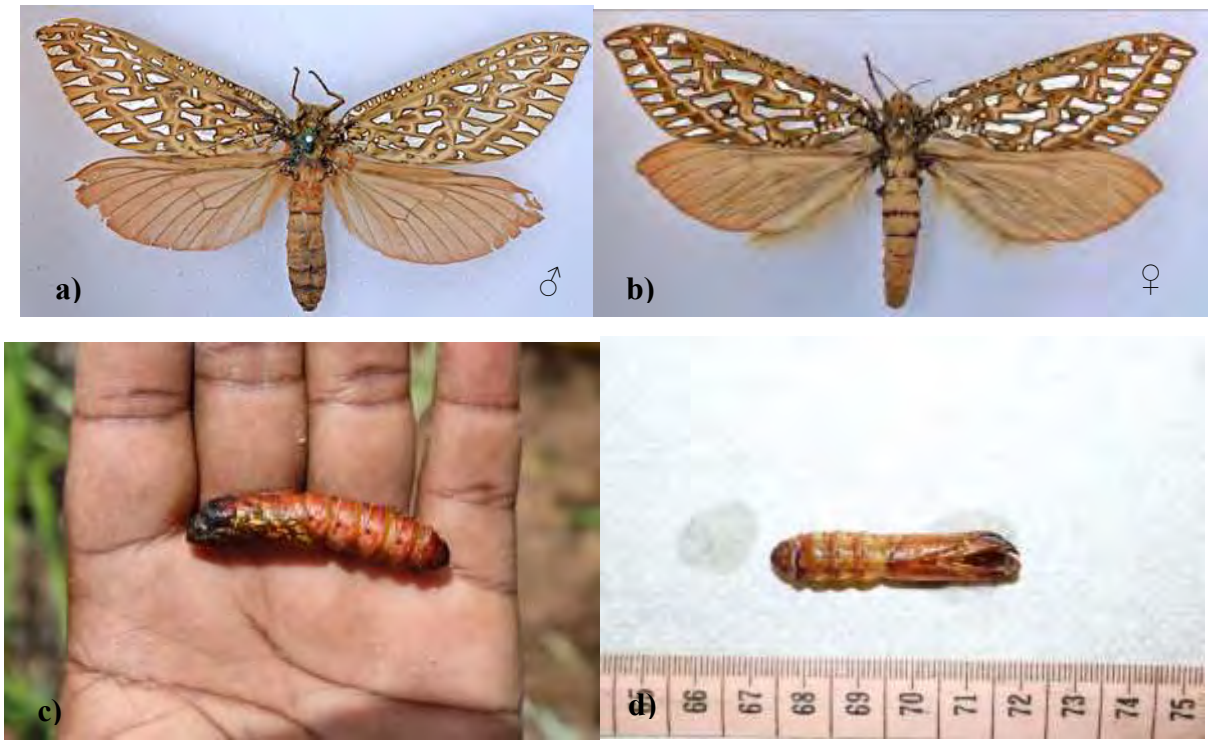

Figure 2.1: *Leto venus* adult male (a) and female (b) with bright and well defined spots (Grehan et al., 2018) and pupa with silver spots clearly visible (c) and pupa casing (d) (Photo Credit: Tapiwa Mushore).

The morphological characteristics of *L. venus* larvae were comprehensively described by Janse (1939). The first three segments or the thoracic segments each have a pair of true legs. The first

segment has a neck shield which is large and extends to just beyond the tip of the spiracle. The spiracle found at the tip of the neck shield is large and well developed. The second segment is also broad and well developed. The mesothorax is found across the anterior dorsal area. It has moderately placed setae which are arranged more centrally all over the body. The abdominal region has ten segments from the fourth segment to the thirteenth segment. The last segment forms the anal shield with one elongate pinaculum (Figure 2.2). Janse (1939) concluded that most segments of the *L. venus* larvae retain their primary and sub-primary setae such that a few secondary setae are formed. Janse (1939) further reported that primary setae in the thoracic region tend to disappear in the abdominal segments.

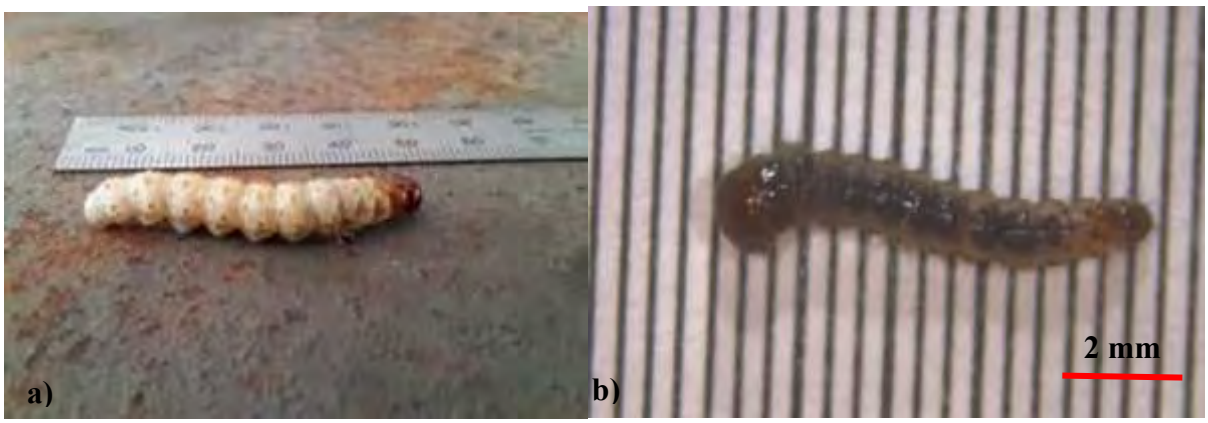

Figure 2.2: A mature larva (a) (Grehan and Ralston, 2018) and an immature larva of *Leto venus* (b) (Photo credit: T. Mushore).

The larvae of most hepialids are phytophagous, and their hosts include mosses, pteridophytes, gymnosperms and angiosperms. They are primarily borers which feed on roots and stems by ingesting host tissue, with some leaf feeding species that bore tunnels in the ground. Host specialisation has also been reported in some species such as *L. venus* which has been associated exclusively with Keurboom trees, *Virgilia* spp. (hence the name Keurboom moth) (Janse, 1945) and *Cyclopia* spp. (Grehan et al., 2018). It is, however, not clear as to whether specialisation arose because of these plant species being locally available, or because they contain desirable properties required by the larvae. Since it is host specific, their distribution is influenced by the availability of their host.

Leto venus is considered a key pest of cultivated honeybush with the larvae exhibiting an oligophagic feeding habit, feeding on *Cyclopi*a spp. and *Virgilia* spp. which both belong to the

same family (Grehan, et al., 2018). Efforts to better understand the larval feeding patterns of *L. venus* were made by Grehan and Ralston (2018), who examined and described feeding patterns on *Virgilia* spp.. Grehan and Ralston (2018) described the larval feeding habits as not primarily wood borers, but grazers of the phloem which then oozes nutrient rich sap. Their findings were consistent to those observed in hepialids and other stem borers (Nielsen et al., 2000). *Leto venus* larvae are thought to initiate tunnelling in stems closer to the ground and do not penetrate the roots hence their classification as stemborers. Tunnels are lined with a mixture of frass, wood shavings and silk, which also covers the opening. Tunnelling pattern was observed to differ from circular to a range of ovoid extensions which may appear broad and flattened at times (Grehan and Ralston, 2018). Grehan and Ralston (2018) also noticed that tunnelling was not entirely confined to cortical tissue, but at times extended to the bark suggesting phloem feeding, as the bark was entirely removed in some cases (Figure 2.3).

Figure 2.3: *Leto venus* just beneath the bark of *Cyclopia subternata* (a) and frass ejection from feeding (b) (Photo credit T. Mushore). Larval tunnelling by *L. venus* on *Cyclopia longifolia* (Grehan and Ralston, 2018).

The feeding pattern exhibited by *L. venus* is similar to that recorded for the genera *Aenetus*, *Zelotypia*, *Schausina* and *Trichophassus* (Grehan, 1989, 1983). Due to the similarities in feeding patterns observed on the tunnelling behaviour of *L. venus* with other hepialid stemborers, Grehan and Ralston (2018), hypothesised that pre-boring stages exhibit an initial microphagous feeding stage among plant detritus on the ground. They also observed that the tunnels are both in the outer and inner cortex. These observations are in sync with those

observed by Geertsema (1964) who stated that fully grown larvae descend and move under the bark of the tree. Larval diet transition between pre-boring stages and boring stages has been observed in species which have been studied, as the early instars are ground dwelling and mycophagous, feeding on fungi and dead decaying matter (Nielsen et al., 2000). The later instars are phytophagous and bore tunnels into stems and roots. It is thought that the developmental transition from mycophagy to phytophagy in Hepialids is a result of partial suppression of mycophagy in a generalist feeding ancestor of Lepidoptera (Grehan, 1983). Larval development is thought to occur throughout the whole year due to observations made on bark damage and presence of lateral growth. Geertsema (1964) suggest that fully grown larvae descend and move just under the bark before emerging as adults, whilst Grehan and Ralston (2018) argued that the mature larva moves to the top of the tunnel where it pupates. Therefore, there is a need for more detailed insect behaviour/ecology descriptions to better inform management practices. The damage caused by the *L. venus* on the host compromises structural integrity and the tunnelling effect destroys water and sap-conducting tissues (xylem and phloem). The resultant effect is stem dieback, girdling, decline in foliage and eventually death of susceptible plants. Moreover, entry sites serve as points for secondary infection by bacterial and fungal diseases (Machingambi, 2013).

During their survey, Knipe and Rosenberg (2008) identified several predators including lady bird beetles. However, literature suggests that predators are rare to find against stem boring larvae (Barbora and Singh, 1994; Hazarika, 2018). Several parasitoids, though not specified, were identified by Knipe and Rosenberg (2008) suggesting a potential control strategy. Stem borers are known to be parasitised by several indigenous and exotic parasitoids and the general assumption is that parasitism is higher on wild plant populations than cultivated populations (Mailafiya et al., 2011). The use of parasitoids for the control of *L. venus* remains unclear due to lack of information regarding the biology and ecology of the moth. The information relating to insects associated with *Cyclopia* spp. is still limited hence the need for more research.

The objective of this chapter, therefore, was to provide more information on the biology and ecology of *L. venus* with reference to cultivated Honeybush in South Africa. Specifically, to, (i) describe and quantify infestation by larvae of *L. venus* in cultivated *Cyclopia* spp., (ii) establish infestation parameters, including host preference through field surveys, (iii) determine the infestation severity, and (iv) determine the mode of infestation.

2.2 Methods and Materials

2.2.1 Study area description

The study was conducted in the Western Cape and Eastern Cape provinces of South Africa where Honeybush is grown. *Cyclopia* occupies narrow ranges in the Fynbos biome, an internationally recognised biodiversity hotspot (McGregor, 2018). The Fynbos biome extends in a broadband, 100 – 200 km wide, from the Northern Cape near Nieuwoudtville to the Cape Peninsula in the Western Cape and eastwards to Gqeberha in the Eastern Cape spanning the Coastal plains to the mountains of the inland Cape fold ranges (North et al., 2017). The Western climatic conditions are dominated by a combination of winter rainfall and hot dry summers. Whilst, in the Eastern region, rainfall is experienced throughout the year but most reliably in winter. The soils are nutrient poor, well drained, highly acidic and low in phosphorous and plant parasitic nematode count. This all facilitates the growth of certain plant species (Lotter and le Maitre, 2014).

Cyclopia spp. grow in the Coastal Districts from Darling to Cederberg, Bokkeveld, Klein Swartberg, Groot Swartberg and Kouga mountains with individual species being localised. According to SAHTA (2017), approximately 30 000 ha of the fynbos biome are dominated by *Cyclopia* spp. with Tsitsikamma, Kouga, Baviaans and Swartberg being the most abundant *Cyclopia* sites. Cultivation of *Cyclopia* spp. is still in its infancy with 300 ha under cultivation and is localised in the Overberg area to the Langkloof. *Cyclopia subternata* grows mainly in sandy loam soils in the Langkloof, Waboomskraw near George and in the Riversdale area (Joubert et al., 2011). *Cyclopia genistoides* have been established in the Overberg and Mossel Bay area where coastal sandy soils favour its production (Figure 2.4) (Mbangcolo, 2008).

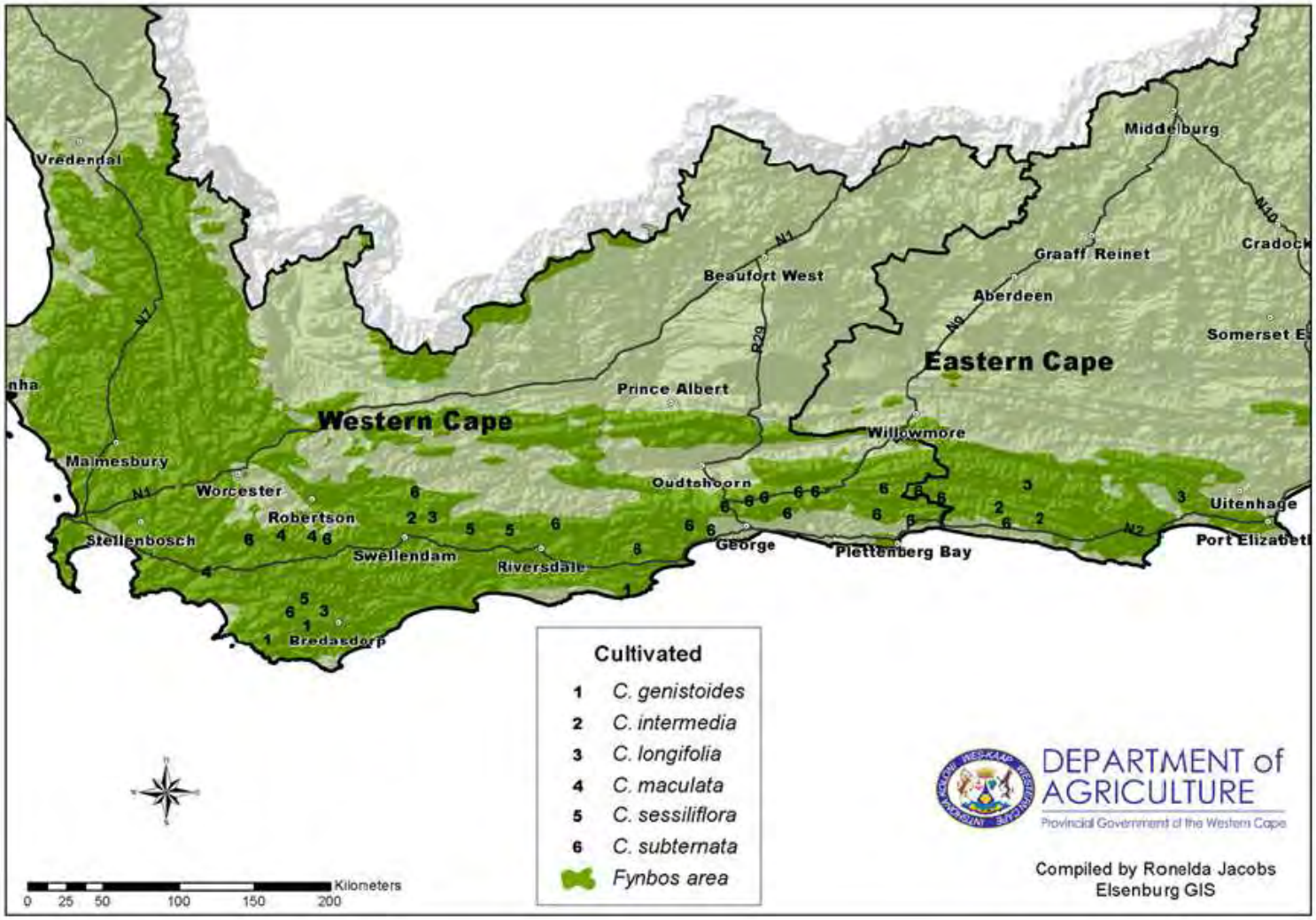

Figure 2.4: Distribution of natural and cultivated *Cyclopia* within the Fynbos biome (adapted from Joubert et al. (2011)).

2.2.2 Study Sites

Data were collected from two farms (Hidden Woods Farm and Treehorse Farm) in Harkerville (Western Cape, South Africa) and one Farm, Lennox Farm, near Sedgefield (Western Cape, South Africa) (Table 2.1;Figure 2.5) . These farms were chosen as considerable *L. venus* infestations have been reported there.

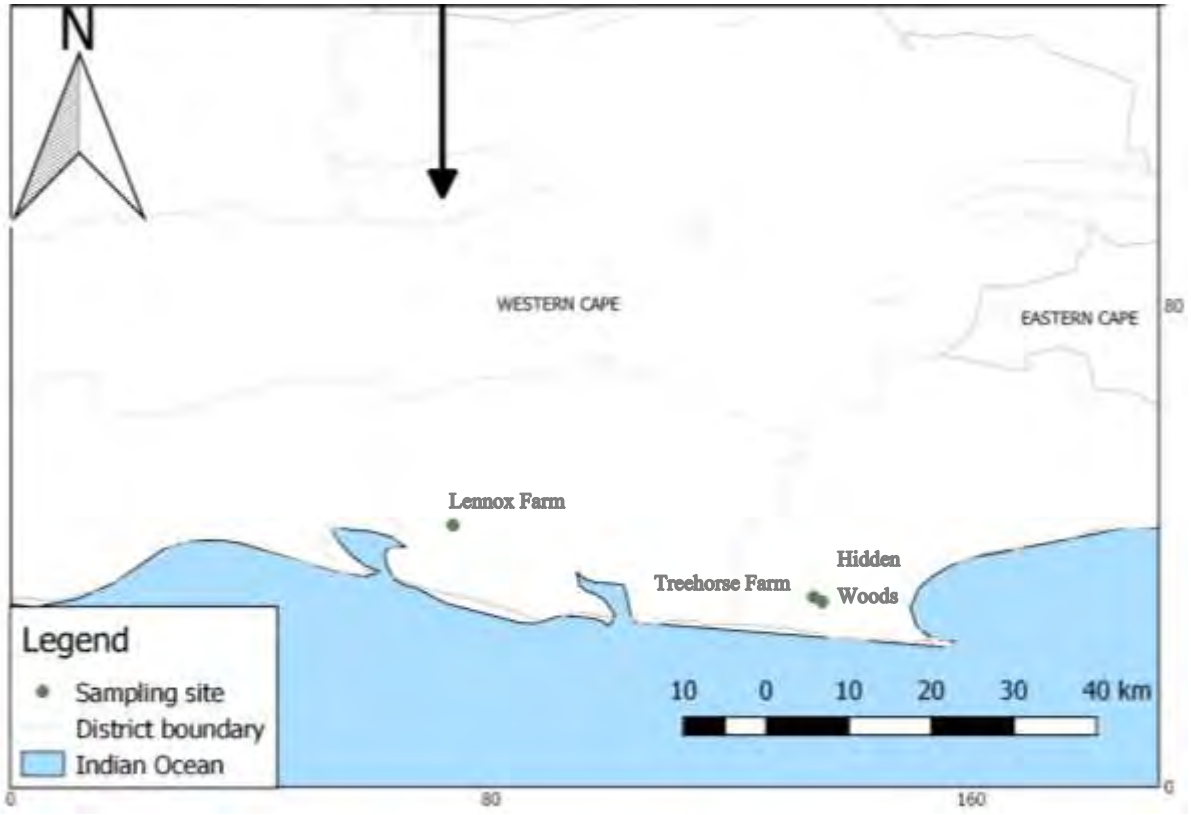

Figure 2.5: Location of *Leto venus* sampling sites. The green dots represent the location of each Farm.

Other farms such as Kurland and Kobus Lessig Farm had low infestations, which were reported by farmers. Data were not collected from these sites as the infestations were too low. Of the six cultivated species, only farmers growing *C. subternata* reported damage by *L. venus*, except for Kurland that cultivates over 30 ha of *C. longifolia*. However, at the time field surveys were conducted, there was no current infestation.

Table 2.1: Location and agronomic data for study sites.

Name of the Farm	Location (Co-ordinates)	Plantation Size (ha)	Species Grown
Hidden Woods Farm	Harkerville (34°03'16"S, 23°15'21"E)	1	*C. subternata*
Treehorse Farm	Hakerville (34°04'40"S, 23°18'49"E)	0.75	*C. subternata*
Lennox Farm	Sedgefield (33°58'36"S, 22°51'50"E)	0.8	*C. subternata, C. longifolia, C. genistoides and C. maculata*

2.2.3 Dynamics of *Leto venus* on cultivated Honeybush

At each Farm, the field was divided into blocks based on the age of the plants. Information on the age of the plants was obtained from the farmers. A minimum of 10 infested plants (due to very low infestation rates) were selected at random from each block. For comparison purposes between infested and non-infested plants, measurements were made on the plant adjacent to the infested one. The approximate total number of plants per block was determined by multiplying the number of plants per block with the number of rows. The total number of infested plants per block was recorded by walking the entire block counting all infested plants (Table 2.2). Infestation status was determined by dividing the total number of infested plants per block by the approximate number of plants in the respective block.

The infested species information was necessary to determine cultivated *Cyclopia* spp. that are prone to damage by *L. venus*. Age of the plants per quadrant was recorded to help determine the "age minima" (age of plants at which they become at risk from pest infestation) (Table 2.2). Infestation status as to whether the observed damage was a "current or old'' infestation was also recorded since scouting was done on plants older than one year. The difference between old and current infestation was confirmed by the presence of exit holes and the texture of

ejected frass. Currently infested plants have entry holes without exit holes and the frass is usually wet with a deep brown colour but, with time, it turns off-white in colour.

Table 2.2: Dynamics of the sampled fields in terms of age of plants in months, species grown and plant population.

Site	Block Number	Age of plants (months)	Species grown	Plant population
Hidden Woods	1	48	*C. subternata*	6 600
	2	40	*C. subternata*	3 600
Treehorse Farm	1	40	*C. subternata*	2 800
Lennox Farm	1	36	*C. subternata*	300
	2	24	*C. subternata,* *C. longifolia,* *C. maculata*	1 200
	3	18	*C. subternata* *C. longifolia,* *C. maculata,* *C. genistoides*	4 400

The number of larvae per individual plant was also recorded by counting the number of entry or exit holes. Stem diameter was recorded by measuring the stem thickness at its maximum (base of the plant) to determine the minimum plant thickness that is required to support and harbour the boring larvae. First branch height and entry hole height were measured to pinpoint exactly where on the plant the insect prefers as its entry point.

2.2.4 Data analysis

Generalised linear mixed effects models (Bolker et al., 2009) were used to investigate potential determinants of infestation of Honeybush plants and severity of infestation by *L. venus* larvae. For both infestation and severity of infestation, a global model was specified with four fixed effects namely; stem diameter, Farm, species and age. The variables were chosen based on the biology of honeybush plants and known dynamics of the *L. venus*. Infestation models were specified with a negative Binomial error distribution, with log link function. Infestation severity models were specified with a Poisson error distribution, with a log link function. A Kruskal-Wallis rank sum test was used to test for differences in the average entry height of *L. venus* on Honeybush plants.

The Akaike information criterion corrected for small samples (AICc) was used to identify predictor variables which influence infestation and infestation severity. The AICc fitted progressively simpler versions of the global model, which were then ranked using scores to identify plausible models (models considered to be within five AICc points of the top-rated model) (Harrison et al., 2018). Furthermore, Akaike weights (w_i) were used to determine the degree of support for each model. The relative importance of fixed effects was determined by summing w_i across all the models contained in the plausible model set to which each predictor occurred (Burnham and Anderson, 2003). Data analysis was performed using R software version 4.0.0 (R Core Team, 2020). Binomial models were specified using the "*glmTMB*" package (Brooks et al., 2017), while Poisson models were specified using the "*lme4*" package (Bates et al., 2015). All model diagnostics were performed using the "*DHARMa*" package (Harting, 2020). Models selection were performed using the "*MuMIn"* package (Barton, 2015). Mode of infestation data tested for normality using the Shapiro-Wilk test.

A negative Binomial GLM was used to identify variables that influenced the probability of infestation of Honeybush plants. The binomial distribution was a good fit for the model as residual diagnostics yielded a non-significant p value ($P = 0.273$) for the KS test. Furthermore, when model predictions were plotted against the standardised residuals, they showed equal variances. A chi-squared test was performed to test if the model with predictor variables was better than the null model. The model with predictors (Resd. Dev = 183.64) was significantly different from the null model, ($P = 0.013$, Resd. Dev 152.36). This means that the model with predictors was better than the null model by chance alone. Model selection for the logistic

regression model to determine the predictor best fit for the data by the delta AICc showed four plausible models. Stem diameter and species were the most important predictors of infestation as they were included in all top four plausible models (Table 2.3). Farm and age were also good predictors of infestation probability as they were included in two of the top four models.

Table 2.3: Plausible models (ΔAICc < 5) explaining probability of infestation of Honeybush plants. Parameter estimate are provided for each term across all plausible models on maximum likelihood estimation.

	Model rank	1	2	3	4
	ΔAICc	0.00	1.88	4.05	4.58
	Akaike Weight (w_i)	0.616	0.240	0.081	0.062
$\sum w_i$					
-	Intercept	-19.63	-19.42	-19.41	-17.50
1.00	Stem	0.2369	0.2670	0.2262	0.2763
1.00	Species	+	+	+	+
0.30	Age		-0.0144		-05535
0.014	Farm			+	+

Infestation severity (number of larvae per plant) was modeled using a Poisson GLM. The model was a good fit as the DHARMa residual diagnostics detected no significant p value ($P = 0.273$) for the KS test and model predictions plotted against standardised residuals showed equal variances across groups. A chi-squared test showed that the model with predictors, differed significantly from the null model ($P = 0.00053$). The null model had a lower residual deviation (94.057) than the model with predictors (119.01), indicating that the model with predictors was better than the null model by chance alone. Plausible models, were selected by the (ΔAICc < 5) which showed six plausible models. Plant species was the most important predictor as it was contained in all the plausible models, stem diameter was the second most important predictor included in four of the top six models. Farm and age of the plants were also good predictors of infestation severity included in three and two of the top six models, respectively (Table 2.4).

Table 2.4: Plausible models (ΔAICc < 4) explaining infestation severity of *Leto venus* on Honeybush plants. Parameter estimates are provided for each term across all plausible models on maximum likelihood estimation

	Model rank	1	2	3	4	5	6
	ΔAICc	0.00	2.17	3.08	3.69	3.81	3.86
	AICc weight (w_i)	0.499	0.169	0.107	0.079	0.074	0.072
$\sum w_i$							
-	Intercept	-18.75	-18.80	-18.27	-16.80	-18.30	-17.66
1.00	Species	+	+	+	+	+	+
0.85	Stem	0.1	0.097	0.079	0.0997		
0.26	Farm			+	+		+
0.25	Age		0.0024		0.0393		

2.3 Results

2.3.1 Infestation

Plant species

Cyclopia subternata had the highest probability of infestation by *L. venus* larvae while *C. longifolia*, *C. maculata* and *C. genistoides* had predicted probability of zero since there was no infestation recorded on these species (Figure 2.6). *Cyclopia subternata* was the only infested species with a likelihood of infestation over 50%. This suggests that *L. venus* prefers to infest a particular species, *C. subternata,* over the other cultivated species.

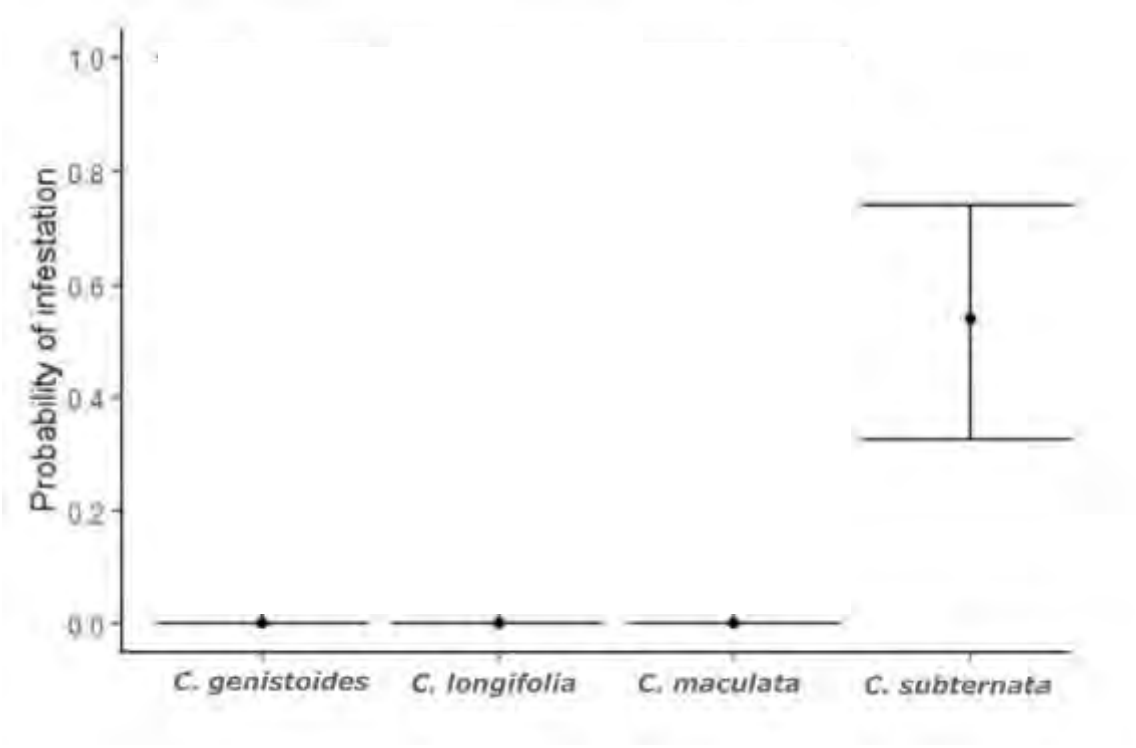

Figure 2.6: A Binomial GLM of the predicted probability infestation of Honeybush plants by *Leto venus* larvae across different species. Error bars represent a 95% confidence interval.

Location

Hidden woods Farm had the highest number of infested plants (1.14%) followed by Lennox Farm and the sparsest infestation at Treehorse Farm (0.02%). Hidden woods Farm also had the highest probability of infestation (0.53) while Lennox Farm had the lowest likelihood of infestation (0.30) (Figure 2.7).

Figure 2.7: Predicted probability of infestation of Honeybush plants by *Leto venus* larvae across different farms. Error bars represent a 95% confidence interval.

Age of the plant

Increase in age resulted in an increase in infestation (Figure 2.8). Plants below 30 months of age were shown to be less susceptible to infestation by *L. venus* than older plants. There is a 50% probability of infestation for plants above 48 months of age. This means that older plants are more susceptible to infestation than younger plants. A narrow confidence interval for plants below 45 months means that the model can predict probability of infestation for this range with higher precision. However, above this age range, the certainty of the model to predict probability of infestation based on age is reduced.

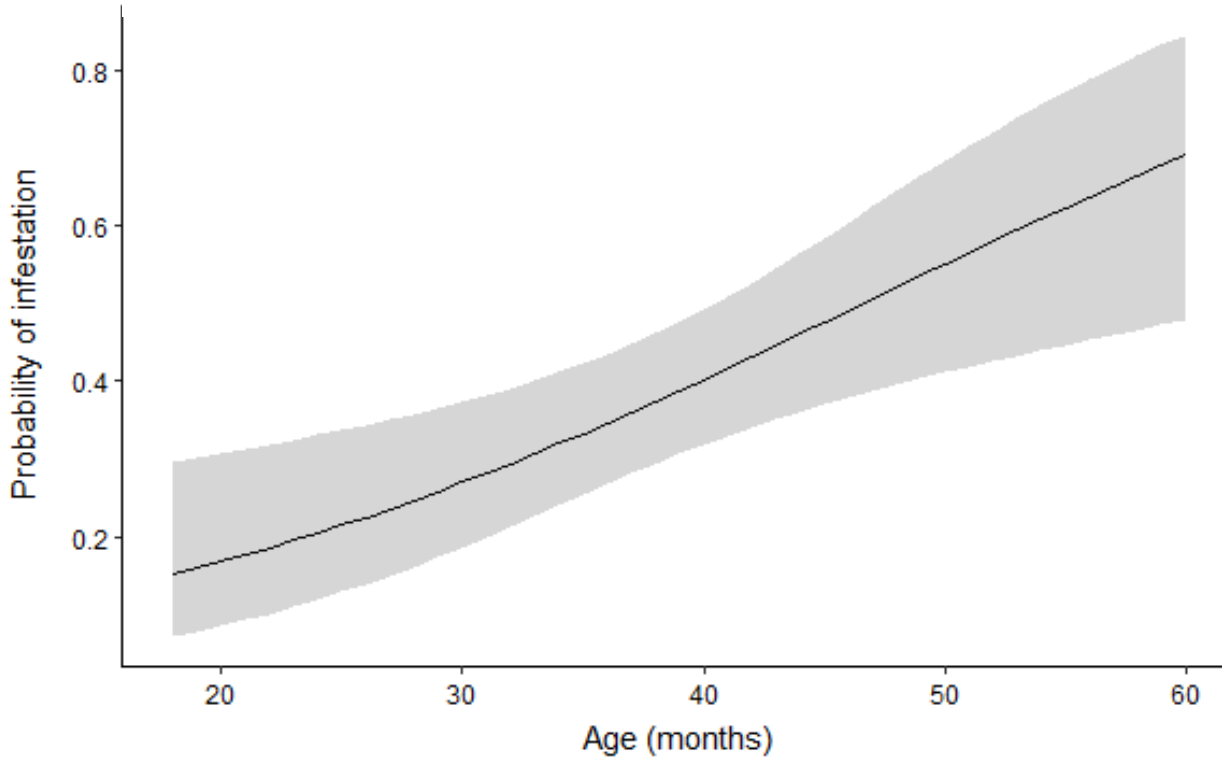

Figure 2.8: A Binomial GLM of the predicted probability of *Leto venus* infestation with age of the plants. The shaded region represents a 95% confidence interval.

Stem diameter

Stem diameter of Honeybush plants was the most significant predictor to have an influence on the likelihood of infestation by *L. venus* larvae. There is a 50% likelihood of infestation on plants with a stem diameter of 12 cm and above. The probability of infestation approaches 1 for plants with stem diameter above 25 cm. Meanwhile, probability of infestation is very low for plants with stem diameter below 5 cm (Figure 2.9).

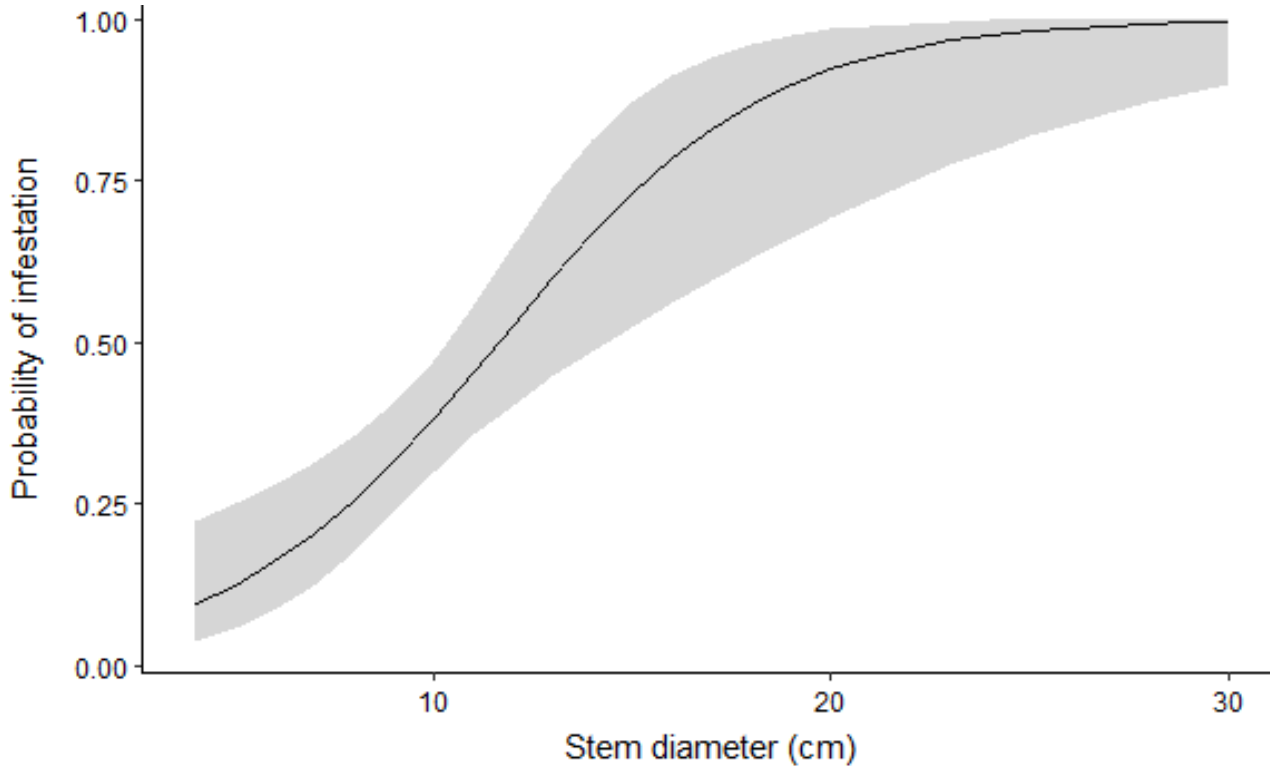

Figure 2.9: A Binomial GLM of the probability of *Leto venus* infestation with stem diameter. The shaded region represents a 95% confidence interval.

2.3.2 Infestation severity

Plant species

Infestation severity denoted by number of larvae per plant showed that *C. subternata*, the only infested species, had high infestation severity of 0.6 (Figure 2.10). The other species did not show severity as no infestation was recorded. This result shows that the severity of infestation is influenced by the species infested.

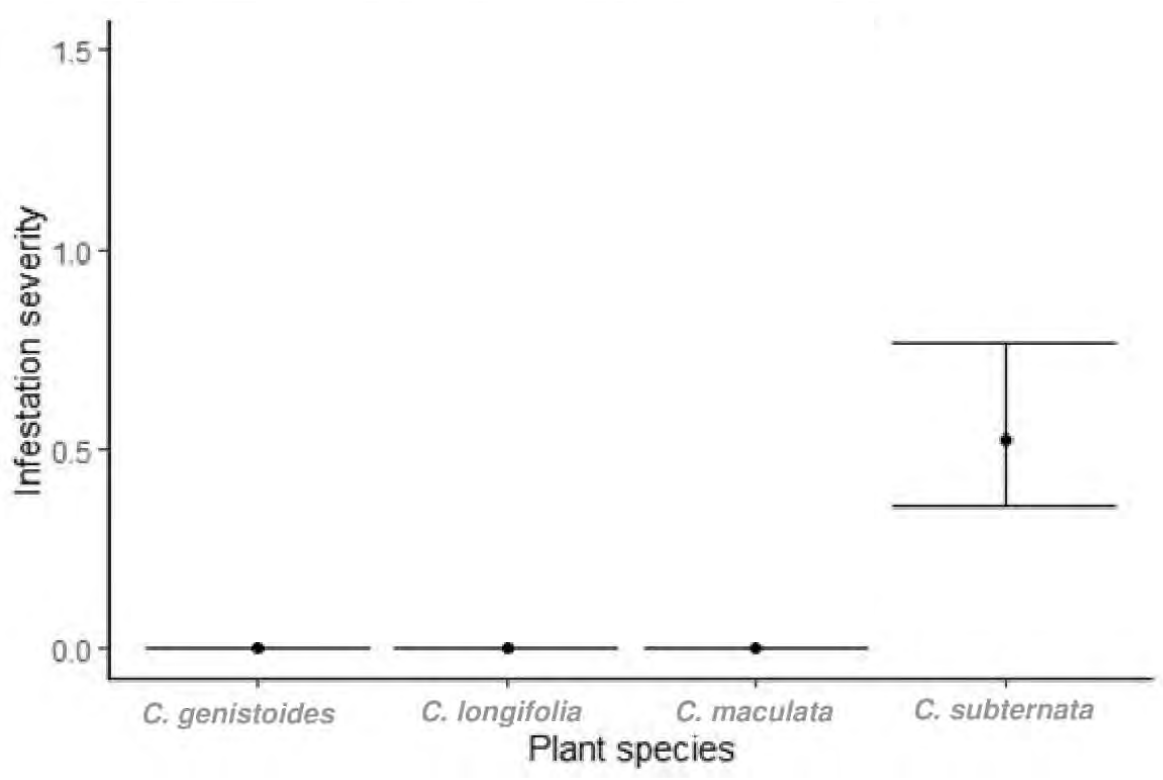

Figure 2.10: A Poisson GLM of infestation severity of *Leto venus* larvae across different species. Error bars represent a 95% confidence interval.

Farm

Hidden woods Farm had the highest predicted severity (0.5) among the three farms (Figure 2.11). Treehorse Farm, although it had the lowest infestation rate, had a much higher severity compared to Lennox Farm though they were no significant differences between these farms

Figure 2.11: A Poisson GLM for average predicted infestation severity of *Leto venus* larvae on Honeybush plants across different farms. Error bars represent a 95% confidence interval.

Stem diameter

The model predicted that below 15 cm stem diameter, severity of infestation is limited to one larva per plant (Figure 2.12). Severity increases with increase in stem diameter, this means that the thicker the Honeybush plant, the more larvae it can accommodate. The model is a good predictor of infestation severity for plants 4,5 – 15 cm because of a narrow 95% confidence interval. The variation of number of larvae per plant increases with increase in stem diameter. This means that thicker plants can accommodate more larvae than plants with small stem diameters.

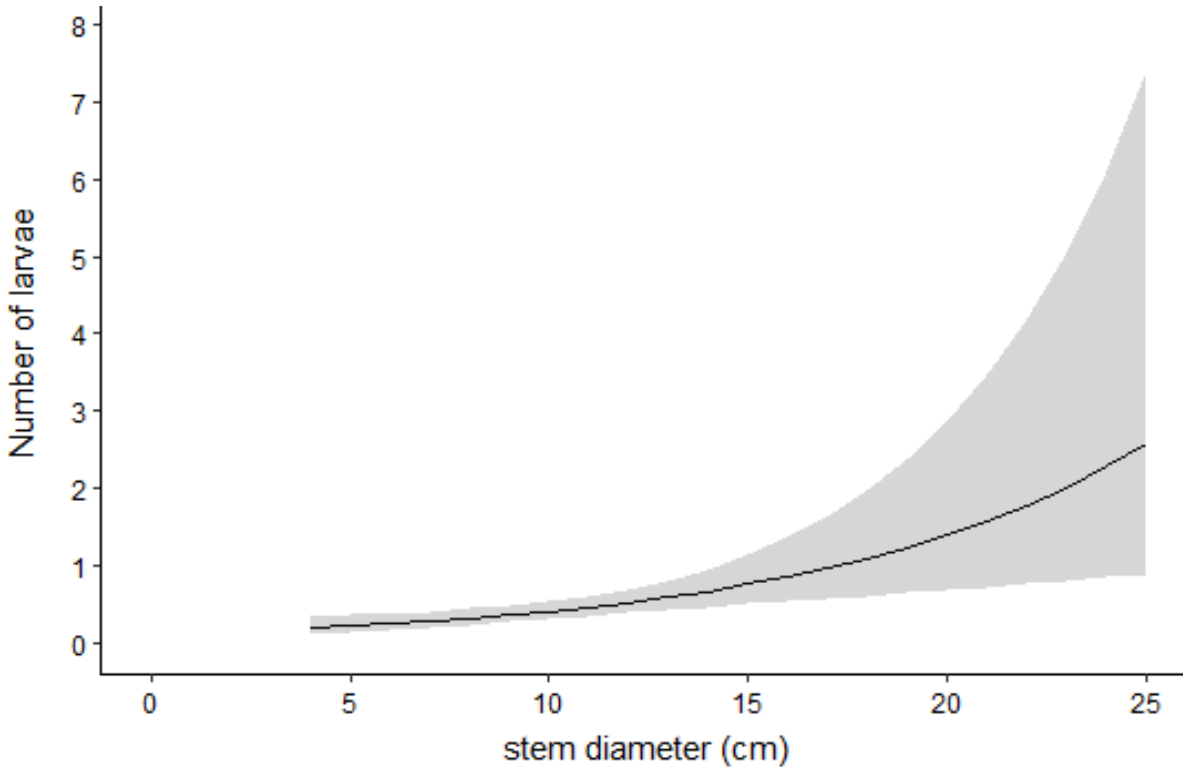

Figure 2.12: Poisson GLM of predicted infestation severity of *Leto venus* larvae influenced by stem diameter. The shaded region represents a 95% confidence interval.

2.3.3 Mode of infestation

Mode of infestation was measured by the position where infestation was initiated (entry hole). Infestation appeared to be initiated at ground level or just above the ground on all the farms (Figure 2.13). A Kruskal-Wallis rank sum showed that there were no significant differences ($P = 0.2239$) in the average entry hole height of *L. venus* on all Honeybush farms.

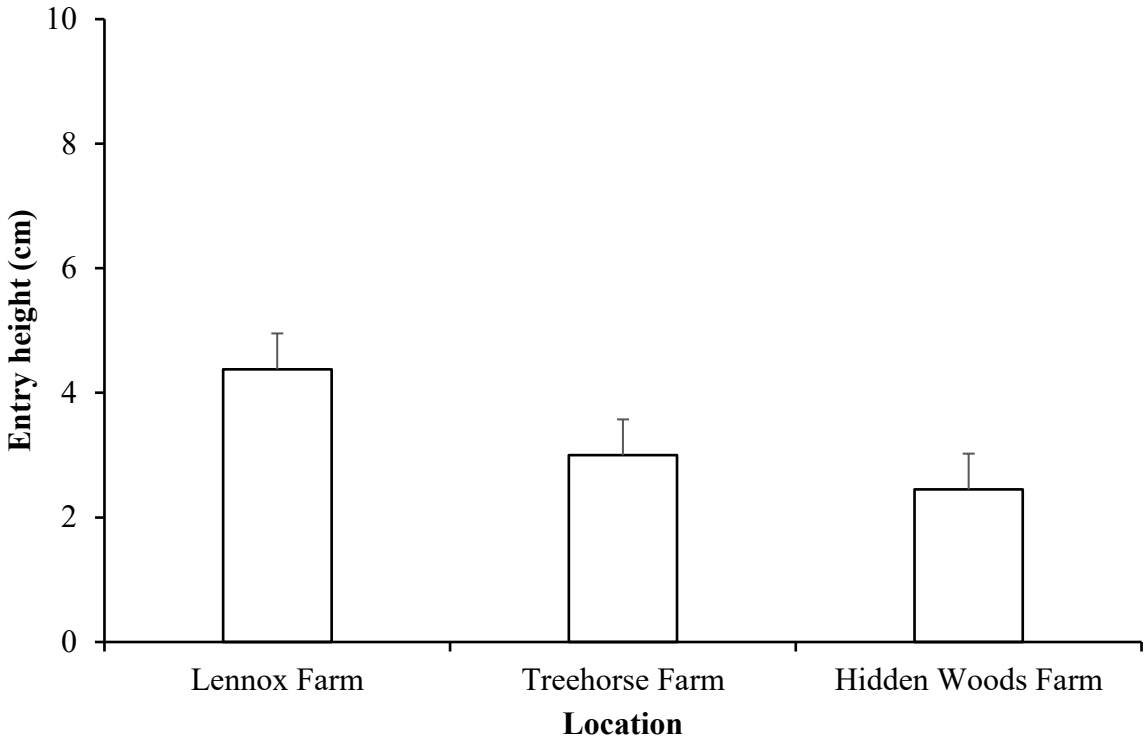

Figure 2.13: The average position of *Leto venus* entry hole on infested Honeybush plants across different locations. Error bars represent standard deviation. No significant difference amongst farms was recorded ($P = 0.2239$).

2.4 Discussion

In this study, *L. venus* showed a significantly strong preference for *Cyclopia subternata.* The results were consistent to findings by Grehan and Ralston (2018); Grehan et al. (2018), in their studies of *L. venus* infestation on *C. subternata* on Hidden Woods Farm disproved earlier reports by Grehan (1989); Nielsen et al. (2000), which reported the larvae as a monophagous insect. *Leto venus* infestation, within the cultivated *Cyclopia* spp. was, however, not exclusively limited to *C. subternata*. Metcalf et al. (2018), reported infestation on *C. longifolia* in their survey in the Eastern Cape and Western Cape, and there are various undocumented reports of *L. venus* infestation of other *Cyclopia* spp.. However, the results show a very strong bias towards *C. subternata*. Metcalf et al. (2018), also arrived at the same conclusion as they reported that *C. subternata* is more susceptible to infestation (90%) compared to *C. longifolia* (10%). The susceptibility of *C. subternata* to *L. venus* infestation may be attributed to ecological monophony (Nielsen et al., 2000) as it may possess both physical and chemical properties that are required by the larvae over other *Cyclopia* species.

Hidden Woods Farm had the highest predicted infestation probability and the highest infestation rate of 1.14%. However, Grehan et al. (2018), reported a decline in infestation levels from 2017 to 2018. Metcalf et al. (2018), also reported infestation as high as 8 – 10% in their survey on the same farms, while the highest recorded infestation in this survey was under 2%. Geertesema (1964) reported high infestation levels of up to 30% on *Virgilia* spp. The low infestation levels recorded in this study could be due to current physical control measures (killing the larvae by poking the entrance holes) implemented by farmers or natural fluctuations in populations of the moth. If the reason for these variations is a natural phenomenon, *L. venus* can be a good indicator species for assessing ecological change as suggested by Grehan et al. (2018). According to da Rocha et al. (2010), ecological consequences of agricultural practices can be explored with biodiversity indicators based on species occurrence.

Our results also showed a positive correlation between age of the plant and the probability of infestation. Infestation was predicted to begin on plants older than 18 months, with the likelihood increasing with age. The older the plant, the longer it is exposed to attack. Since infestation by *L. venus* is strongly hypothesised to occur on a yearly basis as reported by Grehan et al. (2018) and Metcalf et al. (2018), the longer the plant is in the field, the greater the chances

of infestation. Metcalf et al. (2018), reported a case of yield reduction on one of the farms from 36 month old plants and older. However, based on their reported infestation levels, yield reduction could have been due to variety of factors such as the age of the plants and the depreciating soil nutrients. Honeybush tea growth like any other tea plant is dependent on numerous factors some of which are inherent to the plant itself and some external factors such as soil, climate, pests and crop husbandry practices. Yield production in Tea, *Camelia sinensis,* is maintained through addition of organic fertilisers containing major nutrients, Nitrogen (N) Phosphorous (P) and Potassium (K) (Dutta, 2011; Ruan et al., 2020). However, there are no fertiliser regimes in Honeybush production, hence, it is prone to yield reduction over time.

Increase in age also results in increase in stem thickness thus making the plant more desirable to feeding by *L. venus* larvae. The thicker the plant, the higher the probability of infestation. As a stem borer, *L. venus* needs plants that can accommodate it. Host plant location can be achieved through oriented movements towards it or non-directed movements (Ahmed et al., 2019). Both *Virgilia* spp. and *Cyclopia* spp. are very variable in colour and form, making shape a complex cue by which *L. venus* employs host location. The initial *L. venus* survey by Metcalf et al. (2018), reported correlation between stem thickness and infestation and they further noted that mature plants were more susceptible to infestation. The thicker the stem, the better it can accommodate *L. venus* larvae as was shown by our findings, infestation increases with age and consequently, the stem thickens with age also. Thus, host location by *L. venus* adults is not only based on physical properties (stem thickness), as there were also other plants in the field that would equally accommodate the larvae, but it is likely to be also mediated by other cues i.e. visual and/or olfactory.

Infestation severity was predicted to be influenced by species and location. To this end, *C. subternata* was predicted to have the highest severity as it was the species most prone to infestation. Moreover, Hidden Woods Farm had the highest predicted severity over other farms. This could have been a result of successive infestation from previous years. Successive infestation is likely since adult Keurboom moth are short lived and clumsy fliers due to their size and being filled with eggs (Janse, 1945). As such, they are likely to lay their eggs in the same field they eclosed from resulting in increased infestation.

Duke and Taylor (1964), reported severe infestation of *L. venus* on *Virgilia* spp. at Longridge Farm where almost all large trees had more than one larva. Scoble (1992) also noted severe

cases of infestation on *Virgilia* spp. where some trees had over 20 protruding pupal cases. However, these could have been pupal cases from successive years. Machingambi, (2013) investigated the cause of death of *Virgilia* spp. in the Cape Floristic region and concluded that severe infestation by *L. venus* larvae was one of the main causes of death. The infestation severity in cultivated *Cyclopia* spp. does not seem to be comparable with that observed in *Virgilia* spp. as most of the infested plants had one larva per plant. The difference in severity could be because *Virgilia* spp. trees are large with much thicker trunks as compared to *Cyclopia* spp., and as such they can easily accommodate more than one larva. Moreover, they are usually long lived, up to 10 years (Machingambi, 2013), in comparison to cultivated *Cyclopia* spp., all of which were under 5 years. Based on personal observation, only Lennox Farm had *Virgilia* trees showing signs of infestation. However, there were no *Virgilia* spp. near Hidden Woods Farm and Treehorse Farm. The recorded infestation could be a result of subsequent infestation from previous years and what remained after control efforts by the farmers. It is still unclear as to whether *Virgilia* spp., are the preferred hosts of *L.venus* over *Cyclopia* spp.,

Larval establishment in cultivated Honeybush can be ascertained through observations of entry hole height. Grehan and Ralston (2018), in their study of larval feeding habits of *L. venus*, observed that tunnels were within the lower 40 cm of plant stems with frass being ejected from holes 20 cm above ground. Mode of infestation results showed that the larva initiates boring on the ground or just a few centimetres above the ground. Grehan (1989) reported that ghost moth eggs develop on the plant and early larval development occurs on the ground surface before migrating to the plant. Geertesema (1964) hypothesised that *L. venus* eggs are deposited on the ground close to the host plant where they mature prior to infestation. Although inconclusive, the position of the entry hole is highly suggestive that *L. venus* is similar to other boring ghost moths which have both a macrophagous (ground) and a phytophagous (plant) larval development (Grehan, 1989; Nielsen et al., 2000).

In conclusion, fluctuations in population dynamics of *L. venus* show the sensitivity of the moth to human interferences and since it is endemic, it needs to be treated with utmost caution when it comes to control. Furthermore, these fluctuations coupled with very low infestation rates make studies on larval development, biology and ecology of the moth as well as testing of control options extremely challenging. As such, Grehan and Ralston, (2018), used the term “enigmatic” in their description of *L. venus*. This study helped demystify some of the mystery

surrounding the biology and ecology of *L. venus* with respect to cultivated Honeybush. Information on species which are more prone to infestation is useful in implementing control strategies. This will be one of the factors to be considered in selecting *Cyclopia* spp. to grow and where susceptible species are chosen farmers are better able to deal with the infestation. Honeybush tea is a perennial crop where age of the plants influences the level of infestation, farmers can grow their plant up to a certain age and harvest their crop before infestation increases. The best intervention strategy for *L. venus* as a stem borer, is to prevent infestation. The implications of a ground developmental stage (egg and early larval development) present an opportunity to control it before it migrates to the plant and should be investigated further if further control of this pests is deemed necessary in the future.

CHAPTER 3

Bionomics of the Leafhopper *Molopopterus* sp.

3.1 Introduction

As a long-lived bush/shrub, *Cyclopia* spp. create micro-habitats which are ideal for a many phytophagous insect pest that are not well studied (Slabbert, 2016). The leafhopper, *Molopopterus* sp. Jacobi (Hemiptera: Cicadellidae), is a sap sucking pest of agricultural importance among other pests (Gerhard and Prinsloo, 2017). The family Cicadellidae is in the suborder Auchenorrhyncha which comprises four modern Superfamilies; Cercopoidea (spittlebugs), Fugoroidea (planthoppers), Cicadoidea (cicadas) and Membracoidea (leafhoppers). Auchenorrhyncha have an antenna hair-like flagellum (aristoid), the arostrum that arises from the posteroventral surface of the head and a complex sound producing tymbal apparatus (Resh and Garde, 2009). The hind wing coupling apparatus has a long downturned fold on the forewing and a short upturned lobe on the wing (Theron, 1978). Leafhoppers are ubiquitous insects with a worldwide distribution occurring in almost all terrestrial habitats where host plants occur. They are especially important agricultural pests as they are injurious to plants directly through feeding and oviposition and indirectly through transmission of pathogens (Dworakowska, 1973; Hatting, 2017; Theron, 1978).

The genus *Molopopterus* has a wider head than the protonum and a well-developed ocellus. The thorax has a pronotum with conspicuous pits and the forewing has an outer apical cell which is elongate and basally truncate. The veins also form continuous lines, whilst the hind wing apex is broad and rounded (Figure 3.1) (Theron, 1978). The sub marginal vein on the hind wing does not extend to the wing apex. The front femur has two or more rows of fine basal setae. The male genitalia are perhaps the main distinguishing feature of this genus as the pygofer is not extended to the genital plates and are ungulate without setae. In addition, sub genital plates have 2–4 basal macrochaeta, the dorsal apodeme of the adageus has a distinct v-shaped ligament connected to the anal tube. The anal tube is devoid of processes and lateral spines.

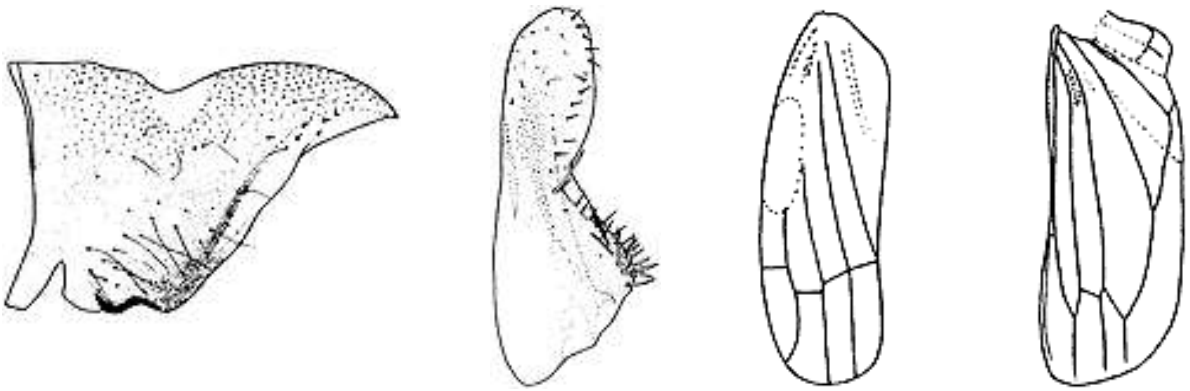

Figure 3.1: Distinguishing features for *Molopopterus* spp., From left to right: the thorax, sub genital plate, forewing and hindwing (Dworakowska, 1973).

The genus occurs exclusively in Africa with only one location reported outside Africa in the South of Asia (Figure 3.2) (Dworakowska, 1973; Hatting, 2017).

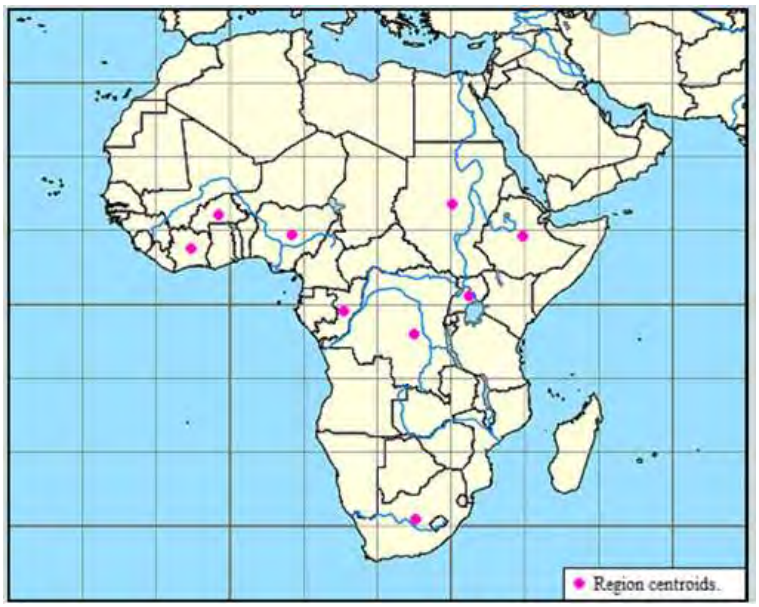

Figure 3.2: Distribution of the genus *Molopopterus* across Africa (Dworakowska, 1973).

Leafhoppers are paurometabolous (Oliver et al., 2011), having a gradual metamorphosis with nymphs and adults displaying morphological similarities (Backus et al., 2005). Their population structures (longevity and abundance) are dependent on geographic area, climate and the plant species and tissues they feed upon. The life cycle involves simple metamorphosis proceeding from egg to adult via a series of nymphal stages. Eggs are deposited inside the leaf and stems from which the nymphs emerge. Hatchlings are wingless and nearly colourless and

just like adults, they have piercing mouthparts, hence, they are restricted to a liquid diet. The nymphs moult leaving their exuviae on the underside of the foliage. In *Molopopterus* spp., abdominal bands become darker and more visible with each moult (Theron, 1978). However, there is limited information regarding the life history traits of this genus prompting the need for further study.

Adults and nymphs suck plant sap mainly form the underside of the leaf causing phytotoxic symptoms termed “hopperburn”, which can result in complete desiccation. Hopperburn is a result of a dynamic feeding interaction between insect feeding stimuli (hopperburn initiation) and a complex plant response (Habibi et al., 2001). According to Backus et al. (2005), the hopperburn symptoms are a plant wound response triggered by the insects stylet, and further intensified by their saliva. Damage is cumulative with the green leaf appearing as if it has been increasingly sand blasted (Figure 3.3). Leafhoppers excrete honeydew (a combination of undigested sugars and excess water) which attract bees, wasps, flies and ants (Brodbeck et al., 2009; Jarrell et al., 2020). At very high humidity, the honeydew also promotes the growth of sooty mould (Jeanike, 1990). The feeding repercussions become one of the limiting factors in terms of economic production of Honeybush tea due to biomass loss. In addition, many leafhoppers are considered to be vectors of several plant pathogens (Beanland et al., 2006; Orenstein et al., 2003). Metcalf et al. (2018), observed streaking and stunted growth on infested plants. Although unconfirmed, they suggested that the *Molopopterus* sp. has potential secondary damage as a vector of plant viruses. The economic injury level of leafhoppers is high since they feed on leaves and new shoots affecting yield potential of tea plants (Meyling and Eilenberg, 2018).

Figure 3.3: Leafhopper damaged leaves with a characteristic "sand blasted" appearance (left) and undamaged leaves of *Cyclopia longifolia* (right) (Photo credit: T. Mushore).

Kathirithamby et al. (2010), did a survey of *Molopopterus theae* Theron in Rooibos from December to April. Natural enemies included *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales), a predatory mite *Namadia floreata* Meyer & Ueckermann (Acari: Anystidae) and a strepsipteran parasitoid, *Halictophagus calcaratus* Pasteels (Strepsiptera: Halictophagidae). Slabbert (2016) recorded seven families of parasitoids within Honeybush stands including Platygastridae, Encyrtidae, Mymaridae, Eulophidae, Pteromalidae, Braconidae and Diapriidae all of which may be potential parasitoids of leafhoppers. However, there is need for further studies to ascertain whether these parasitoids attack the leafhopper. Natural enemies can suppress populations of *Molopopterus* spp. in the field to some extent. However, without augmentation they cannot prevent economic damage (Brodbeck et al., 2009). Healthy plants can tolerate pest damage to some degree, hence, *Cyclopia* spp. plants need to be kept as healthy as possible (Jarrell et al., 2020).

The damage potential of *Molopopterus* sp. in cultivated *Cyclopia* spp., is determined by its movement. Host selection by *Molopopterus* sp. is done predominantly by the adult. According to Jeanike (1990), leafhoppers associated with woody plants tend to be host specific irrespective of nearby potential alternate suitable host plants. The movement from one host to the next is a product of their life history traits, host range and preference, availability and status of the host. Very little is known about this pest regarding host range and preferences. In *Cyclopia* spp. cultivation, awareness of life history traits and damage potential of *Molopopterus* sp. and its interactions with the crop environment are necessary components for a successful management programme. Since there is zero tolerance towards synthetic pesticides in the cultivation of *Cyclopia* spp., there is the need for the most desirable management practices which are effective, compatible and economic.

The future of Honeybush pest management lies in comprehensive understanding of pest species and plant pest interactions. Crop protection is an essential component of tea husbandry; hence the development of economic strategies for pest control, are important. IPM is a robust construction system that in the context of associated environmental and population dynamics of pest species, uses all suitable technologies to suppress pest populations below the economic injury level (FAO, 2009; van Emden et al., 1986). One of the keystones of IPM is a thorough

understanding of the biological cycle of pests. This knowledge enables the understanding of pest population dynamics and determination of community dynamics (Abdel-Baky et al., 2004). Therefore, any successful pest control programme should begin with an in-depth understanding of the pest bionomics.

Therefore, the aim of this Chapter was to contribute to *Molopopterus* sp. bionomics by describing the life history of this pest, determining its host preference and factors that influence its distribution and abundance in cultivated Honeybush fields. The specific objectives of the study were; (i) to determine oviposition behaviour and partitioning of fitness components of *Molopopterus* sp., i.e. oviposition preferences in various plant structures; (ii) to describe the lifecycle of the leafhopper through instar determination through head capsule measurements and determine its generation time; (iii) to determine variations in host preferences of *Molopopterus* sp. to some of the most cultivated species of Honeybush, *C. subternata*, *C. longifolia* and *C. maculata* and; (iv) to conduct a field survey to examine correlations between plant characteristics (age of plants and plant species), harvesting regimes and Farm location on pest densities.

3.2 Methods and Materials

3.2.1 Life history of *Molopopterus* sp.

Life history experiments were undertaken at the Waainek Research Facility (33°19'14.1"S, 26°30'25.5"E), Rhodes University in a polyethylene greenhouse tunnel. Potted insect free *C. longifolia* plants acquired from a *Molopopterus* infested Honeybush Farm (Kurland) were used as host plants. Adult leafhoppers were also collected from Kurland and were exposed to host plants for seven days after which they were all removed. Daily observations were made, and the dates of hatching, moulting and adult emergence were recorded. Eggs were not visible to the naked eye. Thus, to determine the number of eggs laid and oviposition locality, young *C. longifolia* plants were exposed to 20 adult leafhoppers for 10 days (as generally leafhopper eggs are known to hatch in 10 days (Oliver et al., 2011)). After ten days four small branches with both leaves and stems were stripped from the plant and stapled to a cardboard block for better handling during clearing and staining. A lactoglycerol staining procedure was used to stain the plant tissues. Plant tissues were cleared using lactoglycerol (85% lactic acid, glycerol and distilled water 1:1:2). Acid fuchsin dye was added to the lactoglycerol solution at 1 g/L to stain both plant tissues and the eggs. Plant tissues were exposed to the clearing and staining solution at 80°C in a beaker for five minutes before being transferred to hot water for two minutes to remove excess stain. Observation on shape, size and position of oviposition was made under a dissecting microscope (Leica EZ4D) at 10 – 30× magnification with substage lighting (Figure 3.4).

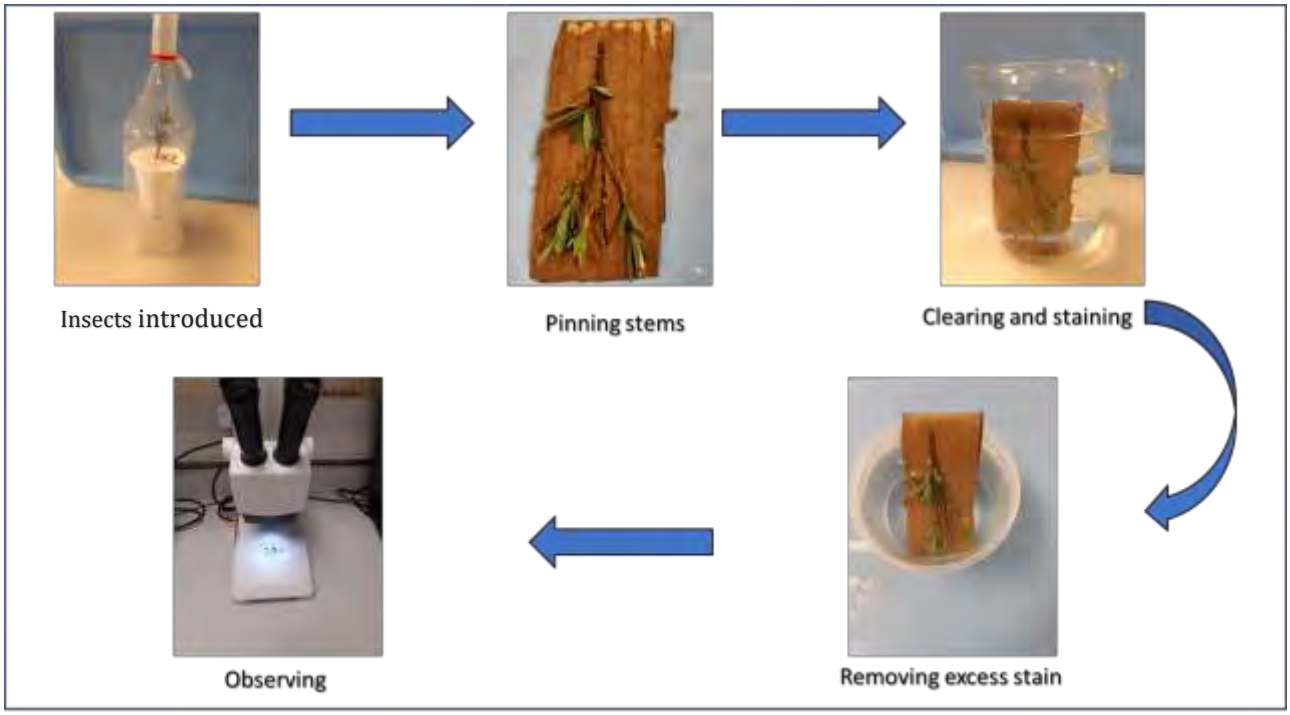

Figure 3.4: Visualisation procedure of egg masses of *Molopopterus* sp., from *Cyclopia longifolia* plant tissues.

Leafhoppers are difficult to handle and easily injured during handling. As a result, only the total development time and incubation period were recorded. The incubation period was determined by recording the time from which the host plant was exposed to adult leafhoppers until the nymphs appeared. Development time was recorded from the time eggs hatched until the final instar was reached and as such, both development time and incubation period are given as a range. To facilitate the determination of number of instars, several nymphs were originally collected in the field at Kurland. Number of instars was determined using the frequencies of the head capsules according to Dyar's Law (Dyar, 1890). The width of the head capsules was measured under a dissecting microscope (Leica EZ4D) at 10 – 30× magnification. Images were acquired using the Leica application Suite (Version 3.3.0).

3.2.2 Host preference to feeding by *Molopopterus* sp.

Host preference by the leafhopper was established through choice tests. They were done by comparing the attractiveness of the three most cultivated species of Honeybush; *C. subternata, C. longifolia* and *C. maculata*. Seedlings of approximately 25 cm in height and of similar architect were placed in individual soil-filled pots. The pots were then placed in a cage

equidistant from one another and the number of leaves per plant was counted. Thereafter, 30 adult insects were collected and released at the centre of the cage (Figure 3.5). Observations were made at 24 hour intervals for the first three days and then every three days until 15 days post-release. Host preference was determined by the total number of leaves with hopperburn symptoms at each time interval and was expressed as a proportion of the total number of leaves on the plant at the onset of the experiment. Furthermore, feeding intensity was accounted for using a scoring system of zero to two, zero being no damage, one being less than 50% leaf surface damage and two being more than 50% leaf surface area damage (Figure 3.5). The number of insects per plant was not considered a good measure of host preference as adults are easily unsettled prompting them to move around.

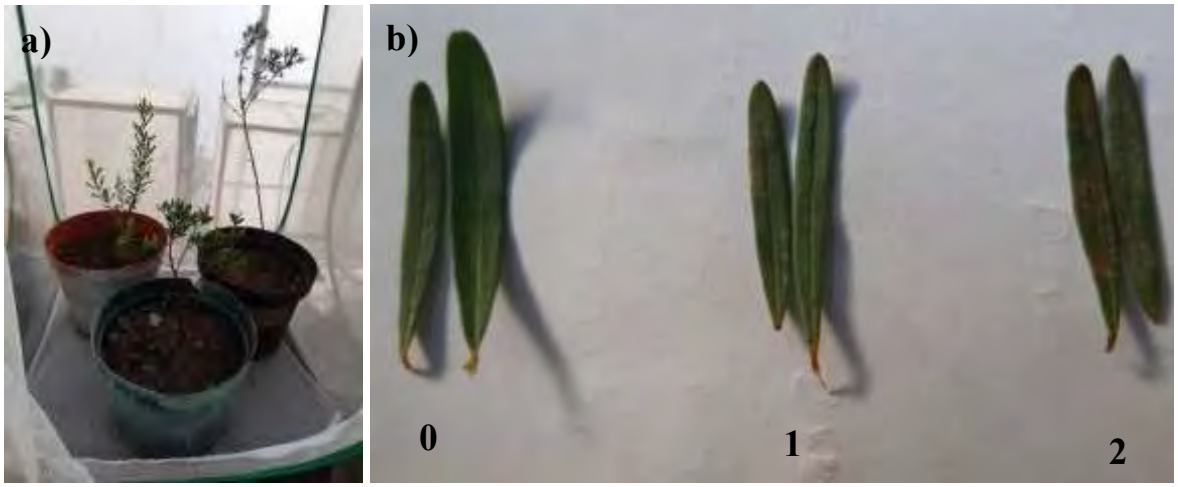

Figure 3.5: **a)** Choice test setup for *Molopopterus* sp. on three different *Cyclopia* species, **b)** rating scale used to quantify feeding damage.

3.2.3 Field survey for leafhopper density in Honeybush plantations

A field survey was conducted to determine *Molopopterus* sp. density, distribution and factors influencing infestation on seven different farms in the Western Cape. Data were collected at Kurland in the Crags, Lodestone Farm in Redford, Hidden Woods Farm and Treehorse Farm in Harkerville, Lennox Farm near Sedgefield, Bitou Plantation in Wittedrift and Jongershoek Farm near George (Figure 3.6).

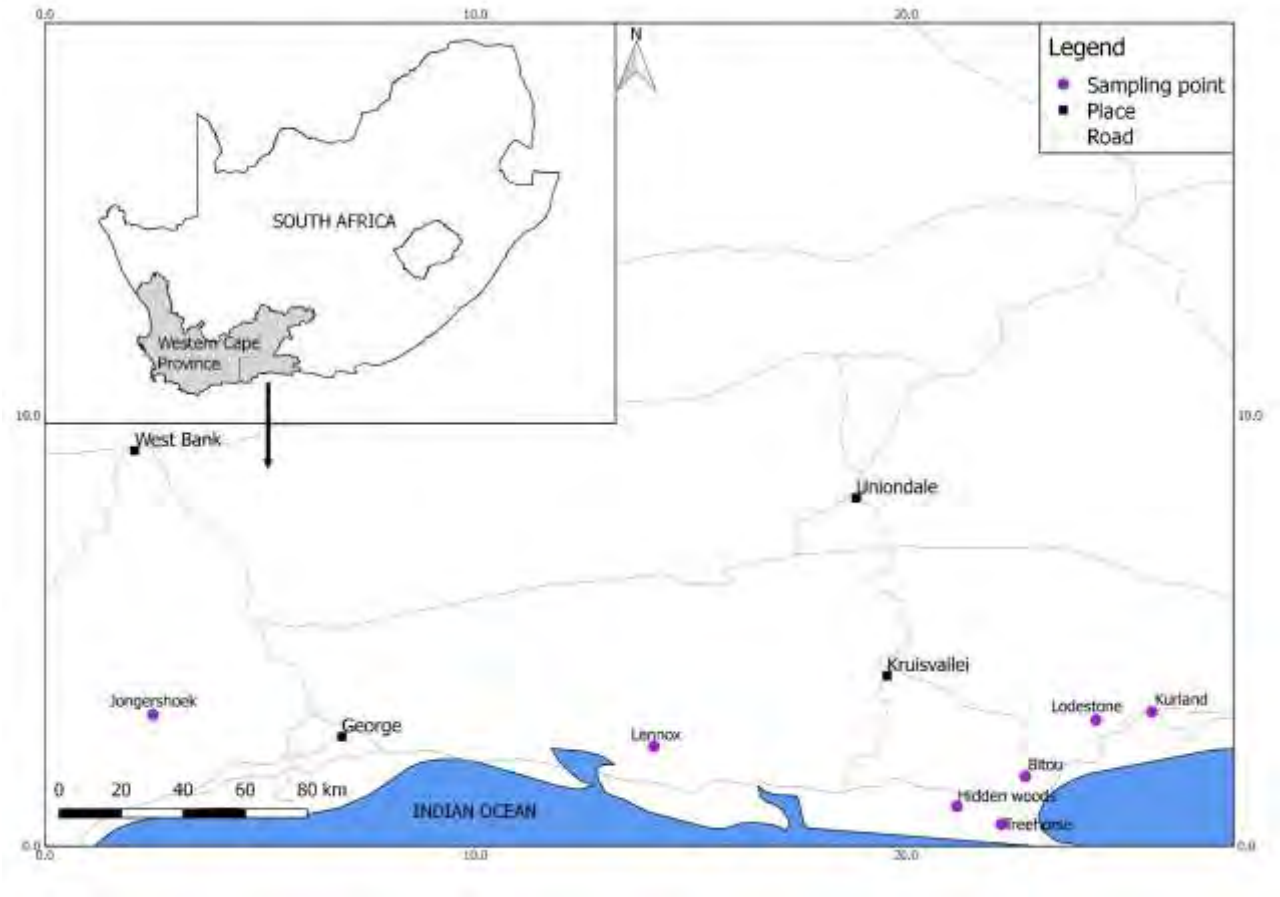

Figure 3.6: Sampling sites for *Molopopterus* sp. distribution and abundance in cultivated *Cyclopia* spp..

At each Farm, Honeybush fields were subdivided into blocks based on the age of the plants. Data on the age of the plants were obtained from the farmers. From each block, a total of 20 individual plants were selected at random and the density of leafhoppers per plant determined by counting the number of *Molopopterus* sp. per branch. Insects were sampled using a modified branch sampling procedure similar to the one described by Peeters (2002). Branches of Honeybush plants were immersed in a sweep net (diameter 32 cm × 55 cm length) then rapidly shook to dislodge the insects into the net. The branch was selected at random and care was taken to minimise disturbance of the branch prior to collection. The number of *Molopopterus* sp. collected in the net was considered as the total number of leafhoppers per plant. After sampling each individual plant, the net was turned inside out to prevent double recording.

Table 3.1: Characteristics of blocks sampled for *Molopopterus* sp. density.

Farm	Block	Age(months)	Species	Harvested
Jongershoek Farm	1	12	*C. longifolia*	No
(33°56'09"S, 22°12'53"E)	2	24	*C. longifolia*	Yes
	3	30	*C. subternata*	No
	4	36	*C. longifolia*	No
	5	42	*C. subternata*	No
Lennox Farm	1	42	*C. subternata, C. maculata and C. longifolia*	Yes
(33°58'36"S, 22°51'50"E)	2	50	*C. longifolia and C. subternata*	Yes
Hidden Woods Farm	1	12	*C. subternata*	No
(34°03'16"S, 23°15'21"E)	2	70	*C. subternata*	Yes
Kurland Farm	1	24	*C. longifolia*	Yes
(33°55'55"S, 23°30'31"E)	2	24	*C. longifolia*	No
	3	30	*C. longifolia*	Yes
	4	36	*C. longifolia*	No
	5	36	*C. longifolia*	No
	6	70	*C. longifolia*	Yes
Bitou Plantation	1	9	*C. subternata*	No
(34°00'57"S, 23°20'41"E)	2	18	*C. subternata*	No
	3	28	*C. longifolia*	No
	4	28	*C. subternata*	No
	5	28	*C. maculata*	No

	6	30	*C. longifolia*	No
	7	42	*C. subternata*	No
Treehorse Farm	1	56	*C. subternata*	No
(34°04'40"S, 23°18'49"E)	2	56	*C. subternata*	Yes
Lodestone Farm	1	36	*C. subternata*	No
(33°56'34"S, 23°26'10"E)	2	98	*C. subternata*	No

3.2.4 Data analysis

Descriptive statistics were used to calculate average incubation and development time of the leafhopper and a regression analysis was used to determine larval instar stage from head capsule width measurements. A two-way repeated measures analysis of variance (ANOVA) was used to test the differences in means of the three Honeybush species preference to damage by *Molopopterus* sp.. Post-hoc tests were conducted using Tukey HSD ($\alpha = 0,05$) to separate means that were statistically significant. Generalised linear mixed effects models (Bolker et al., 2009) were used to investigate potential determinants of *Molopopterus* sp. density (average number of leafhoppers per plant). A global model was specified with four fixed effects namely; the Farm, plant species, age of the plants and harvesting regimes. The pest density model was specified with a Poisson error distribution, with a log link function.

The Akaike information criterion corrected for small samples (AICc) was used to identify predictor variables which influence leafhopper density. The AICc fitted progressively simpler versions of the global model, which were then ranked using scores to identify plausible models (Harrison et al., 2018). The model with the lowest AICc was chosen. Data analysis was performed using R software version 4.0.0 (R Core Team, 2020). Repeated measures ANOVA was specified using the "*nlme*" package (Pinheiro et al., 2013). Homoscedasticity and independence were checked by plotting residuals vs fitted values. Poisson models were specified using the "*lme4*" package (Bates et al., 2015). Model diagnostics were performed using the "*DHARMa*" package (Hartig and Lohse, 2020). Multi-comparison tests at 0.95 confidence level for Farm and species were performed using "*emmeans*" package.

3.3 Results

3.3.1 Instar determination

Incubation and preoviposition of *Molopopterus* sp. ranged from 19 – 23 days (n = 13) with an average of 20.8 ± 1.5 days. The nymphal stage development ranged from 23 – 29 days (n = 12) bringing the average development time to an average of 26.5 ± 2.6 days. The development rate including preoviposition and incubation time yielded an average generation time of 47.7 ± 3.8 days (n = 12). The average head width capsule for the nymphal stages ranged from 0.28 mm for the first instar to 0.93 mm for the last instar (Table 3.2). A linear regression model was used to show the five instars observed using the Dyar's Law of head capsule measurements (Figure 3.7). The head capsule measurements follow a linear regression.

Table 3.2: Head capsule width measurements (mm) of nymphal stages of the leafhopper *Molopopterus* sp..

Stage	Mean (mm)	n	Dyar's Law growth ratio
1st instar	0.28 ±0.046	9	1.5
2nd instar	0.42 ±0.075	13	1.54
3rd instar	0.65 ±0.032	8	1.24
4th instar	0.76 ±0.029	12	1.22
5th instar	0.93 ±0.050	21	_

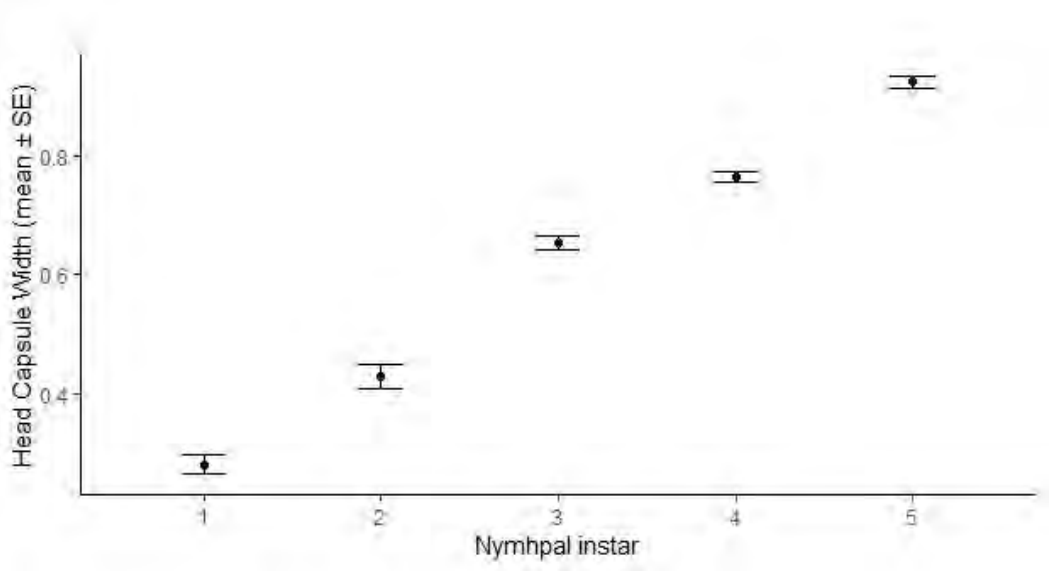

Figure 3.7: Mean of head capsule width (mm) of five nymphal instars of *Molopopterus* sp. conforming to Dyar's Law.

First instars of *Molopopterus* sp. were clear in colour in comparison to the other nymphal stages. The wing buds and abdominal bands were not yet visible at this stage. The second instars began to darken especially around the thoracic region with the bands becoming noticeable but still inconspicuous. Third instars had more evident dark abdominal bands and wing buds began to appear as tiny bulges on the edge of the thoracic collar. Fourth instars had tiny wing buds covering the first two abdominal segments and bore more resemblance to the adult. The fifth instars had more pronounced wing buds covering more than half of the abdomen. The hind legs were much larger in comparison to the fore and mid legs. The adult leafhoppers had well developed wings covering the abdominal region with the characteristic wing cell of *Molopopterus* sp. visible on the hind wing (Figure 3.8).

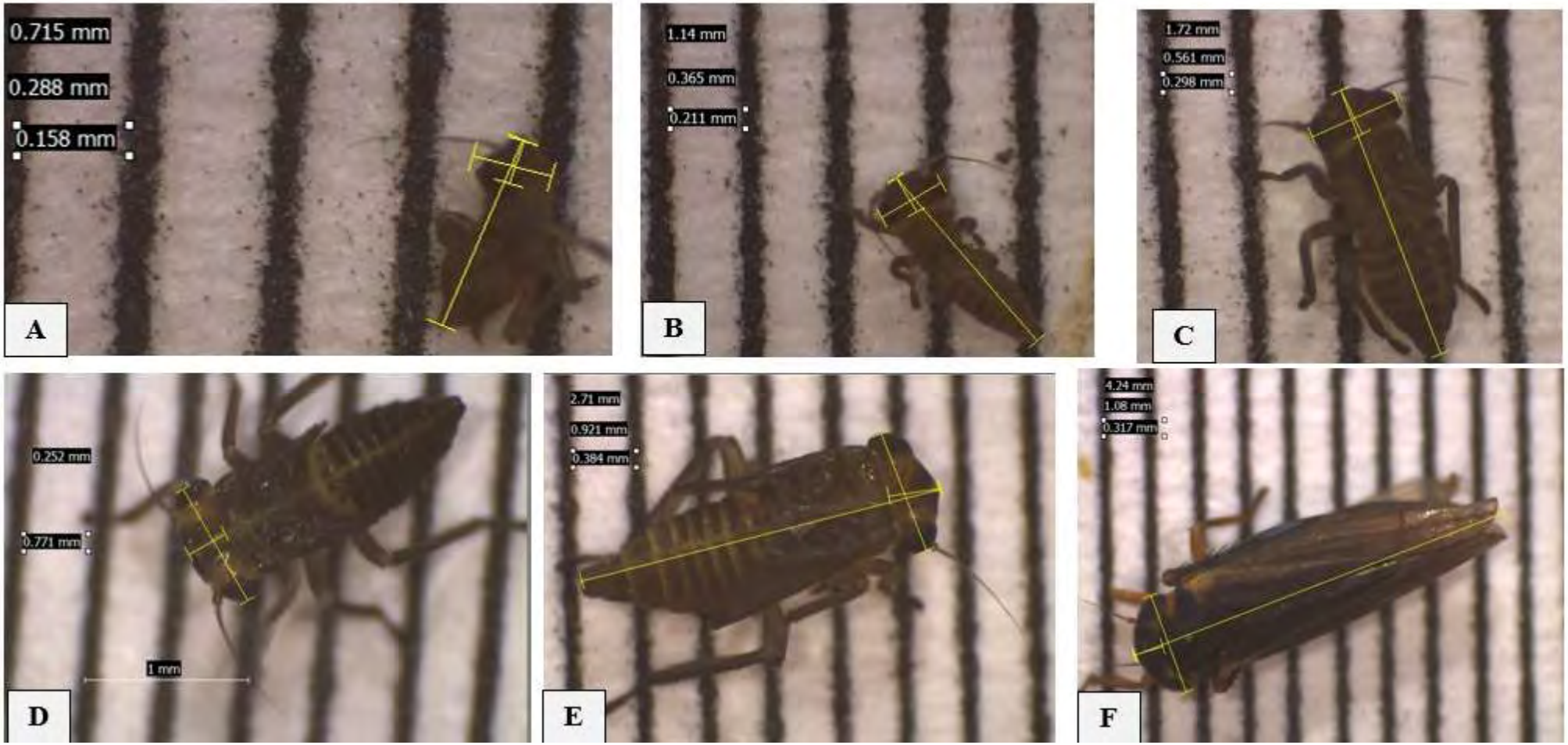

Figure 3.8: Life Stages of the leafhopper *Molopopterus* sp. with five nymphal stages from the first to the fifth instar (A – E) and the adult leafhopper (F). Measurements represent head capsule length, width and body length respectively (Photo credits T.Mushore).

3.3.2 Host preference test

There were significant differences in host preference on the different *Cyclopia* spp., to *Molopopterus* sp. feeding (P = 0.04456), at different time intervals (P = 0.00008) and on both species and time combined (P = 0.0027) (Table 3.3).

Table 3.3: Analysis of variance table of host preference of different Honeybush species to damage by *Molopopterus* sp..

Effect	DFn	DFd	F	*P* value
Species	2	8	26.449	0.04456
Time	6	24	33.160	0.00008
Species: Time	12	48	25.774	0.0027

A Tukey HSD test was used to make post-hoc comparisons between different species and different time intervals. The multi-comparisons test indicated that they were significant differences between different species at an average time at 95% confidence level (Table 3.4).

Table 3.4: Multi-comparisons table of different Honeybush species preference to *Molopopterus* sp.. The mean represent proportion of damaged leaves on the plants.

Species	Time	Mean	SE	Df	Lower CL	Upper CL
C. maculata	6.86	0.0326 **a**	0.02	99	0.0	0.0812
C. longifolia	6.86	0.161 **b**	0.02	99	0.1124	0.2095
C. subternata	6.86	0.4012 **c**	0.02	99	0.3527	0.4498

Means followed by different letters differ significantly at the 5% test level.

An interaction/time series plot showed that *C. longifolia* was the preferred species for the leafhopper with over 50% of the leaves showing hopper burn symptoms after six days and over 70% after 15 days. *Cyclopia maculata* was the least preferred species with 1% of the leaves showing feeding symptoms while *C. subternata* was the second most preferred with slightly over 20% of the leaves exhibiting damage (Figure 3.9).

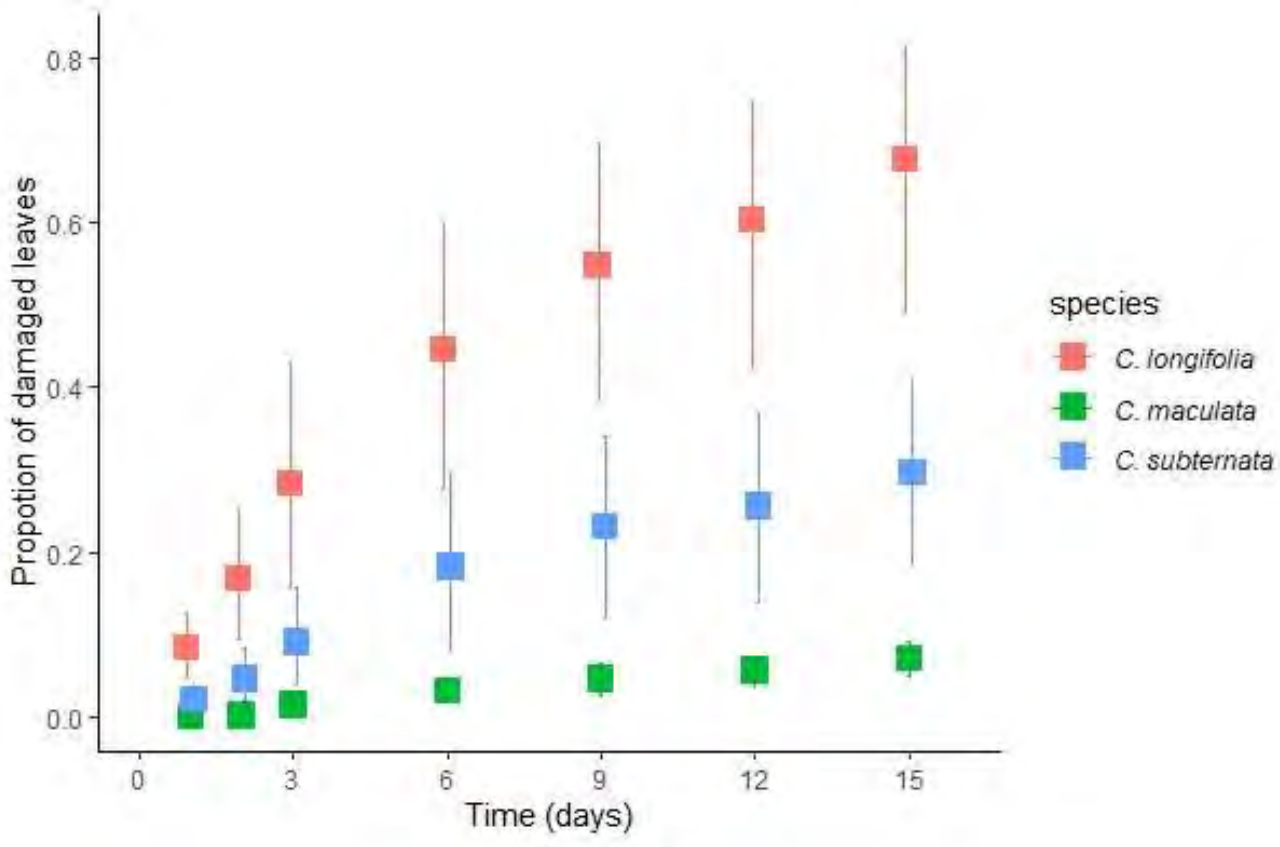

Figure 3.9: An interaction plot showing the proportion of feeding by *Molopopterus* sp. on different species of Honeybush at different time intervals.

3.3.3 Leafhopper density in Honeybush plantations

A field survey showed that *Molopopterus* sp. had a strong preference for *C. longifolia* and that the species can sustain high populations of these leafhoppers per plant. As the most preferred species to infestation among cultivated *Cyclopia* species, *C. longifolia* was the most preferred cultivated species to damage by *Molopopterus* sp. with a pest density of four leafhoppers per plant. *Cyclopia subternata* was the second most preferred species to infestation with pest density just above one, while *C. maculata* was the least preferred species to infestation. Multi-comparison tests with the Tukey HSD at 95% confidence level showed that leafhopper density on *C. longifolia* differed significantly from the other two cultivated species. They were no significant differences between *C. subternata* and *C. maculata* (Figure 3.10).

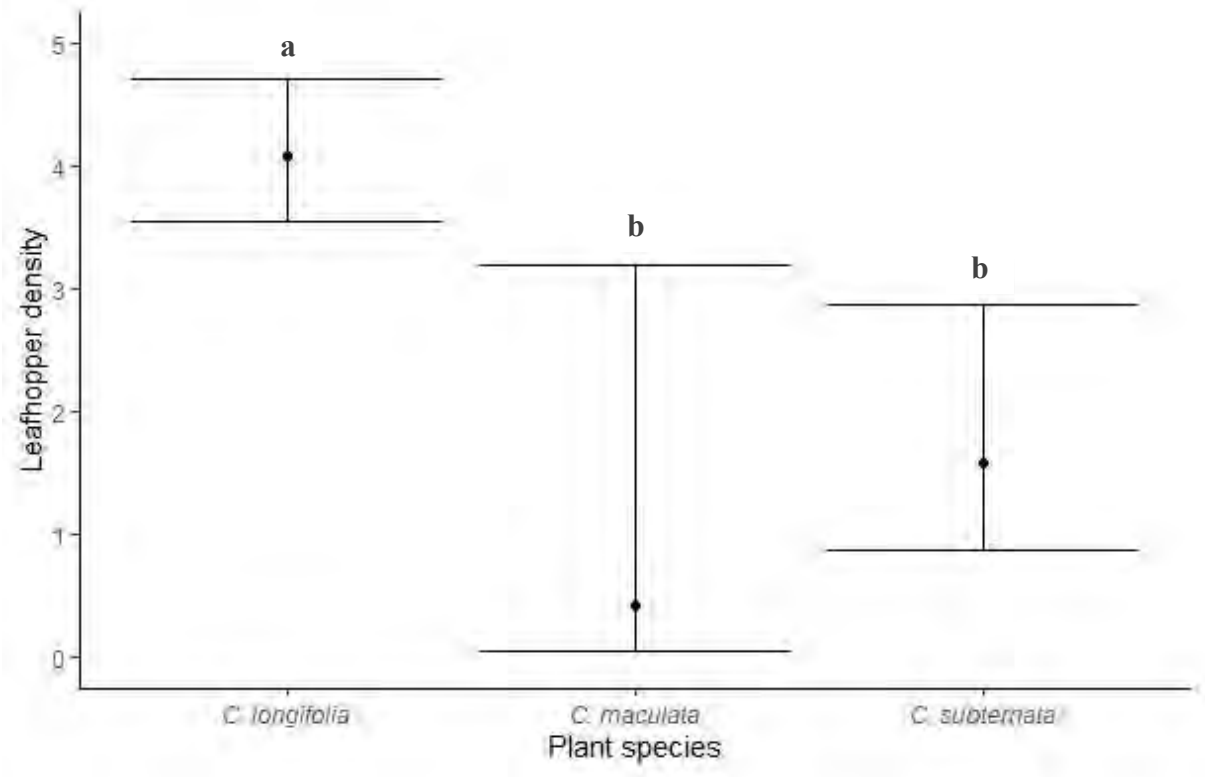

Figure 3.10: A Poisson GLM of leafhopper density (average number of leafhoppers per plant) of *Molopopterus* sp. on different cultivated *Cyclopia* spp.. Error bars represent a 95% confidence interval. Means with different letters differ significantly at the 5% test level.

A Poisson GLM showed that 57% of the sampled plants were infested with the leafhopper. Model predictions suggested Lodestone Farm to have the highest leafhopper density (8 leafhoppers/plant) followed by Kurland (4 leafhoppers/plant), Hidden Woods and Bitou Plantation (Figure 3.11). A Tukey HSD multi-comparison test at the 95% confidence level showed that Bitou plantation differed significantly from Hidden Woods and Kurland, while Lodestone differed significantly from the rest of the farms that had pest infestations.

The results show that Farm location is a significant predictor of *Molopopterus* sp. infestation.

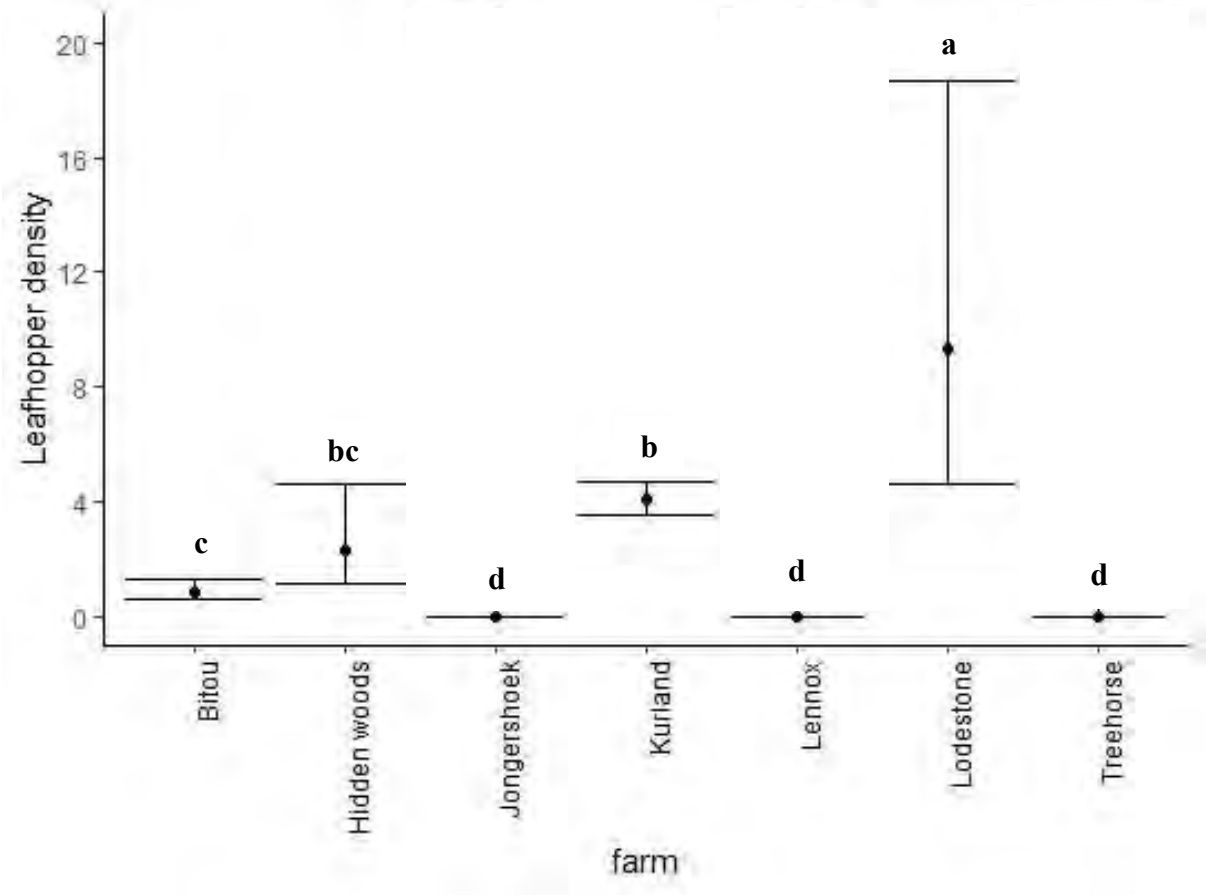

Figure 3.11: A Poisson GLM for average predicted *Molopopterus* sp. density on *Cyclopia* spp. across different farms. Error bars represent a 95% confidence interval. Means with different letters differ significantly at the 5% test level.

Harvested plants had high average *Molopopterus* sp. density of over four insects per plant in comparison to non-harvested plants which had average leafhopper density of one hopper per plant. The 95% confidence interval error bars illustrate that there are significant differences in pest density between harvested and non-harvested Honeybush stands (Figure 3.12). The results clearly show that harvesting Honeybush tea results in an increase in the number of pests per plant.

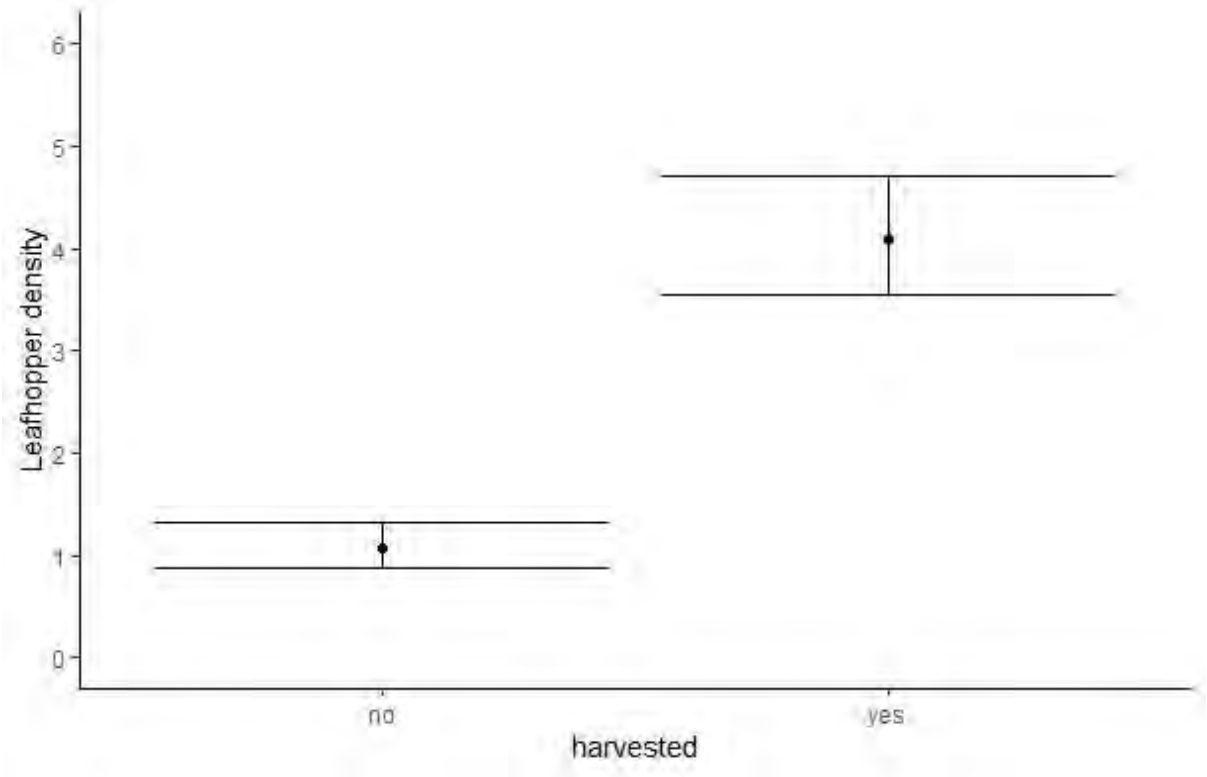

Figure 3.12: A Poisson GLM for average predicted *Molopopterus* sp. density on harvested and non-harvested *Cyclopia* spp. Error bars represent a 95% confidence interval.

The Poisson GLM also predicted an inverse relationship between age of the plant and average *Molopopterus* sp. density. An increase in plant age resulted in decrease in the average leafhopper density. Plants below 18 months had a higher average pest density of over two insects per plant. Leafhopper density on plants above 40 months (one) was significantly lower (Figure 3.13). This means that younger plants were more preferred, but as they grow older, they become less preferred. A narrow confidence interval across all age groups is an indication of how strong the model is in predicting pest density based on age.

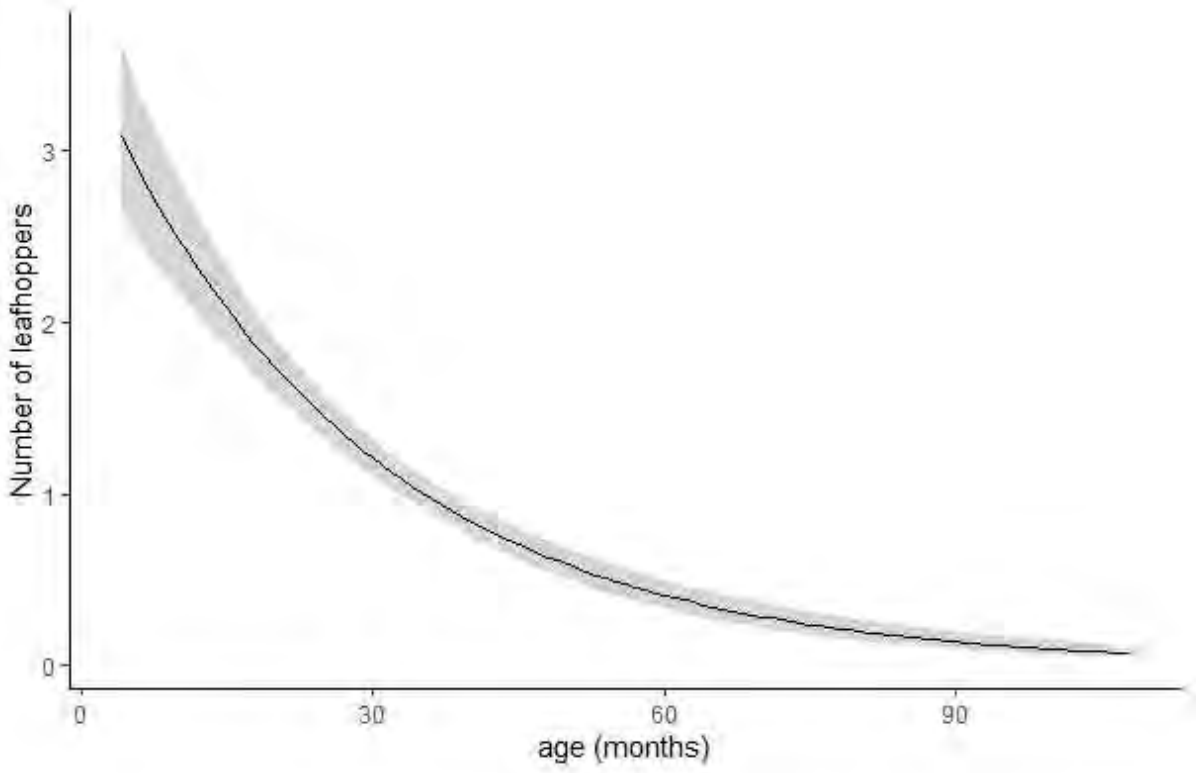

Figure 3.13: Poisson GLM of predicted *Molopopterus* sp. density influenced by age of the plants. The shaded region represents a 95% confidence interval.

3.4. Discussion

The study showed that the leafhopper *Molopopterus* sp. has an average incubation time of 20 days, development time from 1st instar to adult of 26 days and average generation time of 47 days. The study determined five nymphal instars of *Molopopterus* sp.. Incubation time of leafhoppers is known to be temperature dependant. The incubation period is less during the summer when temperatures are high (Jarrell et al., 2020). The study showed development time of 26 days. These findings were consistent to reports by Rakitov and Appel (2012), who reported that several leafhopper species have a development time of approximately 30 days and the results are in line with other studies on different species of leafhoppers (Jarrell et al., 2020; Zimmerman et al., 1996). The first to third instar nymphs were devoid of wings, a morphological feature common in leafhopper nymphs. According to Theron (1978), abdominal bands of *Molopopterus* sp., become more and more visible with each stage. This study showed that the nymphal instars gained colour with bands becoming more and more visible with each stage. However, the eggs could not be visualised using the lactoglycerol staining procedure.

Multi-choice experiments revealed variations in host preference to damage by the leafhopper over a period of 15 days. *Cyclopia longifolia* was identified to be the preferred species for feeding compared to the two other commonly cultivated species, *C. subternata* and *C. maculata*. The results were consistent with those obtained from the field survey, where high pest densities were associated with *C. longifolia*. Structural anatomy and morphology of a plant leaf have potential to impede insect feeding thereby influencing host choice of pests (Peeters, 2002). Murugesan and Kavitha (2010), explored host plant resistance in cotton accessions to the leafhopper *Amrasca devastans* Distant (Hemiptera: Cicadellidae), and discovered that some plant characteristics (height and trichome density) had an impact on leafhopper feeding and oviposition. Lit and Bernardo, (1990), also observed significant differences between susceptible and resistant eggplant varieties to the cotton leafhopper *Amarasca bigutula* Ishida (Hemiptera: Cicadellidae). They discovered a negative linear association between number of branches, leaf length and leaf trichomes on nymphal preferences. According to Awmack and Leather (2002), sap feeding insects respond to host species components of vegetation texture, which may help explain variations in preference of *Cyclopia* spp. to leafhopper feeding, as observed in this study. *Cyclopia maculata*

leaves have reduced surface area as their leaves are terete, with revolute leaf margins. Meanwhile, *C. longifolia* and *C. subternata* leaflets are wide obovate to oblanceolate (Schutte, 1995). The increased surface area of the two latter species is a probable incentive for *Molopopterus* sp. preference over the narrow leaflets of *C. maculata,* which is more likely to discourage the pest.

Leaf mechanics, as discussed above, play a significant role in herbivory (Read and Stokes, 2006), but cannot adequately explain why *Molopopterus* sp. prefers *C. longifolia* over *C. subternata* when they both have almost similar leaf morphology. According to Awmack and Leather (2002), host preference can be a result of the association between a particular plant species and concentration of allelochemicals which can either stimulate oviposition and feeding or daunt the survival of the insect on the host plant. Plants produce a diversity of volatile compounds which are perceived by organisms in their environment (Ahmed et al., 2019). Plant volatiles are made and altered by abiotic factors (e.g. harvesting) and biotic factors (e.g. feeding and oviposition). Plant volatile emissions differ between plant species and also within plant cultivars within species (McDaniel et al., 2016). Host preference in sap sucking insects is a product of the quality and quantity of volatile organic compounds (Vandermoten et al., 2012; Verheggen et al., 2008). Zhao et al. (2020), studied the defensive response of *C. sinensis* against tea green leafhopper, *Jacobiasca formosana* Paoli (Homoptera: Cicadellidae) attack through multi-omics studies. They discovered high concentrations of flavonoid compounds and glycosidically bound volatiles, but reduced levels of amino acids upon leaf herbivory. They also noted an increase in jasmonic acid and salicylic acid concentrations. Phytohormones are major plant defence responses with which different species respond to herbivory facilitating host selection in insects (Yang et al., 2019). *Cyclopia* spp. differ significantly in morphology, it is plausible that they secrete different concentrations of secondary plant metabolites which play a major role in host preference by *Molopopterus* sp..

According to Olson et al. (2009), plant volatiles and herbivore attraction are linked to Nitrogen content of the plant. Nitrogenous contents of the host plant play a significant role in performance and preferences of herbivore pests (Ahmed et al., 2019). Brodbeck et al. (1990), studied host preference determinants of the leafhopper *Hamalodisca coagulate* Germar (Homoptera: Cicadellida) and noted that concentrations of amino acids were greatest when leafhopper numbers were high. They also noted a positive correlation between the amino acid concentration and host

selection. Maseko and Dakora (2016) noted that older *C. subternata* plants are more capable of Nitrogen fixation than younger plants. Differences in Nitrogen fixation potential within species could also exist between species with *C. longifolia* having more amino acids due to the high Nitrogen fixation potential.

Plant nutrients act as attractants or repellents to herbivorous insects based on nutrient stability and their previous experiences (Saad et al., 2015; Wang et al., 2008). Application of Nitrogen fertilisers in rice paddy fields has been observed to increase populations of two planthopper species *Sogatella furcifera* Horváth and *Nilaparvata lugens* Stål (Hemiptera: Delphacidae) (Zhong-xian et al., 2007). Population dynamics of herbivorous insects are determined by abundance, distribution and host plant quality (a product of both nutrient quality and anti-herbivory defences) (Brodbeck et al., 2009). Preferences of *Molopopterus* sp. towards *C. longifolia* in multi-choice tests could be a result of induced selection. Induced selection of feeding hosts was demonstrated by Minkenberg and Fredrix (1989), using the herbivorous fly *Liriyomyza trifoli* Frost (Diptera Agromyzidae). The study established that insects exposed to high Nitrogen content preferred to feed and oviposit on high Nitrogen plants whereas those raised on low Nitrogen content showed no preference. Insects used in this study were collected from Kurland Farm which grows *C. longifolia*. Therefore, preference towards *C. longifolia* over other species could be a result of induced selection.

Due to the influence of biotic and abiotic factors that may explain *Molopopterus* sp. density trends in cultivated Honeybush, it is notoriously challenging to explain the effect of these four variables (age, species, harvesting and location) alone on the observed trends. Nutritional content (Nitrogen) and plant chemistry are potentially the most important factors that can offer plausible explanations for these. Nitrogen content is especially important for herbivorous insects since it is remarkably lower in plant tissue as compared to animal tissue (Ikonen, 2002). As such, pest density as modelled by the four fixed effects is largely influenced by host palatability and olfaction. However, there are other factors, such as leaf structure and management practices which also help explain these trends (Wang et al., 2008).

Age of the plants was shown to influence leafhopper density, which regressed with an increase in age. Adult feeding and oviposition on older plants is a common trend in phytophagous insects

(Blüthgen and Metzner, 2007). According to Shelton and Badness-Perez (2006), herbivore preferences are influenced by various factors including toughness, Nitrogen content, water, primary and secondary plant traits. There is a correlation between plant age and nutritional traits. Older plants often have low Nitrogen and water content and high fibre content in comparison to younger plants due to their high photosynthetic potential (Badenes-Perez et al., 2014). The difference in the nutritional composition between older plants and younger plants is most likely influencing feeding preferences of *Molopopterus* sp., which have shown feeding preference towards younger plants. The plant phenological age hypothesis which seeks to address feeding preferences of sap sucking insects predicts that xylem feeders perform better on younger plants as leaf Nitrogen content seems to universally decrease with an increase in leaf age (Ikonen, 2002; Van Dam et al., 1995). However, there are exceptions to this notion as some insects prefer to feed on older plants. The "reversed" differential feeding is common between generalist feeders and specialist feeders. Young leaves are protected from generalist feeders by plant toxins but are not a deterrent to specialist feeders as they have evolved to neutralise them. Meanwhile, generalist feeders lack specific adaptations to cope with plant toxins (Ikonen, 2002; Singh and Seetharama, 2002). The results of this study, though inconclusive are suggestive that *Molopopterus* sp. can detect Nitrogen content of leaves and this influences their host preference.

It is well established that there is a strong positive association between leaf toughness (morphology) and herbivory (Badenes-Perez et al., 2014; Read and Stokes, 2006; Shelton and Badness-Perez, 2006). As plants grow older, more lignin is deposited into plant tissues and they become "tough" for herbivores to feed on. Herbivores, therefore, tend to prefer younger plants to avoid mandibular wear which accrues from feeding on lignified plant tissues (Clissold et al., 2006; Price and Hunter, 2005). Bellota et al. (2013), also suggested that oviposition preferences in leafhoppers is influenced by leaf toughness since eggs are deposited in leaf tissues. Therefore, they tend to prefer younger plants over older plants. Furthermore, lignification is known to bind amino acids in the plant such that they are not readily available to herbivores. Honeybush is a perennial crop (McGregor, 2018) that does not shade its leaves and as a result, its plant tissues are lignified over time. Decrease in density of *Molopopterus* sp. with increase in age may be a result of insects avoiding older (tough) plants for younger and probably more succulent and nutritious plants. For

example, Hoffman and McEvoy, (1985) found that tough leaves can limit feeding of early instars of meadow spittlebugs.

Harvested plants had higher leafhopper densities in comparison to non-harvested plants. Given the assumptions of the plant stress hypothesis, reduced metabolism in stressed plants often leads to increased concentration of soluble Nitrogen in plant tissues coupled with decreased production of chemical defence compounds (Mattson and Haack, 1987; White, 1984; Wilson et al., 2015). Increase in soluble Nitrogen can be a result of nutrient transfer from uninjured parts of the plants being moved to injured parts of the plant. Honeybush is harvested by cutting the branches close to the ground leaving only a few branches per plant (McGregor, 2018). The harvesting process stresses the plants and brings about chemical responses one of which might be increased level of soluble Nitrogen leading to increased leafhopper density.

Furthermore, herbivorous insects rely on plant chemical cues to identify host plants (Silva et al., 2019). Olfactory cues are known to enhance attractiveness of host plants to leafhoppers (Borkakati et al., 2019). Todd et al. (1990), demonstrated olfactory attraction in leafhoppers using the maize leafhopper *Dalbulus maidis* Delong & Walcot (Homoptera: Cicadellidae) using a combination of plant volatiles. Against this backdrop, we hypothesise that, harvesting of Honeybush plants causes it to release plant volatiles (a phenomenon common in injured plants) which may act in attracting the leafhopper *Molopopterus* sp.. Alternatively, plants are known to emit both primary and secondary plant volatiles to discourage herbivores (Schoonhoven et al., 2005). Due to probable chemical imbalances in harvested plants, they may no longer be producing plant volatiles to deter insects causing the leafhopper to move from non-harvested stands into harvested stands resulting in high leafhopper densities.

The density of *Molopopterus* sp. differed significantly between farms with Kurland and Lodestone having the highest leafhopper density. Differences in density could be attributed to different management strategies employed on different farms, however, both these farms are within proximity to each other which suggests a possible geographic influence on leafhopper density. According to Tscharntke et al. (2005), arthropod assemblages within an agricultural field are a product of the regional species pool surrounding the field. This is because adjacent landscapes

provide habitat features for insects that move between cultivated and non-cultivated areas (Vaidya et al., 2017). Furthermore, seminatural habitats, such as field margins are known to harbour larger number of arthropods, housing more detrimental insects than beneficial ones (Rusch et al., 2010). They maintain populations of alternative hosts of crop pests acting as overwintering sites and a source of pest outbreaks (Saeed et al., 2015). The observed differences could be attributed to a variety of factors ranging from Nitrogenous content of the soil, nature of field margins habitats, and management practices which impact field population dynamics of *Molopopterus* sp.

Successful implementation of management strategies of the leafhopper in cultivated Honeybush relies on knowledge of its bionomics, host preferences and correlations between the pest and plant characteristics as well as production practices. *Molopopterus* sp. has a short generation time; hence it can have several generations in a year and thus has potential to cause significant damage. Findings of this study have implications on formulation of management practices. Host preference towards *C. longifolia* means that farmers need mitigation/control especially for this species. Due to higher pest densities in harvested stands than in non-harvested and high densities in younger plants over older plants, control strategies can be intensified at certain stages or periods. North et al. (2017), investigated the effect of harvesting date on growth, production and quality of Honeybush. This study showed differential densities in harvested and non-harvested stands, as such the knowledge can be used in determining an optimal harvesting date. For optimal harvesting dates, there is need for seasonal occurrence studies of *Molopopterus* sp. in Honeybush fields so that plants can be harvested when pest densities are low. Furthermore, there is need for further studies in the seasonal occurrences of *Molopopterus* sp. in cultivated Honeybush to determine economic injury levels and economic threshold. There is also need for no choice test studies to explore host susceptibility of cultivated Honeybush species and to determine their tolerance to infestation.

CHAPTER 4

Entomopathogenic fungi associated with cultivated Honeybush fields and their pathogenicity towards *Molopopterus* sp.

4.1 Introduction

Entomopathogenic fungi are facultative obligate pathogens found in association with moribund and dead insects in nature (Mora et al., 2018). They have been well studied for use as microbial control agents for soil-dwelling insects. The history of their use in biological control dates back to the 18th century when they were examined by Metchnikoff and later Krassilstschik who studied their efficacy against various pest insects in the former USSR (Zimmermann, 2007). Since then, fungal pathogens have been tested and used with great success against several insect pests (Mora et al., 2018). Currently, entomopathogenic fungi are being used to control a variety of pests including soil-dwelling insects, such as termites, field insects such as lepidopterans, hemipterans and coleopterans, greenhouse pests such as whiteflies as well as medical and veterinary pests; mosquitoes and ticks (Jaronski et al., 2005; Mochi et al., 2005; Ownley et al., 2010). There are over 750 described species of fungi that can cause infections in insects (Meyling and Eilenberg, 2018). Entomopathogenic fungi do not form a monophyletic group, however, most of them are from two fungal divisions, Division Zygomycota, Order Entomophthorales and Division Asycomycota, Order Hypocreales (Khan et al., 2012). The Order Hypocreales contains two of the most common species of insect fungal pathogens *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Mora et al., 2018).

The entomopathogenic fungi have a contact based mode of action (Maina et al., 2018). For successful invasion of insect host, fungal pathogens must overcome a series of sequential challenges which require specific structural and physiological mechanisms. Although not emphasised, the infection process differs slightly between fungal lineages (Bischoff et al., 2006; Hajek and St Leger, 1994). The infection pathway of Hypocreales involves, the attachment of the spores to the cuticle, germination and formation of appressoria, penetration of the cuticle,

overcoming the host response and immune defence, spreading within the host as blastopores and, lastly, outgrowing the dead host and production of new conidia (Boomsma et al., 2014; Shah and Pell, 2003) (Figure 4.1). The fungus penetrates the host through the outer integument specifically via intersegmental folds, such as jointed segments and around mouthparts (Hussain et al., 2014). When the invading fungal spores contact the insect, a series of defence mechanisms are triggered. Triggering of host defence mechanisms occurs just after the host cuticle has been compromised by fungal spores (Shah and Pell, 2003).

Spore adhesion to the insect cuticle is mediated by specific proteins and appressorial structures. Attachment to the insect cuticle is achieved by the hydrophobicity of the conidia and the cuticular surface. When these two are compatible, a series of hydrophobic interactions enables attachment of fungal spores to host cuticle. Subsequently, it can also be aided by interacting with the mucilaginous coat present on the host cuticle (Maina et al., 2018; Vega et al., 2012). The penetration process is mechanically assisted by the production of several cuticle degrading enzymes which include proteases, lipases and chitinases. The appressorium, a thin cellular structure in the form of a peg, is formed and is responsible for breaching host cuticle via mechanical and enzymatic processes (Butt and Goettel, 2009).

Following successful penetration and once invasion has reached the insect haemoceol, the fungus proliferates as blastopores or hyphal bodies which are then distributed throughout the insect body passively by the haemolymph. This allows the fungus to invade and infect other tissues of the insect body (Bischoff et al., 2006; Boomsma et al., 2014). Enough penetrating spores acting quasi-simultaneously must be present to evade the host immune system. Proliferating fungal protoplasts produce secondary insecticidal properties which help to evade the host immune system and ultimately cause host death. For example, destruxins produced by *Metarhizium* spp., achieve host death through synthesis of cyclic depsipeptides which cause host paralysis and other toxins which affect excretion and hinder the insect's ability to move (Boomsma et al., 2014). The emergence of the invading fungus through the host cuticle marks the end of the fungal life cycle (Inglis et al., 2012).

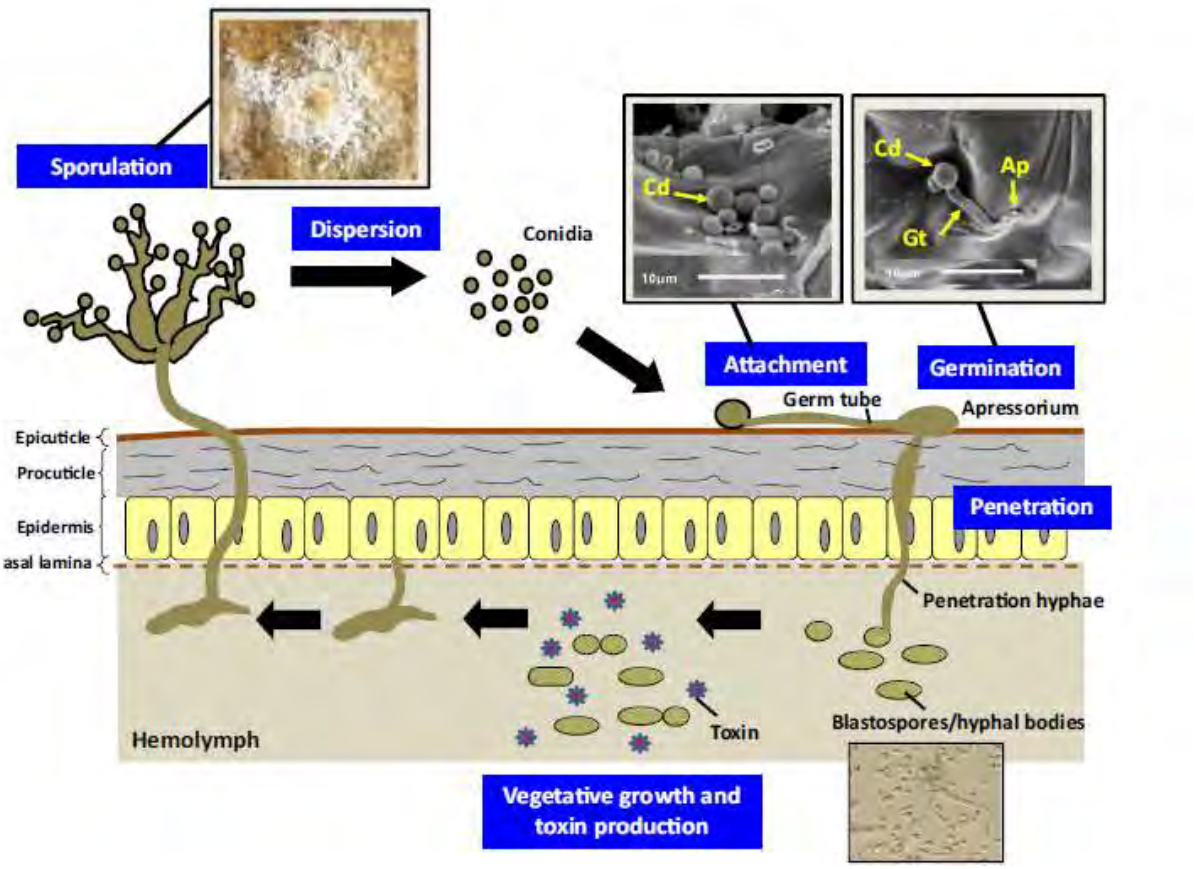

Figure 4.1: Mode of action of entomopathogenic fungi in an invertebrate host (adapted from Mascarin and Jaronski (2016)).

The use of entomopathogenic fungi as biocontrol agents to reduce pest density plays a key role in sustainable pest management programmes as almost all insects are vulnerable to fungal disease (Mochi et al., 2005). These fungi possess facultative abilities which make them ideal candidates for biological control as they can persist in soil until the host becomes available (Bidochka et al., 1998). They are especially ideal in that they are eco-friendly, have a wide host range and they pose little threat to humans with minimal residues (Barra et al., 2012; Bidochka et al., 2001). Entomopathogenic fungi are an important biological control alternative as they can also be used against sap sucking pests since their pathogenesis process does not require ingestion (Khan et al., 2012; Thomas and Read, 2007).

Complex multitrophic interactions exist both in agriculture and natural ecosystems involving predators, herbivores, parasitoids and pathogens all of which regulate arthropod community (Chen et al., 2004; Chen and Chen, 1989; Du et al., 2003). The resulting complex relationships among arthropods and their parasitoids, predators and pathogens represent opportunities for the control of many pests. A thorough understanding of arthropod interactions especially within agro-ecosystems, is important to develop effective biological control strategies and prevent disruption of existing beneficial interactions through the introduction of another natural enemy (Meyling and Eilenberg, 2006). In order to understand insect pathogen diversity in the soil, there is need to study the natural occurrence, ecology and distribution in different ecosystems and different soil types. Numerous studies have thus been conducted on the development of entomopathogenic fungi as inundative bio-control agents in agro-ecosystems (Ahmed and Holmstrom, 2014; Hussain et al., 2014; Meyling, 2007).

Sampling of host individuals for entomopathogenic fungi can also reveal information regarding prevalence and host range of fungal species in natural populations. However, the shortfall in this approach is that several fungal populations only occur as infections in living hosts for a limited amount of time (Meyling, 2007; Shahid et al., 2012). Entomopathogenic fungi are omnipresent in the soil as part of a complex ecosystem characterised by a variety of other microorganisms of agricultural importance (Bruck et al., 2005). Local indigenous species of entomopathogenic fungi are usually adapted to some environmental condition of the agro-ecosystem from which they are isolated (Quesada-Moraga et al., 2007). For example, Goble et al. (2010) isolated and characterised entomopathogenic fungi from citrus orchards for use against citrus pests as an alternative and attractive method of pest control.

Bioassays are tools used for identifying virulence, host range conditions enhancing epizootics and barriers to infection. They can be used to determine and quantify host pathogen associations and the effect of biotic and abiotic parameters (Shah and Pell, 2003; Shahid et al., 2012). Pathogenicity is a qualitative measure of the ability of a pathogen to cause disease to a host (Boomsma et al., 2014). Virulence is the most important indicator of pathogenicity of fungi against insect hosts (Butt and Goettel, 2009). Bioassays are employed to select isolates for field trials and eventually mass production. However, not all selected isolates make it past field trials due to environmental

differences between field conditions and the laboratory in which they were developed (Butt and Goettel, 2009; Sharma et al., 2018).

The cultivation of Honeybush, has resulted in the outbreak of the leafhopper *Molopopterus* sp. as a major pest (Metcalf et al., 2018) (Chapter 3). Currently, there is no recommended pest management strategy for this pest, as farmers rely on natural enemies. Rooibos tea farmers rely on inorganic pesticides for the control of *M. theae*, an option unavailable to Honeybush farmers (Hatting, 2017). Surveys for, and isolation and characterisation of, entomopathogenic fungi associated with tea has received considerable attention with over 40 species of pathogenic fungi being documented (Meyling and Eilenberg, 2018; Wang et al., 2010; Zhang and Tan, 2004). It is against this background that the objectives of this chapter are to: (i) recover, identify and compare indigenous isolates of entomopathogenic fungi from soil samples obtained from Honeybush agro-ecosystems (cultivated and natural refugia) and; (ii) investigate the pathogenicity of the isolated entomopathogenic fungi against the adults and nymphs of *Molopopterus* sp..

4.2 Materials and Methods

4.2.1 Soil sampling

Soil samples were collected from three Honeybush farms in the Western Cape; Kurland in the Crags, Lennox Farm near Sedgefield and Jongershoek Farm near George. Soil samples were collected from Honeybush fields and surrounding refugia. A total of 98 rhizospheric soil samples were collected using the sampling procedure described by Goble et al. (2010). Twelve soil samples, roughly 12 m apart, were collected per field, along two intersecting transects. Twelve samples were also collected on each Farm from the natural refugia within a 2 km radius of the field with each sampling point 18 m apart (Figure 4.2). A cylindrical soil auger (7 cm × 14 cm) with 538 cm^3 volume was used to collect soil to a depth of 15 cm after surface litter was removed. The soil auger was wiped with 70% ethanol between fields to prevent contamination. Samples were collected at the beginning of April 2019. The soil samples were placed in individually labelled plastic ziplock bags, which were stored at 4°C and baited within 2 months of the sampling period.

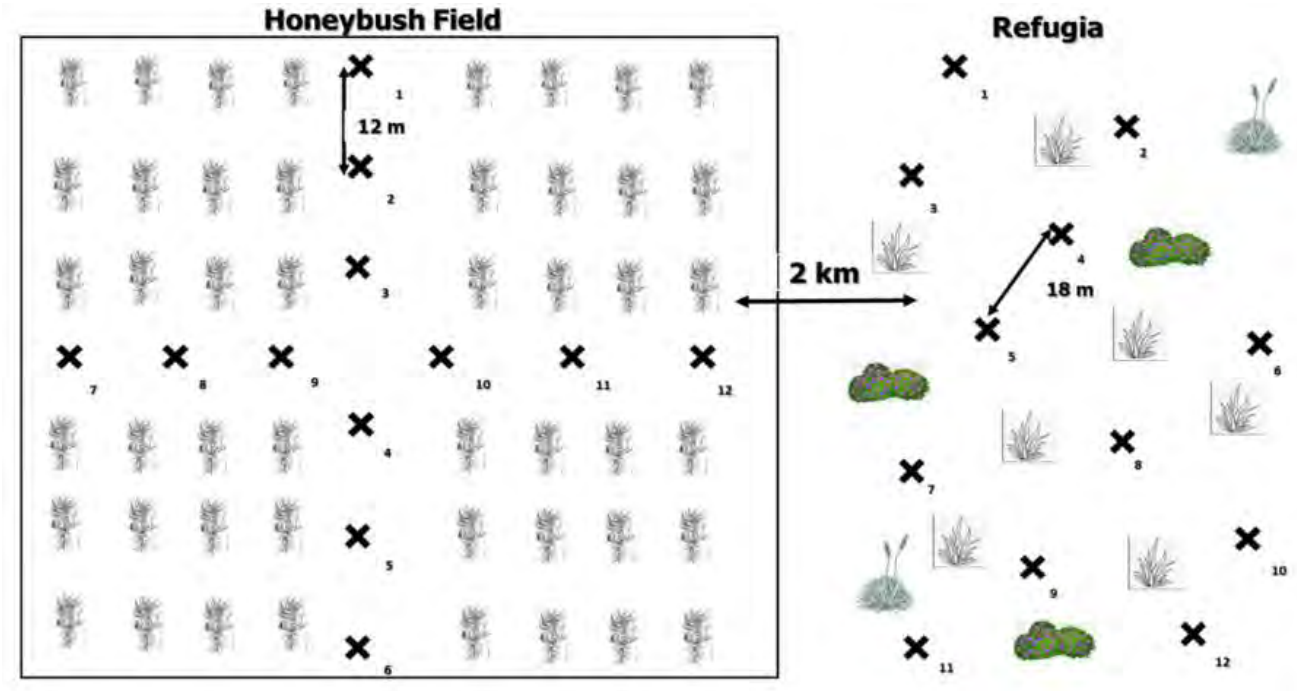

Figure 4.2: Soil sampling procedure employed in Honeybush plantations and in the refugia.

4.2.2 Baiting Procedure

The insects used for baiting were late instar larvae of *Tenebrio molitor* which were obtained from cultures kept at Rhodes University reared in plastic containers on an artificial diet which comprised of a mixture of oats and wheat bran as protein sources. Potatoes were supplied as needed as a carbohydrate source and to provide moisture. Entomopathogenic fungi were isolated using the '*Galleria* bait method' as described by Meyling (2007) and Meyling and Eilenberg (2006), with slight modifications. Soil samples were sieved through a sterile metal sieve with mesh size of 4 mm after which they were thoroughly mixed. If the soil was dry, the samples were moistened with sterile distilled water to ensure enough moisture was present to promote fungal infection during baiting. Two 200 ml portions were measured and transferred to 400 ml transparent plastic containers, previously sterilised with 70% ethanol. The containers were sealed and 10 late instar larvae of *T. molitor* per soil sample were added. No heat treatment was applied to the insects as there was no excessive webbing. Soil samples were then incubated in complete darkness at room temperature.

Containers were inverted daily for the first week to encourage larval movement throughout the soil profile. The soil samples were moistened with distilled water if they became too dry and were checked for dead insects every three days beginning one week after baiting was initiated for a period of 21 days. All dead larvae were surface sterilised by first washing them in in to 1% of sodium hypochlorite and then 70% alcohol for 30 seconds, before being quickly rinsed three times with sterilised distilled water to prevent growth of opportunistic external saprophytic fungi (Oliver et al., 2011). After surface sterilisation, they were placed in an incubation chamber (sterile 90 mm petri dish lined with moist filter paper). Sporulating larvae and/or pupae were placed on Sabouraud Dextrose Agar (SDA) media for fungal isolation (Figure 4.3). Selective isolation growth media adapted from Meyling (2007), SDA supplemented with 50 mg/L Chloramphenicol, was used to isolate fungi. Conidia were scrapped from insect cadavers and diluted in Tween 20 (1: 100) before being spread on prepared media where they were incubated at room temperature. Individual colonies were then cut and transferred to new plates.

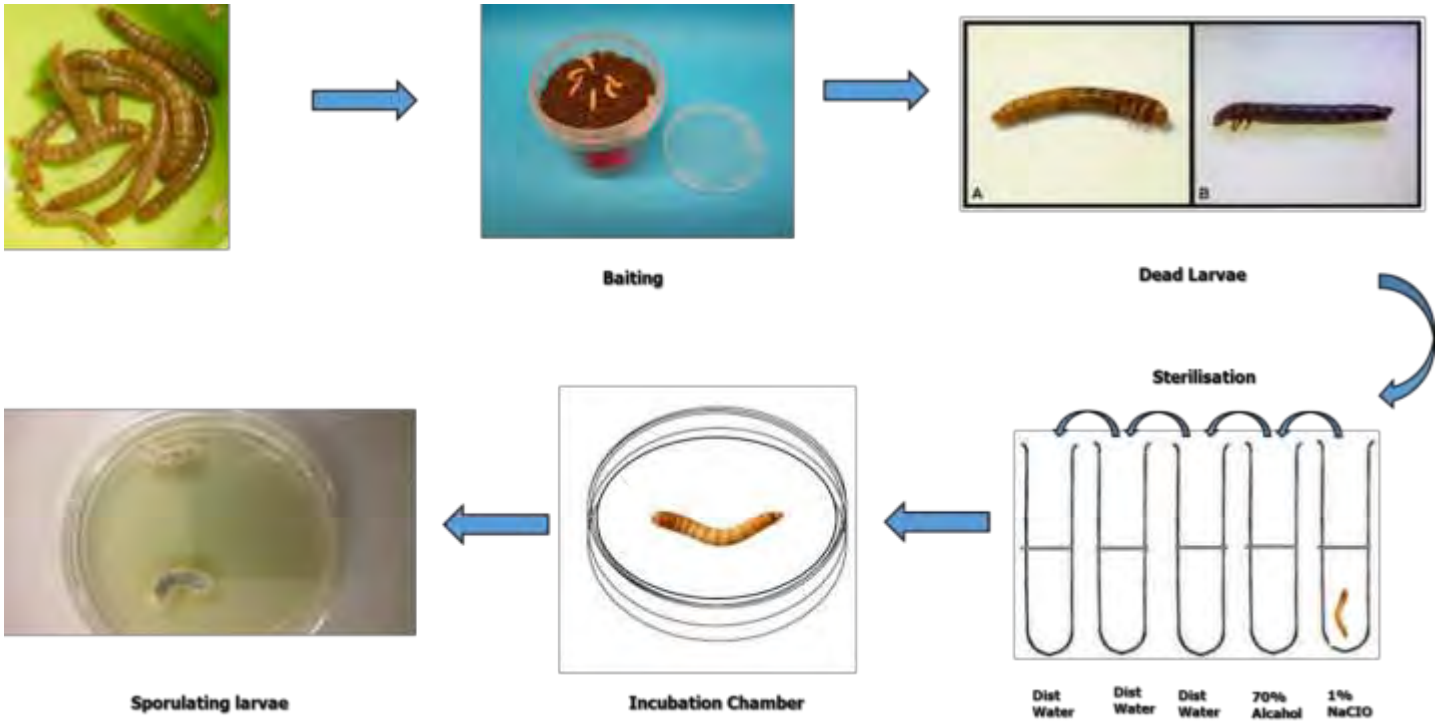

Figure 4.3: Schematic diagram depicting the baiting and isolation procedure.

4.2.3 Molecular characterisation for identification of fungal isolates

The fungal taxa often comprise of cryptic species making biodiversity assessment of entomopathogenic fungi communities challenging. Cryptic species occur for *Metarhizium* spp. and *Beauveria* spp., hence, evaluation of biodiversity of these species must be based on genetic identification (Inglis et al., 2012). Molecular characterisation was done for all the isolates. DNA was isolated following a modified protocol by Marzachi et al. (1998). The fungal samples were extracted from the agar plates using a 1000 µl sterile pipette tip, which was melted with a lighter creating a ball which was then used to macerate the fungi. The samples were macerated in 200 µl sterile dH_2O in a 2 ml microcentrifuge tube after which 180 µl of ATL buffer (Qiagen) and 15 µl of proteinase K were added. The samples were then vortexed for 30 seconds prior to being placed in a heat block overnight at 56°C. The samples were then centrifuged at 13 000 rpm for 5 minutes after which the supernatant was removed and transferred to a new Eppendorf where 65 µl of 5M Sodium chloride was added to the supernatant. The samples were then vortexed for 30 seconds

and again centrifuged for 5 minutes at 13 000 rpm after which the supernatant was removed and transferred to a new Eppendorf, where 150 µl of cooled isopropanol was added. The samples were then placed in the freezer overnight and then centrifuged again at 13 000 rpm for 5 minutes. The supernatant was removed, and the DNA pellet was kept. 250 µl of cooled 70% ethanol was added to the pellet and centrifuged at 13 000 rpm for 5 minutes after which the ethanol was removed. The Eppendorf was placed overnight on a heat block at 50°C to allow any excess ethanol to evaporate. The DNA pellet was then dissolved in 20 – 40 µl of AE buffer (Qiagen).

A nanodrop spectrophotometer (Thermo Scientific®) was used to determine DNA concentration. Universal fungal primers were used to amplify the internal transcribed spacer region. Agarose Gel Electrophoresis (AGE) was used to analyse PCR products to confirm whether amplification of the ITS region was successful (Table 4.1; Table 4.2). The following PCR cycling parameters were used: initial denaturation step of 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds, 72°C for 1 minute and finalised by one cycle at 72 °C for 5 minutes. The samples were run on 1% AGE. The samples were then sent to Inqaba Biotechnical Industries Pty Ltd for sequencing. Chromatograms were analysed in Finch TV v1.4.0 (Geospiza, Inc.; Seattle, WA) and samples were identified using the BOLD and GenBank database. Sequences were aligned using Chromas (v2.6.6) and analysed using the maximum likelihood method) in MEGA-X (v10.2.2). 1000 bootstraps were used to support the topology of the tree using the General Time Reversible model (GTR) and Gamma Distributed with Invariant sites.

Table 4.1: Universal oligonucleotide primers used for the amplification of the internal transcribed spacer region (White et al., 1990).

Oligonucleotide	Sequence (5' to 3')
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

Table 4.2: Reagents used for the amplification of the ITS region of fungal isolates

Reagent	Quantity (µl)	
	Negative control	Samples
TopTaq	12.5	12.5
ITS1(10 µm)	2	2
ITS4(10 µm)	2	2
Template DNA (50 ng/ul)	0	2
$MgCl_2$	1	1
ddH_2O	8.5	6.5
Total	25	25

4.2.4 Fungal culture and preparation for pathogenicity tests

Fungal isolates were cultured for two weeks on SDA before conidia suspensions were prepared. The plates were then flooded with 20 ml of sterile 0.05% Tween 20 solution. A sterile 1000 µl pipette tip was used to gently scrape the surface of the media to dislodge the conidia before transferral into a sterile glass bottle. The suspension was then vortexed for five minutes to ensure homogenisation before determining the concentration of the spore suspension. The concentration of the spore suspension was determined by preparing 1:100 dilutions using 0.05% Tween 20 solution. A Neubauer haemocytometer (Marienfeld, Germany) was used to determine the stock concentration. Prior to use, both the chamber and cover slips were washed with 70% ethanol and allowed to dry. Counts were made for each replicate and the average thereof used to determine the concentration of the stock suspension using the formula;

$Conidia/ml^{-1} = df \times d \times c.$

Where, df = dilution factor; d = dilution; c = average number of conidia counted.

The desired concentration for the bioassay (1×10^7 $Conidia/ml^{-1}$) was calculated using the formula; $C_1V_1 = C_2V_2$,

Where, C_1 is the stock suspension concentration, V_1 is the volume of the stock suspension needed to make the desired concentration, C_2 is the desired concentration and V_2 is the final volume of the desired concentration.

A viability test was conducted on the fungal isolates using the method described by Lacey, (1997). Conidia viability was determined by aseptically spreading 100 µl of 1×10^5 and 1×10^6 conidia/ml^{-1} fungal suspension onto freshly prepared SDA plates. Three replicates were made for each fungal isolate and for each concentration. The plates were wrapped with foil paper and incubated for 24 hours at 26°C. A total number of 100 conidia were counted under a light microscope (OLYMPUS CX2LED) at low magnification. Germinating conidia were identified from non-germinating conidia by the presence of a visible germ tube (Lacey, 1997). The average germination percentage was determined by dividing the total number of germinating conidia by 300 and multiplying by 100.

4.2.5 Fungal bioassay against adult *Molopopterus* sp.

A completely randomised design with 21 fungal treatments, including the control, replicated five times was used for the bioassay. The bioassay test was repeated twice on different dates with a fresh batch of fungi. The experimental setup comprised a modified assay chamber, a sterilised 2 L soft drink transparent plastic container, which was used to cover the treated seedlings to prevent the insects from escaping and an inverted transparent plastic container to help seal the bottom of the assay chamber (Figure 4.4). Seedlings of *C. longifolia* containing 20 – 40 leaves were used for the bioassay. The seedlings were transplanted from trays into 250 ml disposable foam cups. The seedlings were first washed with distilled water and allowed to dry and then sprayed with 0.4 ml fungal suspension using a spray bottle and left to dry at room temperature.

Adult *Molopopterus* sp. were collected from *C. longifolia* plants at Kurland using an active beating method described in Chapter 3. Infested plants were actively beaten by hand to dislodge the insects while a sweep net (diameter 32 cm × 55 cm length) was positioned below to catch the insects. An electric pooter was used to collect the insects from the net before being transferred into pill vials with perforated lids. The vials were placed on ice to lower the temperature during transport. Ten

adult leafhoppers were introduced from the top and closed afterwards to prevent the insects from escaping. The lid of each chamber was opened briefly daily to prevent moisture from building up inside the chamber as this promotes the growth of saprophytic and opportunistic fungi (Oliver et al., 2011). Mortality was checked after seven days by counting the number of dead insects in each treatment.

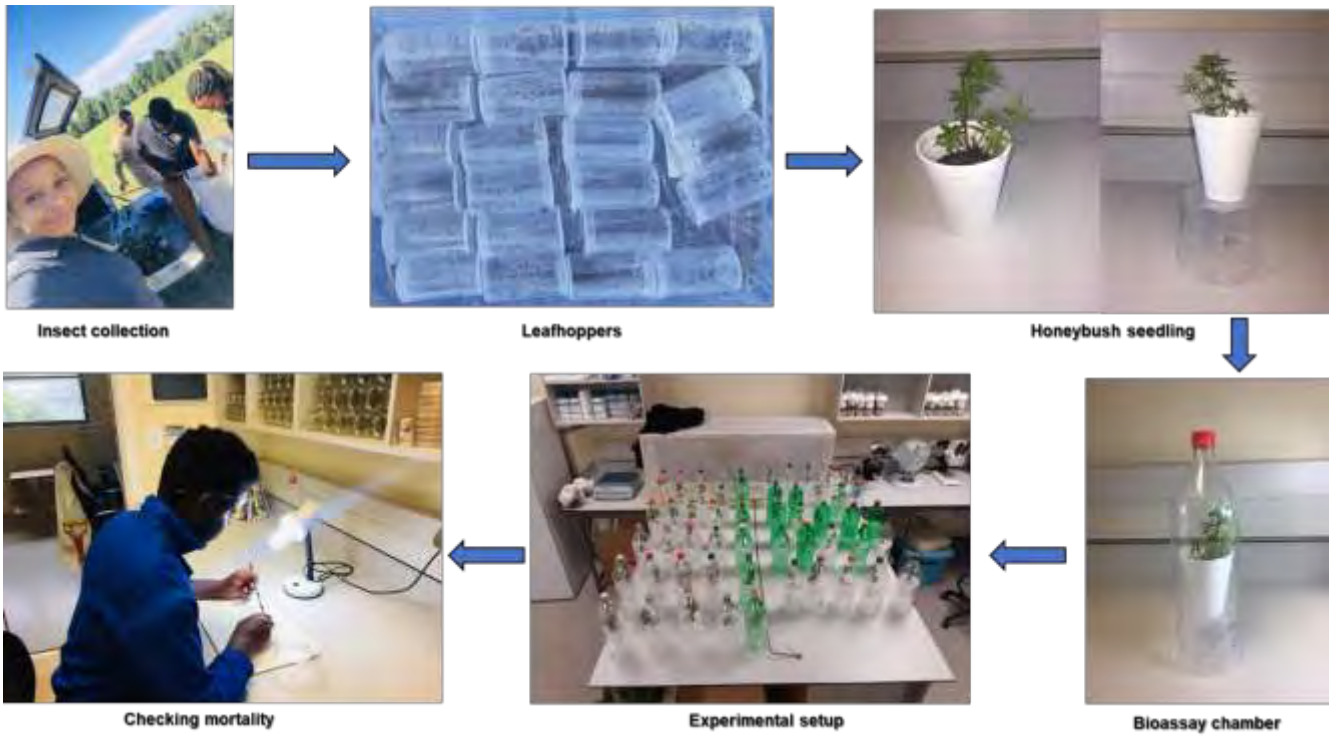

Figure 4.4: Experimental setup of the bioassay of the adult *Molopopterus* sp. from insect collection in the field to the experimental setup in the laboratory.

4.2.6 Fungal bioassay against nymphal stages of *Molopopterus* sp.

The bioassay was also conducted on the immature stages of the *Molopopterus* sp. using the same method as the one previously described but with slight modifications. Late instar nymphs (3^{rd} – 5^{th} instars) were collected from Kurland using the active beating method. The nets were emptied into a tray (40 × 50 cm) and the contents were evenly spread out before being turned up-side-down. The nymphs remained attached to the tray and were then transferred to transparent tubs with perforated lids for transport. Transparent tubs (11 cm diameter × 8 cm height) with perforated lids

were used as bioassay chambers. Branches of *C. longifolia* were inserted into a wet floral foam in a Petri dish, then placed at the bottom of the tub. Ten Crawling nymphs were then transferred to the bioassay chamber using a fine brush (Figure 4.5).

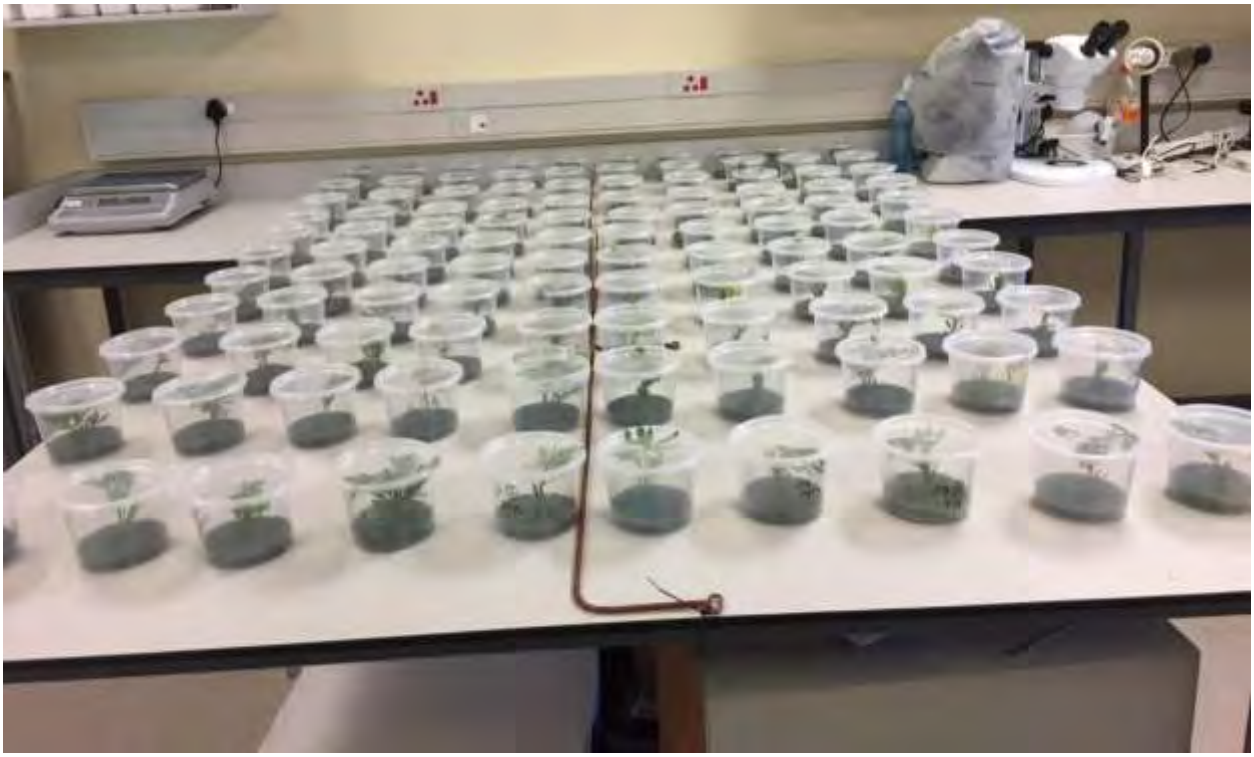

Figure 4.5: The bioassay set up of 20 fungal isolates with control against late nymphal instars of *Molopopterus* sp..

4.2.6 Data Analysis

Abbott's (1925) formula was used to adjust for control mortality. A one-way Analysis of Variance (ANOVA) was used to compare the differences in the mean mortality of *Molopopterus* sp. between 20 treatments of fungal isolates. A Bonferroni Least Significant Differences (LSD) test was used to separate the means. Data were analysed using R software version 4.0.0 (R Core Team, 2020). One-way ANOVA was specified using the "*stats*" package (R Core Team, 2020). The Shapiro-Wilk and the Bartlett test were used to check for normality and homogeneity of variances respectively. LSD tests were specified using the "*agricolae*" package (Mendiburu and Muhammad, 2020)

4.3 Results

4.3.1 Occurrence and identification of entomopathogenic fungi

20 fungal isolates were recovered. Most of the fungal isolates were isolated from Kurland (17) while a further two isolates came from Lennox Farm with the last one originating from Jongershoek Farm. The fungal isolates belonged to only two species *M. anisopliae* and *Fusarium oxysporum* (Table 4.3).

Table 4.3: Isolated fungal isolates according to source and species identification.

Sample	Farm	Source	Species ID
J S1	Jongershoek	Field	*Metarhizium anisopliae*
KF1 S1	Kurland	Field	*Metarhizium anisopliae*
KF1 S3	Kurland	Field	*Metarhizium anisopliae*
KF1 S6	Kurland	Field	*Metarhizium anisopliae*
KF1 S8	Kurland	Field	*Metarhizium anisopliae*
KF1 S9	Kurland	Field	*Metarhizium anisopliae*
KF1 S11	Kurland	Field	*Metarhizium anisopliae*
KF1 S12	Kurland	Field	*Metarhizium anisopliae*
KF1 S13	Kurland	Field	*Metarhizium anisopliae*
KF2 S2	Kurland	Field	*Fusarium oxysporum*
KF2 S3	Kurland	Field	*Fusarium oxysporum*
KF2 S5	Kurland	Field	*Fusarium oxysporum*
KR S2	Kurland	Refugia	*Metarhizium anisopliae*
KR S4	Kurland	Refugia	*Fusarium oxysporum*
KR S5	Kurland	Refugia	*Metarhizium anisopliae*
KR S6	Kurland	Refugia	*Metarhizium anisopliae*
KR S7	Kurland	Refugia	*Metarhizium anisopliae*
KR S8	Kurland	Refugia	*Metarhizium anisopliae*
L S1	Lennox	Field	*Metarhizium anisopliae*
L S2	Lennox	Field	*Metarhizium anisopliae*

Fungal isolates had two distinct clades which were supported by the tree with each of the species having its own clade. Bootstrapping showed that *F. oxysprum* isolates differed (100%) from *M. anisopliae* isolates. Within *M. anisopliae* isolates, isolate J S1 differed (87%) from the rest of the isolates Figure (4.6).

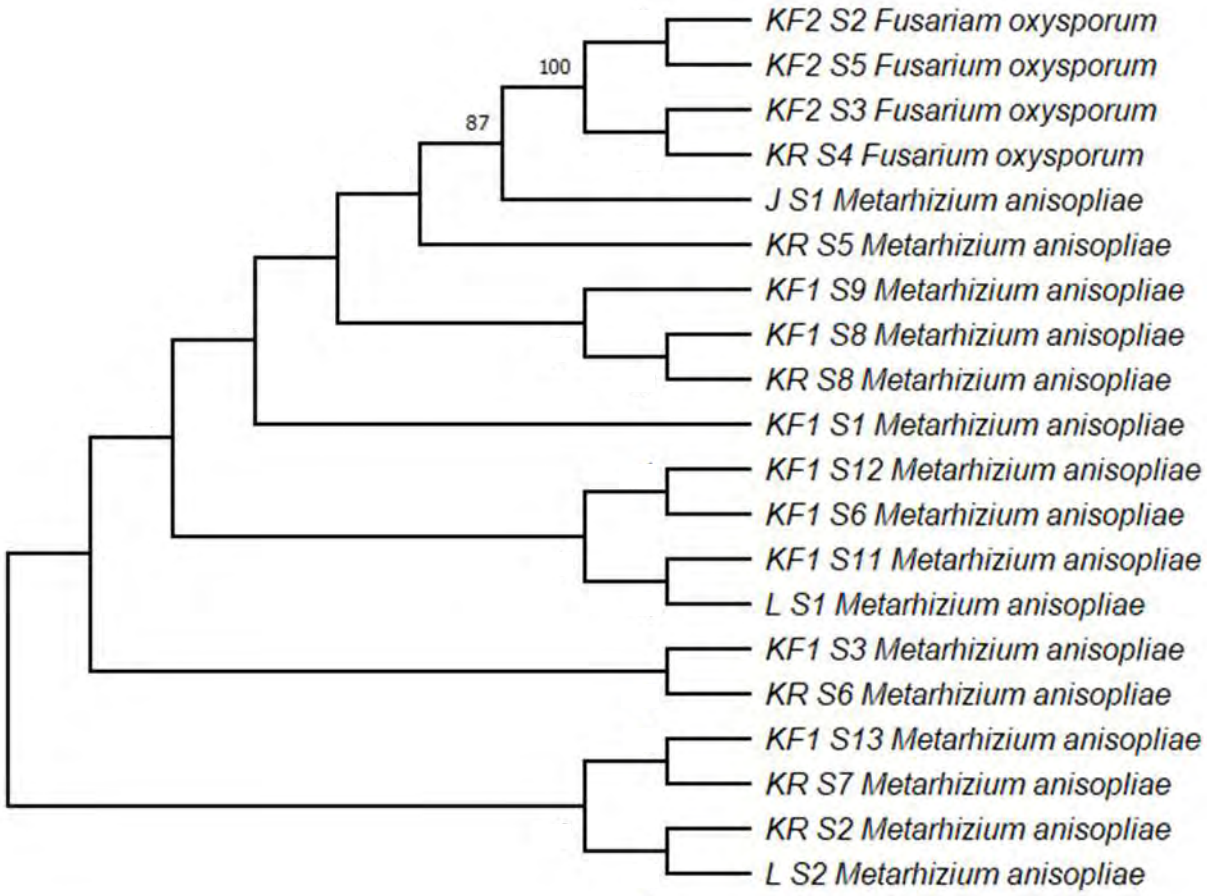

Figure 4.6: Maximum likelihood phylogenetic tree of 20 fungal isolates isolated from Honeybush farms.

Twenty fungal isolates were recovered from 98 soil samples (20.04%). Fourteen isolates, which constitutes 70% of the total isolates, were recovered from the planted field while six isolates (30%), were recovered from the refugia. *Metarhizium anisopliae* was the most recovered species in both the field and refugia (Figure 4.7).

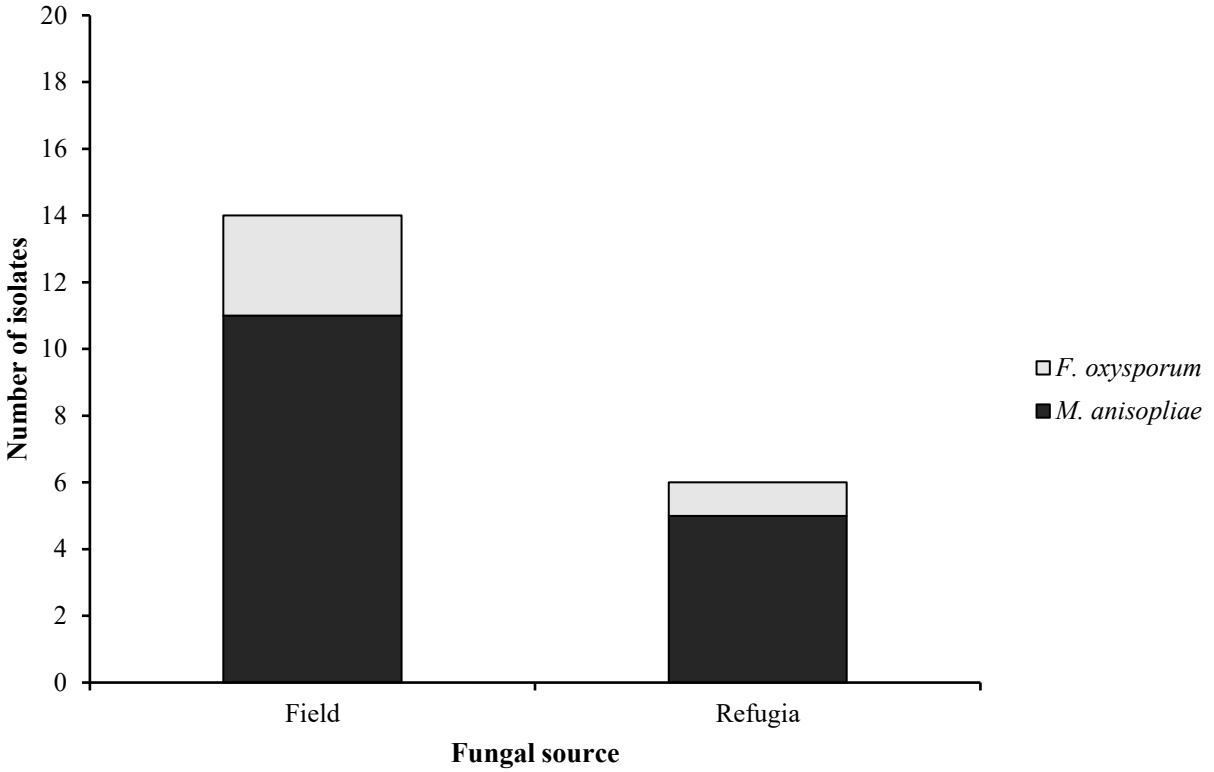

Figure 4.7: The occurrence of entomopathogenic fungi in soils sampled from cultivated Honeybush stands and surrounding refugia.

4.3.2 Pathogenicity of entomopathogenic fungi against *Molopopterus* sp. adults and nymphs

All the isolates had over 85% conidia germination. A one-way ANOVA was conducted to compare the effect of 20 fungal isolates on the mortality of adult *Molopopterus* sp.. Significant differences in the average mortality at $P < 0.05$ level for the 20 treatments was found ($F_{(19, 179)} = 16.892$, $P = 0.0021$) (Figure 4.8). Highest mortality of 72% was reported for *M. anisopliae* KF1 S13, whilst the lowest mortality of 11% was reported for *F. oxysporum* KF2S2. One-way ANOVA showed significant differences between fungal treatments ($F_{(19, 179)} = 5.463$, $P = 0.00097$) on nymphal stages (Figure 4.9). Post-hoc analysis using the Bonferroni LSD criterion (alpha = 0.05) separated the means. Control treatments had low mortalities of 9% and 6% in adults and nymphs respectively which was used to adjust for mortality. There were no marked differences in mortalities between

adults and nymphs, though different fungal isolates differ in their efficacy to both adults and nymphs. *Metarhizium anisopliae* isolates induced higher mortalities against both adults and nymphs (25-80%) than *F. oxysporum* isolates (10-60%) indicating *M. anisopliae* to be more virulent.

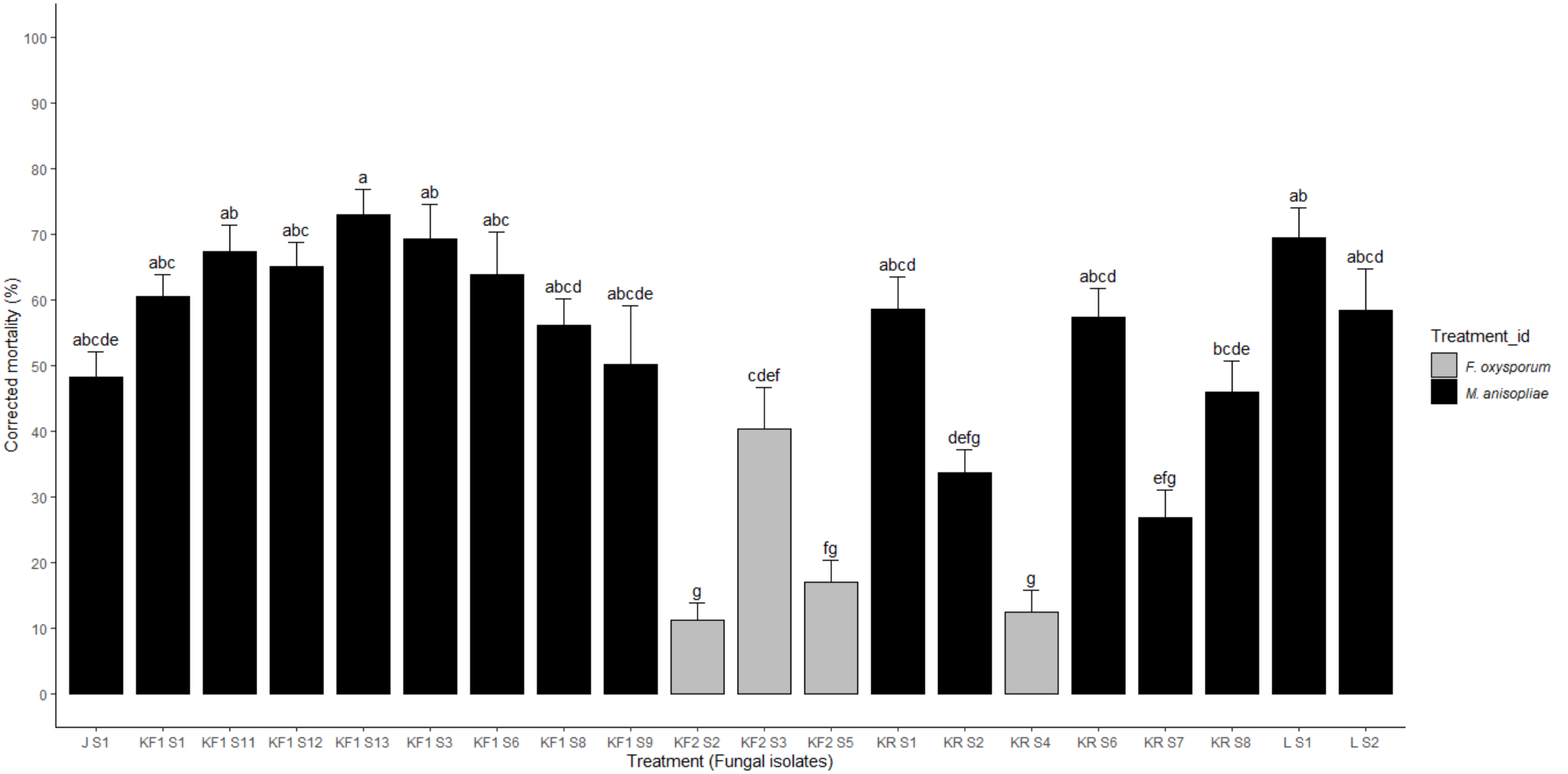

Figure 4.8: Corrected percentage mortality (± standard error) recorded for 20 fungal isolates against the adults of *Molopopterus* sp.. Fungal isolates not with different letters differ significantly at the 5% test level.

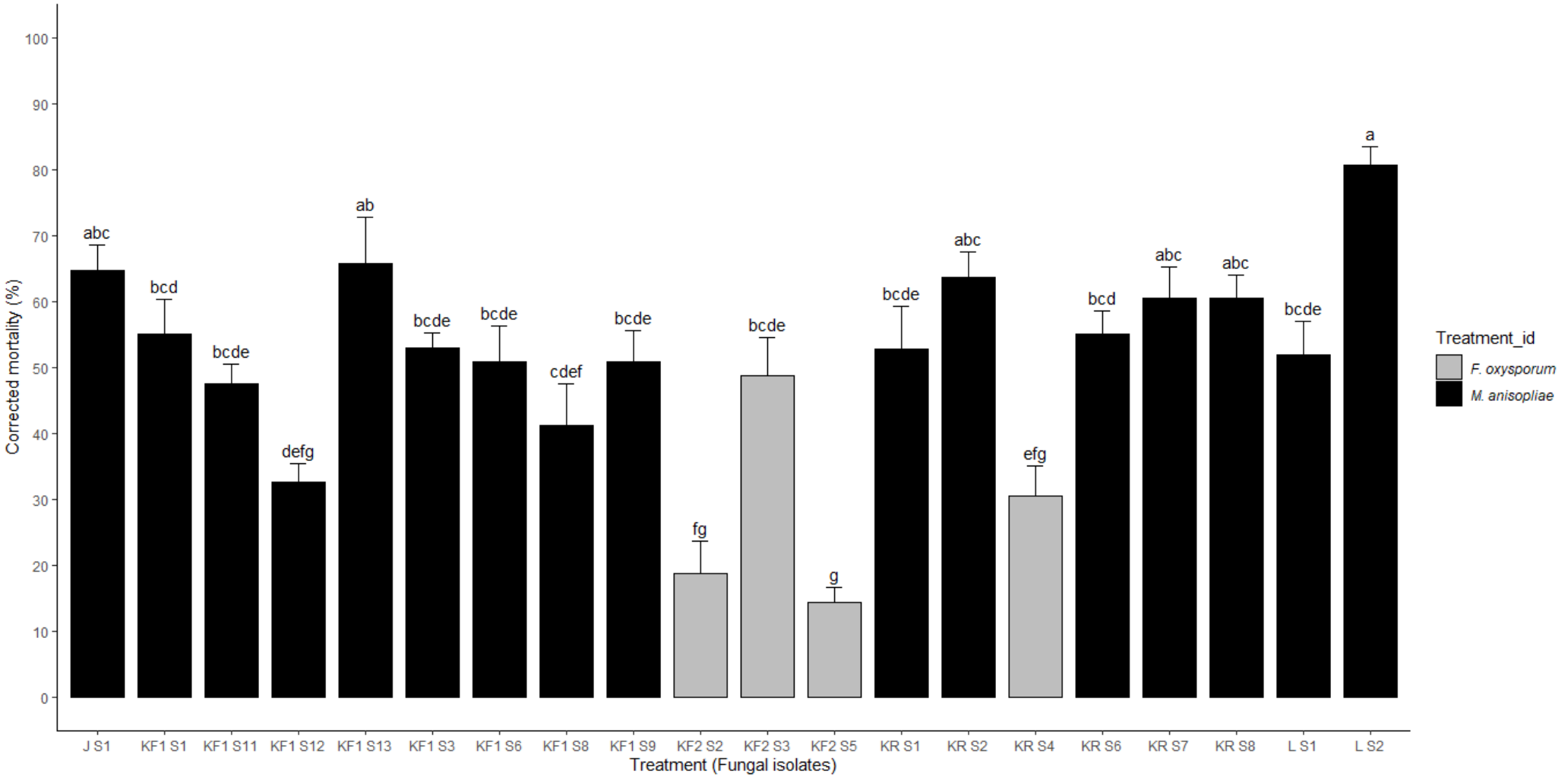

Figure 4.9: Corrected percentage mortality (± standard error) recorded for 20 fungal isolates against the nymphs of *Molopopterus* sp.. Fungal isolates not with different letters differ significantly at the 5% test level.

4.4 Discussion

A low recovery rate of entomopathogenic fungi from soil samples (20.4%) was consistent with findings by Goble et al. (2010) who recovered 21.53% from South African citrus orchards and Rath et al. (1992) 32%, from pastures in Tasmania. However, higher recovery rates have been reported. Bidochka et al. (1998) recovered 91% from temperate soils in Canada, and Keller et al. (2003), had 96% recovery from different habitats in Switzerland. Differences in recovery could be explained by different isolation protocols, variations in the bait insect species, the number of bait insects per sample, baiting temperature and volume of soil. *Galleria mellonella* is the most commonly used insect in the insect bait technique and is very sensitive to isolation of entomopathogenic fungi in the soil. Goble et al. (2010), recovered significantly more isolates from *G. mellonella* compared to *Ceratitis capitata* Wiedemann (Diptera Tephritidae) and *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae). Rath et al. (1992), used selective agar for isolation. The "Galleria bait" method was used in this study, however, mealworms, *T. molitor*, were used instead of the common *G. mellonella*. Furthermore, occurrence and distribution of entomopathogenic fungi is influenced by several factors such as location, habitat type, soil properties and cropping system which may further influence recovery of isolates (Asensio et al., 2003; Klingen et al., 2002; Meyling and Eilenberg, 2006).

The low recovery rate was also coupled with low species diversity as only two species of fungi were recovered. In other agricultural ecosystems within the Western Cape, more species were recovered by Abaajeh (2014) and Goble et al. (2010) who discovered six and five different fungal species respectively, of which *B. bassiana* was the most abundant species. Hatting et al. (2004) did a comprehensive survey of different South African soils and reported high recovery of *B. bassiana* (84%) compared to *M. anisopliae* (13%). Bidochka et al. (1998), suggested low competence ability for *B. bassiana* especially in cultivated soil samples. Furthermore, *B. bassiana* is known to be associated with soils with higher clay content in comparison to *M. anisopliae* (Quesada-Moraga et al., 2007; Rath et al., 1992). Recently, Dhlamini et al. (2020) recovered both *M. anisopliae* and *B. bassiana* isolates from vineyards in the Western Cape. Meanwhile, sampled Honeybush farms are predominantly sandy loam to loam with low clay content (Mbangcolo, 2008). This helps explain the absence of *B. bassiana* isolates in the collected samples. The bait

insect species is also known to influence the recovery of certain species. *Tenebrio molitor* was used in this study as the bait insect. However, Albertyn (2017) used *T. molitor* as one of the bait insects recovered more *B. bassiana* (87.8%) isolates than *M. anisopliae* (11.8%) from citrus orchards in South Africa. This then suggests that other factors are responsible for the absence of *B. bassiana*, rather than the bait insect species used. Honeybush plantations and sorrounding refugia may simply not contain this species of fungus.

The study showed that habitat type played a significant role on both the occurrence and recovery of fungal isolates. Soils harbouring *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen (Hypocreales:Nectriaceae) were more commonly recovered from surrounding refugia, but the presence of *M. anisopliae* alone or both fungal species was more strongly associated with cultivated Honeybush tea. This is in contrast to findings by Goble et al. (2010), who recovered more isolates from refugia than in citrus orchards. Bidochka et al. (1998) recovered more isolates in natural habitats over disturbed ecosystems. Furthermore, Tkaczuk et al. (2013), also discovered more species richness in natural habitats over arable land. Occurrence of entomopathogenic fungi is naturally associated with organic matter in the soil (Bidochka et al., 2001). This may be due to cation exchange in organic matter facilitating adsorption of fungal conidia (Inglis et al., 2012). However, studies by Medo and Cagáň (2011) and Quesada-Moraga et al. (2007) showed that habitat type did not influence recovery of entomopathogenic isolates. Honeybush tea, is a perennial crop that is organically grown; hence it provides a microhabitat that supports a great diversity of arthropod hosts in which fungi can multiply.

Recovery of *F. oxysporum* was quite unusual as many *Fusarium* spp. are well known for their role as plant pathogens and mycotoxin producers (O'Donnell et al., 2018). The genus *Fusarium* is well known to switch between different life stages and act on non-plant hosts (Santos et al., 2020; Van Diepeningen and De Hoog, 2016). Research on fusaria-insect interaction has been limited as they are generally characterised as opportunistic-insect pathogens. However, some *Fusarium* spp. are reported to possess entomopathogenic properties (Santos et al., 2020). Abaajeh, (2014), recovered seven *Fusarium* spp., in the Western Cape from citrus plantations, five of which were *F. oxysporum* isolates. Recently, Sharma and Marques, (2018), did a review seeking to address insect-*Fusarium* pathogenicity under field conditions. *Fusarium oxysporum* spp. complex has

been isolated from soil samples using the *Galleria* bait method (Santos et al., 2020; Sharma and Marques, 2018). Feng-Yan and Quing-Tao (1991), recovered 180 *Fusarium* isolates from 150 different insect cadavers including spiders.

Different *Fusarium* spp. have shown low to high mortality when tested against lepidopterans (Ali-Shtayeh et al., 2003), while showing moderate to high mortality against Coleoptera and Hemiptera (Anwar et al., 2017; Sharma and Marques, 2018). Ganassi et al (2001), recorded 60% mortality induced by *Fusarium* spp. against the aphid, *Schizaphis gramium* Rondani (Hemiptera: Aphididae). Thangam et al. (2014), also recorded 45 – 60% mortality in mango leafhoppers following *Fusarium* spp. application. Moreover, *Fusarium* spp. have also been identified to produce Beauvericin, a cyclic depsipeptide and one of the major toxins produced by *B. bassiana* which exhibits insecticidal properties (Wang et al., 2018; Wang and Xu, 2012). However, there is controversy as to whether they can adequately be classified as entomopathogenic fungi. Navarro-Velasco et al. (2011), studied histological evidence to comprehend its infection process, concluding that larval mortalities were a result of an active infection mechanism. *Fusarium oxysporum* isolates induced low mortalities (10 – 55%) against *Molopopterus* sp., however, they may still retain potential as bio control agents against other Honeybush tea pests. Although *Fusarium* spp. have the potential for biocontrol, there is need to further evaluate negative implications of their use as biological control agents concerning their safety and minimal environmental impacts.

Both adults and nymphs of *Molopopterus* sp. had similar responses to infection by the tested fungal pathogens with some isolates inducing mortalities over 60%. Discrepancies were also observed amongst isolates in the mortalities induced between adults and nymphs. According to Mochi et al. (2005), not all life stages are vulnerable to penetration by propagules, some are more vulnerable than others. For example, Gul et al. (2015), conducted pathogenicity tests against different life stages of the peach fruit fly, *Bactocera zonata* Saunders (Diptera: Tephritidae), and observed higher mortalities in adults than immature and pupal stages. Therefore, the life stage which is targeted can influence the probability of infection. Pupal stages are often most resistant, while nymphs and adults are most susceptible (Aatif et al., 2020). These discrepancies can also be

attributed to behavioural differences, coupled with inoculation technique used in this study (based on secondary pick-up) (Hatting, and Wraight, 2007).

Another important factor is the time of inoculation prior to ecdysis and the length of the intermoult period. If moulting occurs shortly after inoculation, it may remove penetrating conidia before colonisation of the insect host (Boomsma et al., 2014). Previous studies on pests in the order Hemiptera have recorded even higher mortalities using *M. anisopliae*. Sain et al. (2019), recorded high mortality (89%) on *Bemisia tabaci* Genadius (Hemiptera: Aleyrodidae) while, Bayissa et al. (2017), recorded comparable mortalities (73%) against aphid pest species of crucifers and okra which were consistent with the findings of this study. Tounou et al. (2003), discovered that increasing time of exposure in bioassays to 15 days resulted in even higher mortalities (97%) when they evaluated entomopathogenic fungi against the green leafhopper *Empoasca decipiens* Paoli (Homoptera: Cicadellidae). Furthermore, susceptibility of an insect is dependent on several factors that directly or indirectly impact pathogen performance. It is known that there is a threshold for a certain number of propagules necessary to overcome host defenses hence, conidia dosage is an essential factor (Jaronski, 2010). Most studies have shown correlation between number of infective spores and mortality by mycosis (the greater the spore density, the higher the mortality) (Butt and Goettel, 2009; Inglis et al., 2012).

The study demonstrated that the soil is an important source of entomopathogenic fungi, hence potential for controlling insect pests. The study also demonstrated that the bioassay procedures developed can play an important role in conducting future bioassays on Honeybush tea pests. The overall mortality of the isolates should be considered for further development of effective bioformulations for control of *Molopopterus* sp.. Development of the most virulent strains might help reduce pest densities in the field. *Metarhizium anisopliae* isolates which induced high mortalities against both adults and nymphs of *Molopopterus* sp., need to be further evaluated in terms of their dose response and time response before being considered for field application. The *M. anisopliae* isolates, J S1, KF S3, KF S11, KF S13, LS1 and LS2 are recommended for further evaluation.

CHAPTER 5

General Discussion

5.1 Introduction

There is a lack of knowledge regarding Honeybush tea pests and their successful management (Chapter 1). But, management of key pests is imperative to ensure the success of this industry especially in its early stages of commercial production. This study sought to understand the biology and ecology of two pests of Honeybush; the keurboom moth, *L. venus* and the leafhopper, *Molopopterus* sp.. Pest biology and plant-pest interactions are crucial in developing management practices (Faust, 2008). As such, the objectives of this thesis were to improve the understanding of the biology of *L. venus* including the quantification of damage, mode of infestation, infestation severity and determinants of infestation. Further, pest bionomics of *Molopopterus* sp. were determined. As an added component, entomopathogenic fungi were also collected from cultivated Honeybush fields and surrounding refugia as these microbial agents may offer a control option for both the pest species investigated.

In Chapter 2, field surveys for *L. venus* revealed low infestations of the pest with only a few farms recording active infestations. This study showed a link between age of the plant and stem diameter on the probability of infestation. As plants grow older, their stems increase in diameter, thus increasing the probability of infestation. Boring preference was for *C. subternata*, which was the only recorded infested plant species. Infestation severity determined by the number of *L. venus* larvae per plant was also shown to be driven by plant age, plant species and location. Infestation was close to the ground suggesting that eggs are laid on the ground and that the larvae migrate to the plant where it initiates feeding.

Laboratory studies on the biology of *Molopopterus* sp. showed a short generation time with five nymphal instars (Chapter 3). Multi-choice tests to establish host preferences showed a preference for *C. longifolia*. The results were further confirmed by field surveys which showed high pest

densities in *C. longifolia*. Field surveys also revealed that population densities were determined by plant species, age of the plants, location and harvesting practices.

Entomopathogenic fungi were collected and isolated as a possible management tool (Chapter 4). Soil samples collected from Honeybush tea farms and surrounding refugia were baited with *T. molitor* late instar larvae resulting in the recovery of 20 fungal isolates belonging to two species, *M. anisopliae* and *F. oxysporum*. Pathogenicity tests of these isolates against nymphs and adults of *Molopopterus* sp. showed that *M. anisopliae* induced higher mortalities ranging between 30 – 80%. *Metarhizium anisopliae* isolates J S1, KF S3, KF S11, KF S13, LS1 and LS2 induced mortalities above 60% in both nymphs and adults.

5.2 The Honeybush Industry in South Africa

Honeybush, *Cyclopia* spp., is currently being commercially cultivated to meet the ever-growing market demand which wild harvesting can no longer sustain (Metcalf et al., 2018). The Honeybush tea industry has the potential to develop and contribute significantly to the economy of South Africa due to its unique and competitive advantages as an endemic organic herbal tea with numerous health benefits (Toit et al., 2008). Honeybush is amongst other indigenous plants which have been exploited as an alternative crop for commercialisation in South Africa including medicinal plants such as Rooibos, those with floricultural uses such as *Protea* spp. and *Leucadendron* spp., and vegetables such as *Amaranthus* spp. (Akinola et al., 2020). Rooiboos is a predecessor of Honeybush which underwent a successful commercialisation process. Homogeneous stands achieved through the commercialisation of indigenous crops raise interesting problems (Reinten and Coetzee, 2002).

Following pest concerns raised by growers, Metcalf et al. (2018), conducted an initial survey to establish key pests of Honeybush, which prompted this current study. Pest status of an insect can be a product of abundance of individuals as well as the damage they inflict (van Emden and Service, 2004). *Leto venus* is considered a pest due to its deleterious feeding effect on Honeybush (Metcalf et al., 2018). Considering the nature of *L. venus* damage, it is indeed a pest, as pest status can be given to an insect which causes some level of damage to a particular host (Hill, 1990).

Moore (1967) developed a model to categorise crop pests based on previous human encounters with potential pests. The model identified four groups; pre-agriculture pests, pre-pesticide agriculture, pesticide agriculture and pesticide resistance agriculture. Following this model and low infestation, *L. venus* can be considered a pre-agriculture pest since it was never reported to cause any significant damage to plants of economic value prior to the cultivation of Honeybush. This is further supported by reports from researchers who have previously worked with the moth and acknowledged its rare occurrence (Grehan and Ralston, 2018; Janse, 1945). Pest status is subject to change due to ecological and economic changes. An ecological change that may affect pest status is the nature of damage caused by the pest to the host and the value of the damage as assessed by humans (Hill, 1997). Hill (1997) further asserts that change in demand of a crop results in altercation of pest status of some insects. If a crop is not in demand, some damage can be tolerated. Conversely, if a crop is in short supply then damage is not tolerated. The same principle can be applied in the case of Honeybush and *L. venus*. However, whether it can currently be considered an economic pest is still ambiguous given the observed low infestation rate (Chapter 2) suggesting control may be uneconomical.

The leafhopper *Molopopterus* sp. however, causes considerable damage to Honeybush tea as it compromises host usefulness in terms of quality and quantity of the yield. Quantitative measures have been employed to allow assessment of pest status of different agriculture crops (Hill, 1997). This study established high leafhopper population densities of *Molopopterus* sp., especially at Kurland and Lodestone Farm. The value of tea as a perennial crop in agriculture is much higher than in other crops (Mamun and Ahmed, 2011). In Rooibos tea, the leafhopper *M. theae* is one of the major tea pests where it discolours the leaf surface reducing the leaf photosynthetic area. Minor infestations are managed through registered contact and systemic pesticides, while extreme infestations are controlled by crop rotation practices. Considering the nature of damage and high population densities of *Molopopterus* sp., pre-emptive control measures are, therefore, required for the pest due to high population densities especially in cultivated Honeybush stands.

Honeybush is a certified organic industry (SAHTA, 2017). Organic farming is defined in international standards codex such as the International Federation for Organic Agriculture Movement (IFOAM) as a holistic approach which promotes and enhances the ecosystem health

including soil bio activity, biological cycles and biodiversity (Bonsignore and Vacante, 2018). According to FAO (2007), organic farming is a near closed nutrient energy cycle that maintains the ecosystem, soil and people. Generally, commercial farming in tea, necessitated phasic changes from organic to chemical tolerated production. However, chemical tolerated production has caused drastic changes to the soil and ecology, crop productivity and quality in tea farming. These adverse effects have prompted some organisations to revert back to organic farming to combat these adverse effects and regain lost resources (Zhao et al., 2020). Wong (2010) undertook a comparative study between organic and conventional managed tea and discovered that organically manged fields produced better tea in terms of yield quantity and quality and increased soil pH than in conventionally managed fields. Organic tea is now being grown in some parts of China, Japan, India and other tea growing areas in Asia (Xiao, 2018). Pest management in organic farming is a holistic approach rather than simple reductionist control approach that depends on the ecological processes and biodiversity (Daniel et al., 2018). Pest control in organic farming is preventative rather than curative and uses several approaches in an IPM strategy. IPM is an ecosystem based pest management programme and techniques currently being employed include improvement of natural enemy complexes through land management practices (conservation biology), augmentation biological control, cultural control and the use of greener pesticides with low toxicity and low pesticide residues (Figure 5.1) (Bhattacharyya and Sarmah, 2018; Roy et al., 2015). IPM in organic systems is guided under four thematic areas namely monitoring, avoidance, prevention, and suppression (Xiao, 2018). It is under these four principles that possible management strategies of *L. venus* and *Molopopterus* sp. in cultivated Honeybush will be discussed based on findings from this study.

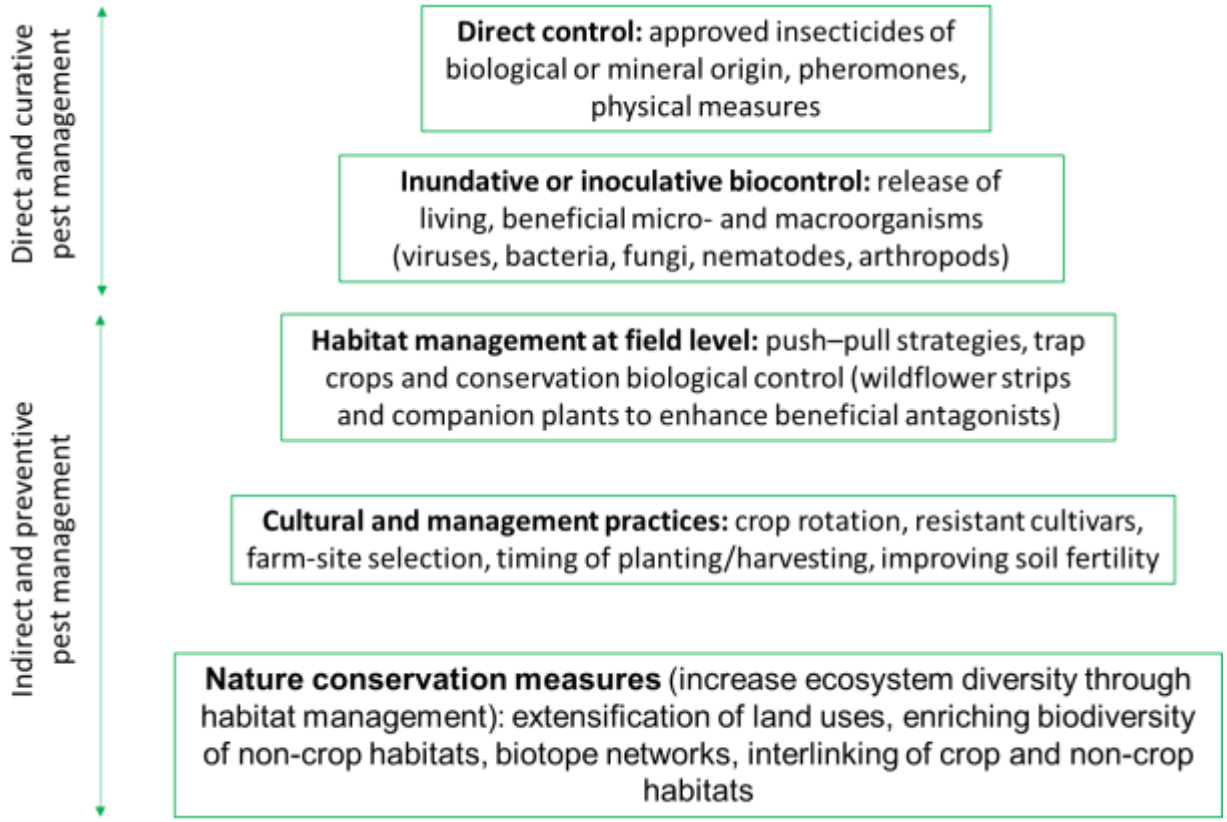

Figure 5.1: Conceptual framework model for pest management in organic farming (Adapted from Daniel et al. (2018).

5.3 Monitoring of Honeybush Pests

Pest monitoring is the backbone of IPM and it is fundamental to assess pest dynamics in the field to determine pest status (Daniel et al., 2018). Monitoring in Honeybush ensures that farmers are aware of the presence or absence of the pests. Monitoring serves as an early warning system allowing decision making regarding evaluation of control methods and management action (Nandagopal et al., 2010). As demonstrated in this study, both *L. venus* and *Molopopterus* sp., populations are variable from one Farm to the next even on a geographic scale. In most cases, stem borers are monitored using a combination of sex pheromones and traps (Togola et al., 2016). The combination of pheromones and insect traps serves not only towards monitoring and detection but

also as plant protection tools for attracting and killing insects, mass trapping and mating disruption (Barzman et al., 2015). Literature on *L. venus* as a ghost moth is suggestive that it relies on mating displays (lekking) rather than pheromones due to its silver spots (Nielsen et al., 2000). However, it can effectively be monitored through pest surveillance at fixed intervals by checking for frass at the base of the plant as was done in Chapter 2 of this study assisting farmers to make timely interventions. Aldini et al. (2003), used yellow sticky traps and sweep nets to monitor the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) in vineyards in Italy. Sweep nets were used in Chapter 3 of this study to effectively monitor pest densities of *Molopopterus* sp. using an active branch beating method. This method can also be used to monitor population dynamics of *Molopopterus* sp. in cultivated Honeybush. Atakan and Canhilal (2004), evaluated yellow sticky traps at various heights for monitoring leafhopper populations in cotton in Turkey with considerable success. Pest monitoring needs to be coupled with record keeping (Barzman et al., 2015). Records will assist in the development of predictive models which predict pest incidence on various Honeybush species. Furthermore, it will enable farmers to make choices on which species to grow in certain localities. Going forward, these records could also be used in the formulation of an area wide IPM.

5.4 Prevention of *L. venus* and *Molopopterus* sp. in Honeybush production

Prevention can be defined as a practice of keeping the pest from infesting a crop and is often desired as the first line of defence in organic farming systems (Barzman et al., 2015). It is often used when the pest presence/abundance can be predicted in advance. Meanwhile, avoidance is practiced when the pest population is already in the field but its impact can be negated through some cultural practices (Bonsignore and Vacante, 2018). Both these tactics strive to create a cropping system that is less likely to experience crop losses due to pest infestations (Barzman et al., 2015; Bellon and Penvern, 2014). Tactics for prevention and avoidance strategies tend to overlap, hence, they will be discussed together. Moreover, a combination of these tactics into management strategies generates more sustainable results rather than single tactic approaches (Barzman et al., 2015).

This study was able to establish that *L. venus* is exclusively associated with older *C. subternata* plants with thicker stems. The implication of these findings therefore suggests that the control of *L. venus* may be restricted to stands of *C. subternata* only when stem diameter exceeds 12 cm, or approximately 36 months of age. As a certified organic industry, one of the most plausible ways of controlling *L. venus* is using cultural control practices on egg laying sites. Entry holes were found at ground level, lending further evidence to the theory proposed by Grehan and Ralston (2018), that the moth lays its eggs on the ground. Literature on other hepialid species supports this finding and further suggests that eggs require high humidity for hatching (Nielsen et al., 2000). This could potentially be exploited for control. Control can be achieved at this stage through the manipulation of land management practices that are aimed towards incubation or habitat destruction for the *L. venus* eggs and larvae respectively. Field sanitation is a prevalent control approach for the control of African cereal stem borers, the maize stalkborer, *Busseola fusca* Fuller (Lepidoptera: Noctuidae) and the spotted stem borer, *Chilo partellus* C. Swinhoe (Lepidoptera: Pyralidae) (Togola et al., 2016). Destroying crop residues at the end of the season has been observed to limit the number of diapausing larvae of stem borer and similarly, the concepts can be applied for controlling *L. venus* by removing all leaf litter from around the base of plants which will help expose the eggs to bio control agents and weather elements preventing them from hatching.

According to Moolman et al. (2014), stemborers rarely infest healthy growing plants in their natural environments. However, when there are environmental stresses such as drought, plants become susceptible to infestation (Wright, 1995). The same concept might be true in the case of cultivated Honeybush since it was not previously considered a host of *L. venus* until its cultivation began. Honeybush is cultivated in a semi-arid environment where water is a scarce commodity and often subjected to severe drought conditions. Most farmers only irrigate their crop the first two weeks after planting (DAFF, 2014). The semi-arid environment coupled with shifting weather patterns being experienced globally may potentially be increasing the susceptibility of Honeybush to infestation. Once again land management practices such as controlled irrigation (Honeybush is a drought resistant plant) during drought season might help reduce infestation and help those already infested to recover. However, irrigation needs to be effectively managed as it creates a humid environment which encourages hatching of *L. venus* eggs, and unwanted fungal plant

pathogens such as *Phytophthora*. During the field surveys, it was discovered that most farmers rarely, if ever, irrigate their crop.

This study demonstrated host preferences of *L. venus* towards *C. subternata* while *Molopopterus* sp. preferred *C. longifolia* over other cultivated species. Sustainable genetic and morphometric variability exist in *Cyclopia* spp.. Thus, host plant resistance can be considered as a management strategy (Schutte, 1995). Host plant resistance is a vital component of IPM in organic tea production systems (Xiao, 2018). Assay and selection methods have been exploited for selection of pest resistant cultivars in organic tea production in Japan (Furuno et al., 1997). This led to the development of the late developing cultivar "Minamisayaka" which is resistant to white peach scale, *Pesudaulacaspis pentagona* Targioni (Hemiptera: Diaspididae). However, cultivar choice criteria are often dictated by market demand with plant pathogens being the second criterion (Dorais, 2007). Pest resistance plays a subordinate role and is merely addressed in breeding programmes as plant-pest interaction are often influenced by very complex interactions and often inherited in a quantitative manner (Daniel et al., 2018; van Emden, 1991). With several species of Honeybush, there are several different flavour profiles which have an effect on consumer preferences (Theron et al., 2014). Market demands are therefore more likely to affect choice of species to grow by Honeybush growers than host plant resistance to certain pest species.

Zhang and Tan (2004), studied host plant resistance in tea cultivars to the Tea green leafhopper *Jacobiasca formosana* Paoli (Hemiptera: Cicadellidae) and concluded that the thickness of palisade and horny layer cells were resistant factors to leafhopper infestations. Further studies on leaf structure and biochemical components in resistance to black spiny whitefly *Aleurocanthus spiniferus* (Quaintance) (Hemiptera: Aleyrodidae) were investigated by Zou et al. (2006). They discovered negative correlations with stoma density and spongy tissue. As already alluded to, there are 23 *Cyclopia* species with only seven being cultivated. Thus, there is need for further research to determine preferences and susceptibility to different pests provided they are also what the market dictates. In addition, there is need to describe likely changes in proteomes of the pest in response to different species and insect resistance to add a new dimension to management programmes for Honeybush pests as the information will be used to influence the choice of species to commercially cultivate. Exploitation of host plant resistance, though subject to market demands,

is a good alternative pest control strategy for Honeybush due to its capacity to reduce pest populations at no cost to the farmer and without interfering with the environment.

The control of the *Molopopterus* sp. needs a pro-active approach due to high population densities especially in recently harvested stands as was indicated in this study. In organic tea production, tea green leafhopper *E. decipiens* populations are controlled through cultural means, by harvesting shoots as they are known to be nutrient rich (Ruan et al., 2020; Xiao, 2018). Harvesting typically disrupts pest habitat and reduces insect food abundance, whilst at the same time increases the spider complex (Du et al., 2003). Harvesting in Honeybush was shown to have an opposite effect on *Molopopterus* sp. populations with significantly more leafhoppers recorded on recently harvested Honeybush plants compared to adjacent non-harvested stands. In this regard, it is imperative to consider alternative options of habitat management using trap plants, a rather unpopular approach in tea pest management, due to limited studies (Gurr et al., 2004). The use of traps may be further modified into a push/pull strategy involving the behavioural manipulation of the pest insects (Aluja et al., 1997; Khan et al., 2010; Silva et al., 2019).

A model example of the push/pull strategy was developed by Khan et al. (2010) at the International Centre for Insect Physiology and Ecology (ICIPE) for stem borer management in maize production. The strategy exploits two grasses, Napier grass *Pennisetum purpureum* Schumach and Sudan grass, *Sorghum vulgare* Pers. var. *sudanense* Hitchc. These grasses are highly attractive to adult stem borers for egg laying, but larval development on these grasses is poor. Legumes, *Desmodium* spp., and molasses grass, *Melinis minutiflora* P. Beauv are used as the push component within the field. *Molopopterus* sp. preference towards *C. longifolia* as established in this study (Chapter 3) can thus be exploited for control in a modified push pull strategy. *Cyclopia longifolia* plants may be used as trap crop on the edges of Honeybush fields and periodically harvested to attract *Molopopterus* sp. from the main field. Since trap crops won't be harvested for marketing purposes, they may be treated with systemic pesticides to help reduce field population densities.

5.5 Suppression of *L. venus* and *Molopopterus* sp. in cultivated Honeybush

Suppression entails the reduction of pest incidence and severity and their impact complementing prevention and avoidance tactics. The intention of suppression is to reduce the pest densities to acceptable levels where the damage they cause is insignificant (Barzman et al., 2015; Gerwick and Sparks, 2014). The damaging larvae of *L. venus* on *Cyclopia* spp., is challenging to control since it lives under the bark or within the stems. Once adults emerge, they are difficult to detect and trap as pheromones and host attractants for adults are poorly understood (Grehan et al., 2018). Due to lack of optimal control strategies for *L. venus*, infested Honeybush plants once detected, are often removed and destroyed by growers as a preventative measure. In large plantations, however, the removal of infested plants is uneconomic, especially if infestation is high necessitating the need for alternative control options.

Physical control has also been exploited for lepidopteran pests in small tea plantations and large plantations with bigger labour force (Mamun and Ahmed, 2011). In our field surveys we observed that farmers physically mutilate *L. venus* larvae by inserting flexible wires in entry holes thereby killing it. This method of control is currently viable and may be the cause of observed low infestations, however, with increasing efforts at commercialisation of *Cyclopia* spp. there is need for more practical and sustainable pest control strategies against the pest. Miyama et al. (2009), designed an insect blowing apparatus for blowing and collecting the Tea green leafhopper off tea shoots with considerable success (Figure 5.2). Such an apparatus can be used on an interval basis to reduce *Molopopterus* sp. populations in cultivated Honeybush especially after harvesting when the population density tends to increase.

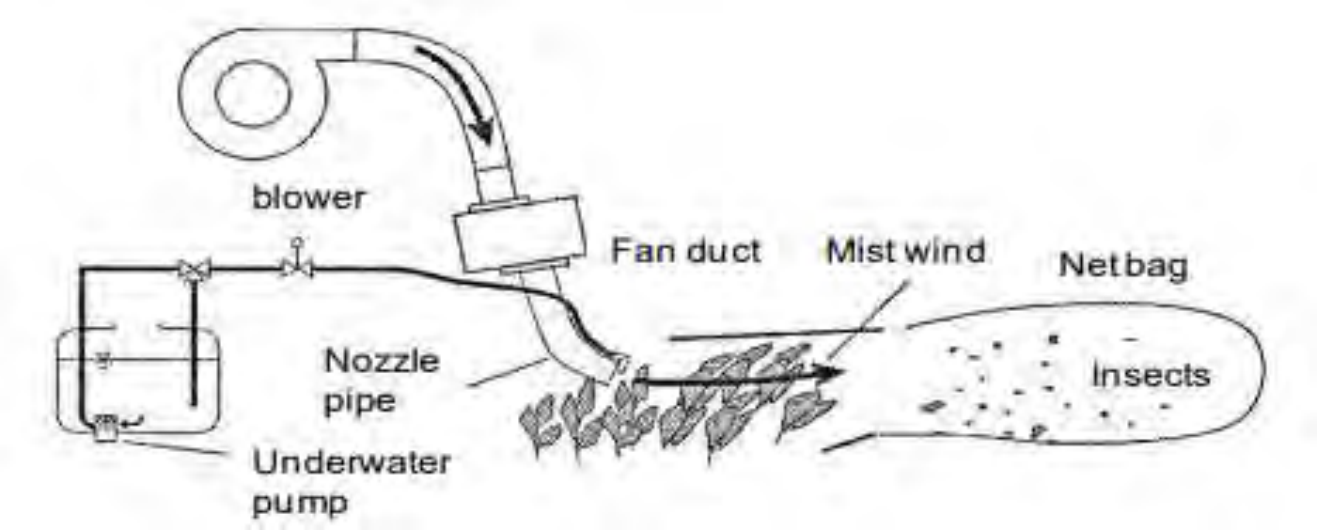

Figure 5.2: Schematic presentation of an insect trapping machine (Miyama et al. (2009)).

Biological control is on the forefront of organic IPM strategies, an outcome of intensive research towards understanding the biology and ecology of natural enemies on pests (Chidawanyika et al., 2012). Biocontrol, as defined by Roy and Muraleedharan (2014), is a method of reducing pest populations using other organisms through predation, parasitism and herbivory but typically involves an active human role. Adoption of biocontrol strategies is pertinent for sustainable Honeybush production and ideal agriculture growth with minimal adverse environmental impacts that may negatively impact the ecosystem, water resources and human lives. The success of biocontrol depends on the efficacy of selected candidates against target pests as well as their adaptability and long term establishment in the habitat (Debnath et al., 2012; Nakkeeran et al., 2005). Thus, selection of a biocontrol agent necessitates a thorough understanding of its dynamics and occurrence with special reference to the intended crop (Trivedi et al., 2012).

In some instances, farmers inject formulations of commercially available mycopesticides (Broadband® BASF) in tunnels with considerable success. Microbial pesticides and mycorrhizal fungi have raised agriculture productivity in tea plantations (Ahmed and Holmstrom, 2014). Entomopathogenic fungi are considered candidates for incorporation into an IPM programme where their effect would be minimal against natural enemies (Lacey et al., 2001). Both *B. bassiana* and *M. anisopliae* have been successfully used in tea production. *Beauveria bassiana* has been

used to control the mosquito tea bug *Helopeltis theivora,* a major pest of tea in India, reducing field populations by 50%, whilst *M. anisopliae* has been examined against live wood tea eating termites (Debnath et al., 2012; Xiao, 2018). Results from this study showed that many fungal isolates, mainly of the species *M. anisopliae,* occur in Honeybush plantations (Chapter 4). Isolation of virulent entomopathogenic fungi is a prime criterion for good agriculture practices as it provides insight into naturally occurring fungal biodiversity and provides a pool of potential biocontrol agents (Hussain et al., 2014; Sevim et al., 2010). Grehan and Wigley (1984), studied entomopathogens of the Puriri moth *Anetus viscrens* which has a developmental form that is synonymous to *L. venus*. In their study, Grehan and Wigley (1984) isolated *B. bassiana* from infected larvae collected in the field and bacteria from larvae collected from the tree stage. In this study, isolated fungal strains could not be screened against *L. venus* due to low populations. However, against *Molopopterus* sp., pathogenicity tests showed variability among fungal isolates with *M. anisopliae* having high mortalities against both nymphal and adult stages. Observed fungal induced mortalities in this study provide a foundation for further studies towards improvement of efficacy and screening against other pests such as thrips.

5.6 Recommendations for Future Studies

The threat of insect pests in Honeybush is insidious but compelling with each plant species appearing to be susceptible to one and not the other pest. The industry sanctions a non-synthetic pesticide approach given the uniqueness of the product and market expectations (SAHTA, 2017). Several entomopathogenic fungi showed potential to induce mortalities of *Molopopterus* sp.. Future research should be oriented towards integration of these isolates in pest management. These isolates can be screened against other Honeybush pests such as thrips which were identified by Metcalf et al. (2018) in their survey and were reported by growers during our field studies as problematic. Moreover, certain species and isolates of entomopathogenic fungi are known to colonise plant tissues as endophytes. This natural colonisation has been reported to be beneficial to plants through improvement of plant growth and reduction of pest infestation (Akutse et al., 2013; Jaber and Enkerli, 2016). Due to their ability to induce systemic resistance as well as reduce diseases caused by soil-borne plant pathogens such as *Pythium* spp. and *Fusarium* spp., they have been artificially introduced in crops (Khan et al., 2012; Ownley et al., 2010). Entomopathogenic

fungal endophytes have been identified in several economic crops such as wheat, bananas, soya beans and tomatoes as beneficial rhizosphere and plant growth promoters (Hu and St Leger, 2002; Jaber and Enkerli, 2016; Ownley et al., 2010). *Beauveria bassiana,* the most studied endophytic fungus, was reported to colonise numerous crops such as cotton, soya beans, and citrus where it reduced the infestation of serious pests predominately in the Orders Coleoptera and Hemiptera (Clifton et al., 2018). Induced systemic resistance in Honeybush may be possible through further exploitation of entomopathogenic fungi. In Rooibos, several species of endophytes of *B. bassiana* have been identified (Hatting, 2017).

Biocontrol of tea pests is largely premised on native predators and parasitoids (Du et al., 2003). During our field surveys, several species of spiders were observed in Honeybush plantations. Spiders are some of the most abundant natural enemies in terrestrial ecosystems (Nyfeller and Benz, 1987). They are considered the most dominant predator over ladybirds, lacewings and mantids with dominance range of 65 – 97% in Chinese tea gardens (Chen et al., 2004). Analysis of population dynamics between spiders and pests have shown that they play a significant role as predators (Yang et al., 2017). There is need to build on the potential ecosystem services and disservices of arthropods (Slabbert, 2016), by looking at actual predator-prey interactions. Specifically, there is need for research to quantify spider complexes associated with Honeybush and predator efficiency to understand their role in pest suppression. Occurrence rates, temporal and spatial overlap between predator and prey as well as dominance of predators have been used as indicators by which predators control prey (Chen, 2003; Sibarani et al., 2017; Sunderland et al., 1999).

Pest outbreaks are often associated with main stages of plant production such as harvesting, distribution and storage (Hill, 1997). Production stages influence dispersal of pest population structures, availability of food and space as well as limit the presence of natural enemies (Hazarika, 2018; Mamun and Ahmed, 2011). For effective pest control measures, there is need to integrate production practices and pest management strategies. Thus, there is need for amalgamation of cultivation and propagation research and pest control research to establish production management that maximise yield whilst at the same time minimises pest pressure. This can be achieved through

studies on the seasonal occurrence and distribution of pests e.g. *Molopopterus* sp. to establish their peaks. Such information can then be integrated into the Honeybush production calendar.

Coupled with seasonal occurrence is the need to establish other alternative hosts for *Molopopterus* sp. as well as overwintering behaviour. Studies on seasonal and spatial distribution of leafhoppers in peach orchards and surrounding wild plants suggested that wild plants acted as sources of infestation (Grange et al., 2017). Further investigations are necessary to identify alternative hosts of *Molopopterus* sp. and to delineate the phenology of such hosts. Habitat management strategies can then be carried out to formulate strategies that farmers can use to minimise infestation and rate of dispersal. Furthermore, olfactory cues are known to play an important role in host location in certain leafhopper species (Orenstein et al., 2003). There is need for further study on *Molopopterus* sp. to establish whether plant volatiles induce behavioural responses. A behavioural manipulation method can thus be developed through trap crops or use of a push pull strategy, where volatile compounds are used to push insects away from the plant of interest and attract them to sink plants (Grange et al., 2017). Research in this regard has been considered for the planthopper *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) in grapevines (Sharon et al., 2015). Sounds and vibrations have been used as a physical control strategy (Daniel et al., 2018). Eriksson et al. (2012), demonstrated the use of disruptive vibrational signals for mating disruption of *S. titanus* on grapevine plants under field conditions and managed to reduce mating to 9%.

5.7 Conclusion

Crop protection of Honeybush against insect pests is, and always will be challenging as this group of plants is indigenous. Nonetheless, intensive research and implementation of management strategies, especially within the context of IPM, will help accelerate the commercialisation efforts. Organic farming complemented with biological control aids biodiversity conservation and soil functionality while ensuring food security. Through this study, it is concluded that considerable strides have been made towards understanding the biology and ecology of *L. venus* and *Molopopterus* sp.. The knowledge gained in this study showed variations in pest status of the two pests due to differences in densities and degree of impact necessitating the need for a rational approach to categorising pests. Different Honeybush species differed in their preferences to pest

infestations, *C. subternata* being associated with *L. venus* while *C. longifolia* being preferred by *Molopopterus* sp.. Pest densities were affected by several factors including harvesting practices, plant species, location and age of the plants. For the control of *L. venus*, we recommend periodic scouting of fields as damage is clearly visible at the base of the plant and physically destroy the larva, a solution that some farmers are already employing. Control of *Molopopterus* sp. with entomopathogenic fungi is quite promising, however there is need for further research.

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