Managing releases of *Anagyrus vladimiri* (Triapitsyn) to augment biocontrol of the citrus mealybug *Planococcus citri* (Risso) in South African citrus orchards

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By
Wayne Trevor Mommsen

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Abstract

In May 2019, South Korean inspectors rejected numerous grapefruit consignments from Letsitele, Hoedspruit and Onderberg in South Arica, because of live mealybug found on fruit. Growers expressed deep concern as mealybug management to a phytosanitary level was almost unattainable. Regular spray interventions for control of citrus black spot fungus, *Phyllosticta citricarpa*, and citrus thrips, *Scirtothrips aurantii,* cause repercussions in mealybug populations because they undermine the naturally occurring biocontrol complex. As part of an Integrated Pest Management (IPM) strategy, release of commercially produced parasitoids is common practice, to augment the naturally occurring beneficial insect populations. Prior knowledge of the harmful effects of insecticides on parasitoids is essential to IPM planning and the success of the biocontrol component in such a programme. Timing of augmentative releases to coincide with the phenology of citrus and the mealybug pest is also considered important for the successful establishment and control. Consequently, field trials were conducted to compare efficacy of early vs. late releases of *Anagyrus vladimiri* (Triapitsyn), an effective parasitoid of the citrus mealybug, *Planococcus citri* (Risso). Semi-field bioassays were conducted concurrently to determine the impact of various thripicides on *A. vladimiri*. The impact of sulfoxaflor, spinetoram, spirotetramat and prothiofos were rated harmless, as *A. vladimiri* mortality was lower than 25% after coming into contact with aged residues between 7 and 14 days old. October and November releases of *A. vladimiri* resulted in early parasitism and lowered peak-infestation of mealybug. January releases are possibly too late in grapefruit and lemon, open field, orchards, considering parasitism by *A. vladimiri* peaked in February. In mandarin orchards under net, percentage parasitism of 3rd instar mealybug increased a month later. Notably, at harvest, the difference in efficacy between treatments was not clear. This could be explained by high levels of natural parasitism observed in the treated and untreated orchards, which emphasises the importance of conservation biocontrol. In a second season, the proportion of hyperparasitoids captured (61%) from samples of mealybug-infested fruit was larger than the proportion of primary parasitoids, *Anagyrus vladimiri, Coccidoxenoides perminutus* (Girault) and *Leptomastix dactylopii* (Howard) (39%), which was far lower than the captures of eclosing primary parasitoids the previous season, which was 60%. The new discovery of *Pseudaphycus* sp. in citrus orchards in South Africa could be a key in explaining the uncontrollable levels of mealybug experienced and has drawn attention to a need for further understanding of ecological factors that influence biological control in citrus.

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Declaration

I, Wayne Trevor Mommsen, hereby declare that the following thesis has not been submitted to any other Academic institution other than Rhodes University, Grahamstown, South Africa and the work presented here is that of the Author.

Wayne Trevor Mommsen
13/12/2023

Problem Statement

In recent years citrus producers in southern Africa have expressed concern over the increased incidence of mealybug in citrus orchards. It has become a challenge to control mealybug populations, which includes several species of mealybug. Although the adult females for most species of mealybug are routinely identified through morphological keys, it is more problematic to distinguish between eggs and nymphs (crawlers) of the different species (Pieterse et al, 2010). If unacceptable levels of mealybug are present on fruit at the time of harvest, particularly in early varieties such as grapefruit and lemons in the northern production regions, export consignments are regularly rejected, based on interceptions of unidentifiable mealybug nymphs or eggs. In April 2019 the citrus industry was warned about alarming mealybug levels intercepted during port inspections on citrus destined for South Korea (CRI Cutting Edge 268, 2019, unpublished). At the end of the season the southern African citrus industry was cautioned and needed to improve. However, in May 2020 mealybug interceptions had not been prevented and the citrus industry was again warned (CRI Cutting Edge 298, 2020, unpublished), thereby placing the future of the South Korean programme in jeopardy.

The citrus industry has been left with a very important question. How can mealybug pest management be improved under these circumstances? Mealybug populations have the ability to rapidly attain economically damaging levels and are extremely difficult to control chemically (Bedford et al., 1998). The incorrect application of pesticides will not control mealybug in the long term and will have an adverse effect on natural enemies (Franco et al., 2009). But no matter how well insecticide control works, sustained mealybug control cannot be achieved without an effective biological control complex for mealybug. In order to overcome constraints and limitations on practical control tactics for mealybug control, there is a need for a more effective, target specific and environmentally safe approach to mealybug control.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 Citrus Production in South Africa

1.1.1 Historical Overview

All species belonging to the genus *Citrus* and related genera originated in the tropical and subtropical regions of south-eastern Asia/north, eastern India, southern China, the Indo-Chinese peninsula and the Malay Archipelago (Talon et al., 2020).

Immigration and trade saw the distribution of citrus to south-eastern Europe and Africa, as early as the 10th Century AD (Reitz, 1984). The first citrus fruit (*Citrus medica*) to arrive in Europe and the Middle East were primarily valued for the ability of their fruit to produce a sweet scent and were used for treating linen; it was sometime later, during the 16th century, around the time of Portuguese trading activities, that sweet oranges (*Citrus sinensis*) were introduced. During this period citrus fruit was associated with the upper-class society and it was these sweet tasting fruit that were regarded as a delicacy (Wearn et al., 2016). Sweet oranges are believed to have arrived in South Africa in 1654 on a Dutch ship bringing supplies from the port of St. Helena and oranges from these trees were harvested in the gardens of the Dutch East India Company in 1661 (Marloth, 1939).

Over years, many citrus trees were planted in home gardens ranging from the Cape Province in Hex River and Clanwilliam district to Rustenburg in, what was then, the Western Transvaal. These early plantings of citrus were made from seedlings (Marloth, 1939) and the trees would grow well, producing good yields, up to the age of 100 years old. It wasn't until the late 1800s that the world made significant improvements in the budding of citrus nursery trees. It was discovered that splicing and inserting a single bud between the bark and trunk of a seedling rootstock, was a very successful procedure (Reitz, 1984). Budding avoided long juvenile periods of seedlings, allowed selectivity of the desired scion and rootstock combinations and paved the way for propagation of trees free of budwood-transmitted tree decline diseases. In 1854 Mr. W. Tuck of Grahamstown distributed the first seedlings grafted with budwood of the Bahia Navel, which originated in Brazil (Marloth, 1951). Later reports described Washington

Navels in Fort Beaufort and budded lemon trees in Groot Drakenstein that were 35 and 40 years old respectively (Marloth, 1939).

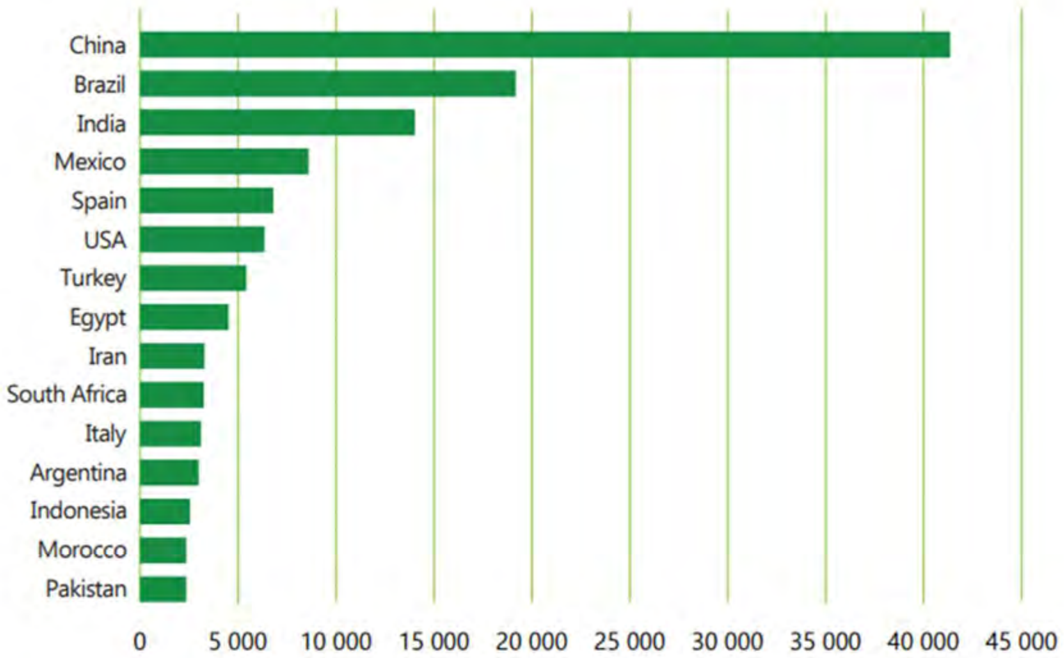
By the early 1900s citrus improvement was well under way and considerations for commercial production and export were realising. Guidelines for citrus production were published in South African agricultural journals as early as 1903 (Davis, 1903) and in June 1906 citrus fruit from what was the Transvaal were exported to London and placed on display along with the deciduous fruits from the Cape (Davis, 1906). As a crop very adaptable to soils, terrain, planting and cultural arrangements citrus was destined to become a worldwide success as the leading tree-fruit crop, with annual world citrus production growing to 15 million metric tonnes in the 1950s and nearly a fourfold increase to 56.5 million metric tonnes by 1980 (Reitz, 1984). Domestic consumption was the greatest market with a few major citrus producing countries described as active in exporting citrus, namely Spain, United States, Israel, Morocco and South Africa (Reitz, 1984).

Today the South African Citrus industry is highly reliant on exports (Hattingh, 2003) supported by a research and technical base with a corresponding market sensitive approach. In 1998 the South African Citrus Growers Association (CGA) was established as the representative body for the citrus growers, funding research by voluntary levies paid by growers. Some 20 years later South Africa is ranked second to Spain as the world's biggest citrus exporting nations, with 2.1 million metric tonnes of citrus being exported to the most discerning markets around the world (Edmonds, 2020).

1.1.2 Commercial citrus production

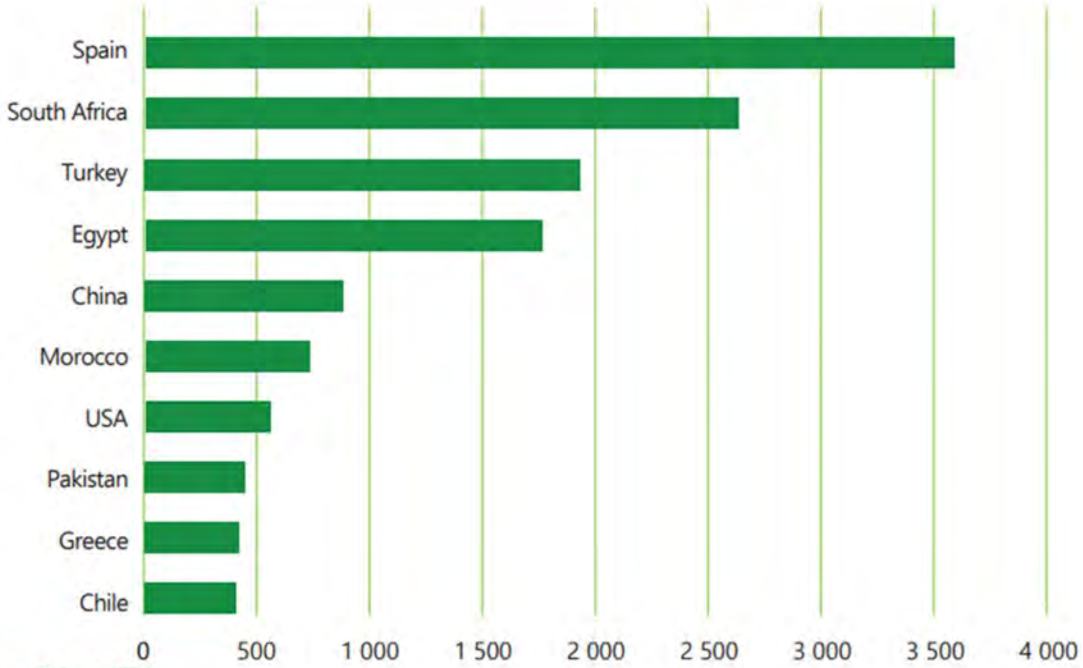
Citrus fruits are highly ranked in international trade in terms of value. Several species and their hybrids, considered under the more general term "citrus", are of great commercial interest. Examples include lemons (*Citrus lemon* (L.) Burn), limes (*Citrus aurantifolia* (Christm.) Swing), sweet oranges (*Citrus sinensis* (L.) Osb.), mandarins (*Citrus reticulata* Blanco), satsumas (*Citrus unshiu* (Mak.) Marc), clementines (*Citrus clementina* Hort.), grapefruits (*Citrus paradisi* Macf.), and pummelos (*Citrus maxima* (L.) Osb) (UNECE, 2010).

Citrus fruit is produced all over the world. According to the Food and Agriculture Organisation (2020), there are just short of 100 major citrus-producing countries and around 70% of the world’s total marketable citrus is grown in the northern hemisphere, in particular Brazil, including countries around the Mediterranean and the United States. The greatest production in Europe is in Spain, which accounts for more than 55% of the European citrus output (Figure 1.1). There are two clearly differentiated markets in the citrus sector: the fresh citrus fruit market, with a predominance of oranges and mandarins, and the processed citrus products market, mainly for orange and grapefruit juice. The current annual worldwide production of citrus is estimated at over 130 million tons (World Citrus Organisation, 2023), with more than half of this being oranges. About a third of citrus fruit production goes for processing, and more than 80% of this is for the production of orange juice (Blasco et al., 2016).



World Fresh Citrus Production 2021/2022 (Thousand Tons)

Source: USDA, FAO



World Fresh Citrus Exports 2021/2022 (Thousand Tons)

Source: Trademap, USDA

Figure 1.1 World citrus production and export volumes for the 2021/22 citrus season (Citrus Growers' Association, Key industry statistics, 2023).

The most suitable traits of citrus fruit for processing are related to internal fruit quality, which is a function of flavour and palatability. Citrus fruit for processing must have no decay symptoms, elevated juice content, high level of TSS (total soluble sugars, mostly sucrose, glucose and fructose), low TA (titratable acidity, mostly citric acid) and minor amounts of bitter components, such as the triterpene limonin and the flavonoid naringin. The external appearance of fruits, however, is not particularly important for processing although an attractive internal colour in oranges is usually required. Citrus fresh fruit quality standards, on the other hand, are largely dependent on consumers' preferences that in addition may change substantially between countries. In general, in fresh markets, consumers place a lot of emphasis on external fruit quality standards (Abouzari et al., 2016), provided fruit are of internally sound quality.

Mandarins, navel oranges, valencias, pummelos, and grapefruit are grown mainly for fresh markets. Lemons are grown for both fresh and juice markets. Internal fruit quality is essential in the fresh market where suitable fruit shape, deep peel colour, and smooth and shiny appearance are the most attractive external quality characteristics for the consumers. Easy peeling, pleasant flavour, seedlessness, and fragrance are the most desired internal quality parameters (Jenks et al., 2011). The objectives of citrus fruit production and the methods applied to achieve it are different, depending on the final destination of fruit, that is, the processing industry or the fresh market (Davis et al., 1994).

Standards of commercially grown citrus for fresh consumption are mainly based on the absence of blemishes, bruises and rotting, as well as adequate shape, colour, size and maturity (Department of Land Reform and Rural Development, 1991). Maturity of citrus fruit is defined by parameters specified for each species concerning minimum juice content, minimum total soluble solids content (sugar contents), sugar-to-acid ratio, and colouring. Certain parameters related to taste and maturity are commonly estimated both in packhouses and in the field in order to assess the quality of the fruit (Blasco et al., 2016). These are the Citrus Colour Index and the Maturity Index (MI), which is a ratio between the soluble solid contents and the acidity expressed as citric acid. Fruits with an MI value of more than a certain threshold (which depends on the species and variety) are considered to be ripe and to have a taste that makes them suitable for commercialization or in the case of southern African citrus, long distance shipping. The minimum juice content is also calculated in relation to the total weight of the fruit and after extraction of the juice by means of a hand press.

Consumer preferences depend largely on cultural habits and many other socioeconomic characteristics. Muslim countries, such as the United Arab Emirates, use lemons at religious gatherings, while Hong Kong and Russia are reliant on importations of fresh fruit and consume citrus as a source of nutrition (Potelwa, 2017). So "quality" is a term that when applied to fresh fruit may be subject to a number of interpretations. Usually within the markets it refers to the external appearance of the product and products imported from countries outside Europe and the US must conform to essential standards. In these standards, fruit are packed according to their grade (i.e. Class I, Class II etc.) with the aim to be virtually free of blemishes and to be uniform in shape, size, colour, and maturity. Fresh fruit quality does not only relate to appearance, but also some other characteristics, for example, taste, richness in nutrients, vitamins, odour, etc. must be considered (Jenks et al., 2011). Irrespective of the yielding potential of a newly developed cultivar, demand for a product and its success in agriculture will be determined by the end-use, overall quality of the final product in the market. It is the end-user who will mostly determine if that crop will be grown in future years. There are two main types of end-use quality. Firstly, "organoleptic", which is consumer acceptance or preference of taste, size, texture, and colour. Although many people differ in their preference, there is a general agreement on taste preference towards certain levels of expression of these attributes, thus 'liking' some genotypes over others (even disregarding 'off -tastes'). The second is "chemical", which is a physical measure of composition or nutrient value. Consumer preference is continually changing and quality standards of today may be superseded by a new set of standards in the future (Abouzari et al., 2016). It is therefore, imperative that cultivar development remains flexible and addresses the requirements for yield, quality, and other factors that are important in the long term (Brown et al., 2008).

As is the case with other commodities, citrus fruits can become damaged in the field or during handling and processing (Blasco et al., 2016) in the packinghouse. However, not all of these defects have the same economic importance. Some types of damage do not evolve, but others do, especially those related to fungal or bacterial infections. It is thus extremely important to detect them in their earlier stages, otherwise they can spread infections to other fruits or they may be invisible during inspection but appear later in the destination marketplace, thereby causing the consignment to be rejected by the buyer. In addition, insect pests that cause cosmetic blemishes on fruit, such as mealybug, will also result in downgrading of fruit quality, or can lead to fruit decay and ultimately rejection of consignments for export (Urbaneja et al., 2020).

The southern African climate is favourable for citrus production. Due to a broad climatic range, from semi-tropical to Mediterranean, a diverse range of citrus cultivars are produced from late February through mid-October (Barry et al., 2010). South Africa is the 10th largest citrus producer in the world and the 2nd largest citrus exporter worldwide (Citrus Growers' Association, Key Industry Statistics, 2023). South Africa produces roughly 2.65 million tonnes of fresh citrus fruits per annum and exports 85% of this volume, second only to Spain, as the world's leading citrus exporters (Figure 1.1). In the 2022 citrus season 2.13 million pallets of citrus, accounting for 69% of all production, was exported to foreign markets. Remaining citrus is either sold to local markets (6%) or processed into other citrus related products (25%). The highest proportion of South African citrus is exported to Europe (EU) (33%), South East Asia (13%) and the Middle East (19%) (Citrus Growers' Association, Key Industry Statistics, 2023). Although citrus crops are grown across seven of the nine provinces in South Africa, the key citrus producing areas are in Limpopo, Eastern Cape, Western Cape and Mpumalanga. South Africa grows valencia oranges, navel oranges, lemons, grapefruit and mandarins.

The citrus fruit industry is an important contributor to the South African economy. The industry comprises a distinctly heterogenous grouping of citrus producers (Sinngu et al., 2014), ranging from large commercial farmers to small scale rural farmers, and is a significant foreign currency earner, contributing considerably to the country's GDP.

In southern Africa, the citrus growing regions differ geographically and climatically, which defines the area suitability for the production of certain citrus types (Figure 1.2). The variability in climate also influences the presence of different insect pests in an area or on a particular farm (Bedford et al., 1998).

Citrus Producing Regions of Southern Africa

Province	Area (ha)
Limpopo	39 524
Eastern Cape	24 508
Western Cape	19 208
Mpumalanga	8 127
Zimbabwe	2 706
Kwa-Zulu Natal	2 350
Northern Cape	1 818
North West	730
eSwatini	714
Free State	12
Grand Total	**99 697**

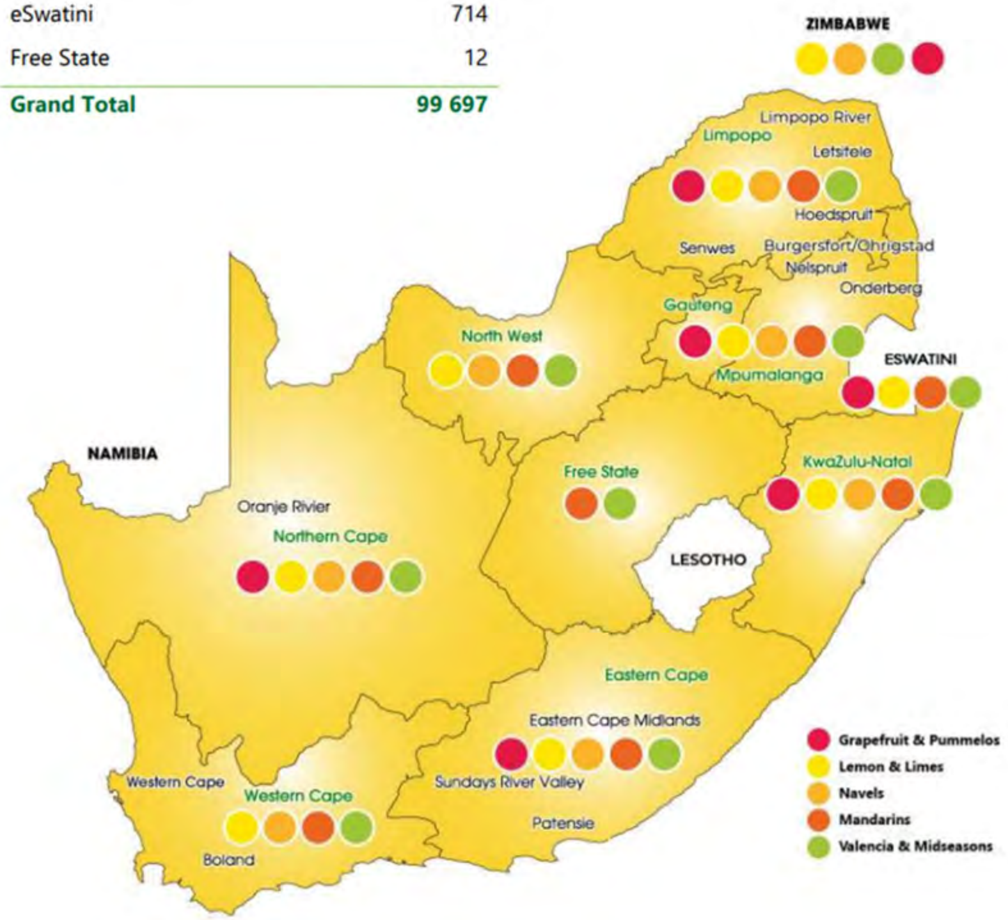


Figure 1.2 Citrus growing regions in southern Africa and citrus types grown per region (Citrus Growers' Association, Key industry Statistics, 2023)

1.2 Citrus Pests

1.2.1 Taxonomy

Citrus pests have been widely studied around the world. These pests are able to establish populations in citrus orchards and are associated with the flowers, fruit, shoots and roots of citrus trees (Tennant, 2009). Some of these pests have gained status as “major” pests of citrus as a result of the suitability of citrus as a host (Table 1.1).

The citrus industry in South Africa currently has close to 100 insect pests, however, there are only a few different insect species that are classified as economically important pests (Moore, 2021; Grout, 2012; Manrakhan, 2023; Moore et al., 2022;). These pests are: the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), Natal fruit fly, *Ceratitis rosa* Karsch (Diptera: Tephritidae, false codling moth (FCM), *Thaumatotibia leucotreta* Meyrick (1912) (Lepidoptera: Tortricidae), red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diapsidae), citrus psylla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), citrus thrips, *Scirtothrips aurantii* Faure (Thysanoptera: Thripidae) and citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae). Although there are six other mealybug species that occur on citrus in South Africa, the oleander mealybug, *Paracoccus burnerae* (Brain) (Hemiptera: Pseudococcidae) and long-tailed mealybug, *Pseudococcus longispinus* Targioni-Tozzetti (Hemiptera: Pseudococcidae) are of greater economic importance than the others (Grout et al., 2015; Moore et al., 2022).

Table 1.1 Key pests on citrus in southern Africa and around the World (Urbaneja et. al, 2020).

Asia	**Mediterranean Basin**	**North America**	**South America**	**Southern Africa**
Panonychus citri	*Ceratitis capitata*	*Diaphorina citri*	*Diaphorina citri*	*Ceratitis capitata*
Diaphorina citri	*Aonidiella aurantii*	*Phyllocnistis citrella*	*Brivipalpus spp.*	*Ceratitus rosa*
Phyllocnistis citrella	*Pezothrips kellyanus*	*Phyllocoptruta oleivora*	*Phyllocoptruta oleivora*	*Thaumatotibia leucotreta*
Aphis citricola	*Delottococcus aberiae*	*Diaprepes abbreviatus*	*Polyphagotarsonum latus*	*Bactrocera dorsalis*
Toxoptera citricidus	*Aphis spiraecola*	*Aonidiella aurantii*	*Gymnandrosoma aurantiana*	*Trioza erytreae*
Toxoptera aurantii	*Tetranychus urticae*	*Coccus pseudomagnoliarum*	*Ceratitis capitata*	*Aonidiella aurantii*
Aphis gossypii	*Prays citri*	*Scirtothrips aurantii*	*Anastrepha fraturculus*	*Scirtothrips aurantii*
Papilio xuthus		*Eutetranychus banksi*	*Phyllocnistis citrella*	*Planococcus citri*
Papilio polytes		*Panonychus citri*	*Cicadellinae (Sharpshooters)*	
Anoplophora chinensis				
Bactrocera dorsalis				

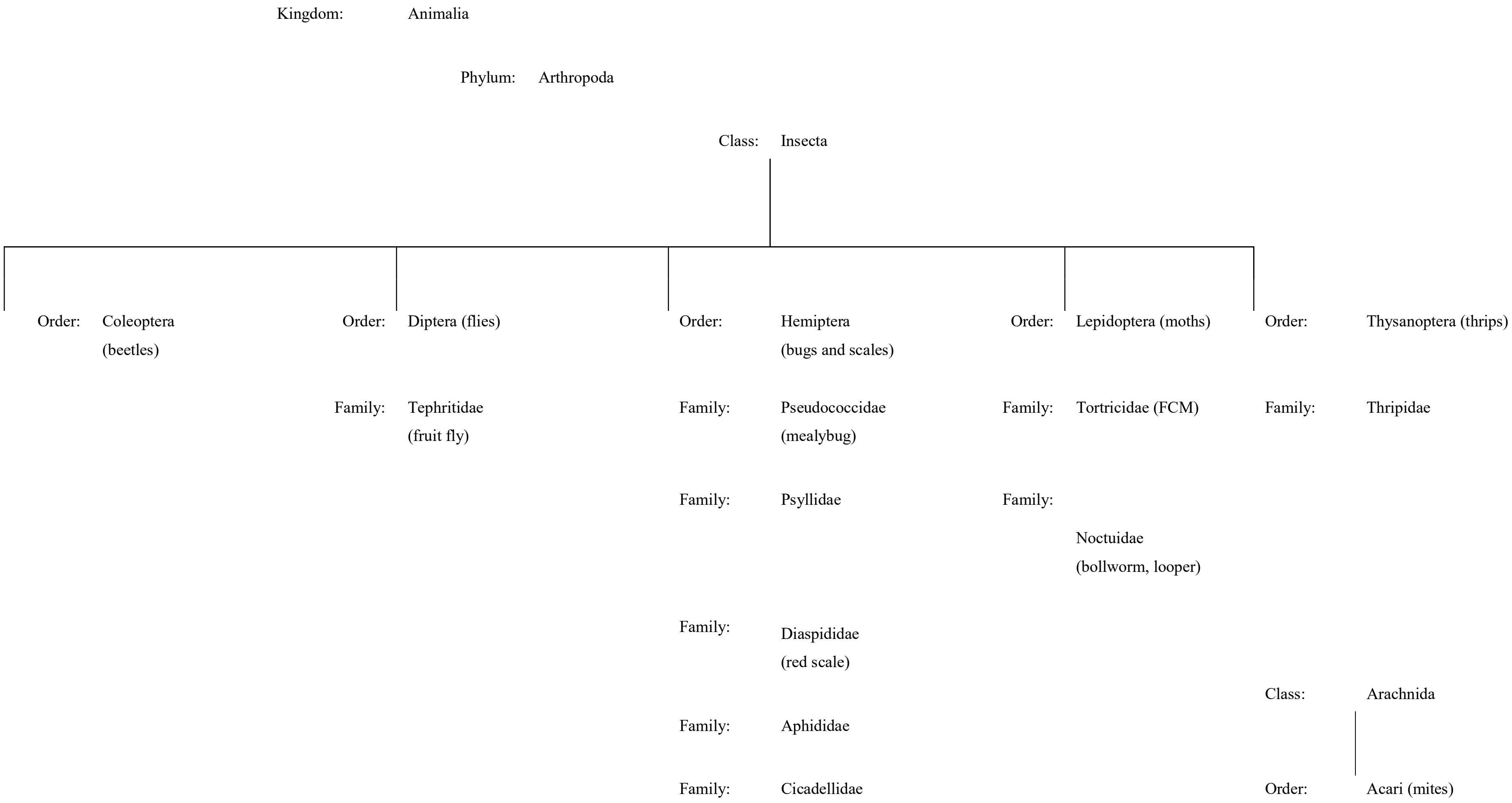


Figure 1.3 Insect Orders of commercial importance on citrus in southern Africa (Bedford et al., 1998).

Bedford et al. (1998) provides a well summarised compilation on the biology and control of citrus pests in South Africa and highlights the most important taxa relevant to the understanding of pest management in citrus orchards in which the major pests of importance have been described. They belong to the Animal Kingdom and are classified within the Phylum, Arthropoda. The pests can be further classified into two classes, namely Insecta and Arachnida, with the vast majority of pests belonging to the Class, Insecta (Figure 1.3).

1.2.2 Economic Impact

A variety of insect pests have established as major pests on citrus with the same species presenting as problematic in different parts of the world (Tennant, 2009). The citrus industry globally is in a dynamic phase of change as a result of diverse consumer demands, consumer health awareness and the drive towards development of innovative technologies and new product characteristics worldwide. According to Ndou et al. (2011), sanitary and phytosanitary requirements in the international markets pose ever increasing challenges for the citrus industry. A sanitary measure is applied to protect animal or human health or life within a country from risks arising from additives, contaminants, toxins or disease-causing organisms in their food. Phytosanitary measures are applied to protect animal or plant life from pests, diseases or disease-causing organisms and to prevent other damage to a country from the entry, establishment and spread of pests (WTO). International regulations further stipulate in the Sanitary and Phytosanitary (SPS) agreement, that every country has a right to apply the appropriate level of protection, which in some cases may be a level of zero risk. Quarantine pests must be identified and a risk assessment carried out to justify the level of protection. Phytosanitary agencies are bound by the SPS agreement to ensure that despite best efforts to protect the country from harmful invasive pests, the requirements stipulated may, in turn, cause the least possible disruption to trade.

However, phytosanitary requirements have become more onerous and complex. These regulatory requirements can be divided into several categories, namely, those that impact production, price and marketing and the impact on trade, food security, human health and the environment (Evans et al., 2002). Millions of Rands in revenue are generated annually from the export of citrus produce. Citrus fruits are exported mostly to Europe, U.S.A., South Korea and China. These countries have strict biosecurity laws in place, which prohibit the import of specific problem insects and in the context of this study, this is one of the most important regulations, which is also a condition for market access. Maintaining market access is a high priority for the citrus industry so the impact phytosanitary requirements have on marketing and trade is very important. Citrus pest diversity is increasing and pest control methods have been intensively studied in order to adapt, resulting in continuously changing management models (Urbaneja et al., 2020).

An increase in attainable yields is often associated with an increased vulnerability to damage inflicted by pests (Jaouad et. al., 2020). The use of pesticides has allowed growers to increase crop productivity without having to endure higher losses caused by a corresponding increase in pest pressure. Citrus pests can be separated into three important categories, namely production pests, cosmetic pests and phytosanitary pests (Urbaneja et al., 2020). Production pests would be more important than cosmetic pests for fruit destined for local consumption. In contrast, cosmetic pests are more important for fruit being exported, requiring more intensive control measures where costs are offset by higher production yields and number of first-class cartons packed. In addition, some of the pests of minor importance for local production may be of phytosanitary concern for destination countries with a low or zero tolerance for the same pests. FCM is a good example of an important pest in South Africa, due to its phytosanitary status (Moore, 2021). Unfortunately, the status of mealybug has recently escalated and the industry now has the responsibility to improve control measures to ensure continued market access.

1.3 Integrated Pest Management (IPM) and Biocontrol

1.3.1 IPM

The concept of integrated pest management includes a threshold concept for the application of pest control measures and reduction in the frequency of pesticides to an economically and ecologically acceptable level. The key to successful citrus production relies on the effectiveness of pest management strategies (Jaouad et al., 2020). The challenge is that not all management strategies are compatible, i.e. the use of non-specific insecticides together with biologically based technologies, such as the releases of predators and parasitoids to target specific pests. Therefore, much investigative research has been done on various aspects of pest management, including novel approaches for the management of insect pests (Franco et al., 2009), use and impact of active chemical ingredients on insect pests (Mamoon-ur-Rasheed et al., 2014), natural enemies of insect pests (Wakgari et al., 2003), approaches to biological control (Gurr et al., 2000), and non-target effects of pesticides on biological control agents (van Driesche et al., 2016; Mgocheki et al., 2009; Hattingh et al., 2000), in order to find practical solutions for improving pest control.

Most of the available technologies have roots in pest management and with higher efficacies required, the integration of several tactics is needed, using compatible tools, often simultaneously, to target different life stages of pests (Suckling et al., 2013). By far the focus area with the biggest scope of research is IPM, which is a vital component of citrus production. It entails the complimentary integration of various pest control measures into a system that strives to balance maximisation of returns, long term sustainability and minimise environmental impact. The emphasis lies in the maximisation of the potential of the biocontrol component of the control strategy with supplementary use of chemical intervention only when necessary. IPM programmes are designed to keep plants healthy and economically productive (Lee, 2009). An important aspect of IPM is biological control, which is simply described as the use of a population of one organism to reduce the population of another organism (van Lenteren et al., 2018). Biological control has been a valuable tactic in pest management programmes around the world for many years, but has undergone a resurgence in recent decades that parallels the development of IPM as an accepted practice for pest management. The potential for using "augmentative" biocontrol to supress arthropod pests has been recognised for many years (Collier et al., 2004). However, one must not overlook the importance of natural biocontrol, which already exists in ecosystems worldwide without human intervention (van Lenteren et al., 2018).

Pest population monitoring is a cornerstone of many IPM programmes. For example, in Greece, red scale *Aonidiella aurantii* does not have a good biocontrol complex and it is necessary to control chemically on occasion. The strategy is to apply spot-treatments if a less target selective pesticide is used (Karamaouna et al., 2010), which considers all the natural enemies present, thereby conserving them in the untreated areas. Monitoring for hot-spots and careful selection of pesticide use practices can lead to more successful implementation of IPM. Monitoring natural enemy populations or their effect on pests can be used to identify economic thresholds to more accurately determine the need for treatment and should be used as far as possible (Jacas et al., 2010).

1.3.2 Classical biological control

Despite the long history of utilizing natural enemies, it wasn't until 1919 that the term biological control was apparently used for the first time by the late Harry Smith of the University of California. There has been debate regarding the scope and definition of biological control brought about by technological advances in the tools available for pest management. Biocontrol is described as the "study, importation, augmentation, and conservation of beneficial organisms to regulate population densities of other organisms". Biological control with predators and parasitoids can be organized under three general approaches: importation, augmentation and conservation of natural enemies (Orr, 2009). Compared to chemical control methods, biocontrol has the key benefit of becoming permanent in nature. The co-evolution of the biocontrol agent and the target pest is ongoing so the chance of resistance developing is unlikely (De Clerq et al., 2011).

Importation of beneficial insects for biological control of a particular pest species is what is often referred to as "classical biological control". Classical biological control requires that a suitable biological control agent, that is not native to the area where pest control of a specific pest is needed, is brought in. Thus, classical biological control requires the introduction of an 'exotic' organism (Eilenberg et al., 2001). This approach, although sometimes very effective, has been strongly, yet ineptly criticized for its potential non-target impacts. Some recent studies have reported that the percentage of agents that establish is between 20 - 55%, and the percentage of introductions contributing to success falls within the range 5 ± 15% (Gurr et al., 2000). However, in broad terms, in excess of 5000 introductions of more than 2000 species have taken place in the past 100 years, resulting in overall low levels of establishment and only a few reports of negative, non-target effects (De Clerq et al., 2011).

1.3.3 Augmentative biological control

Augmentative biological control includes activities in which natural enemy populations are increased through mass culture, periodic release and colonization, for suppression of native or non-native pests. Augmentative biocontrol can be subdivided into inundative and inoculative augmentation. Inundative augmentation describes the release of biocontrol agents in large numbers which will themselves interact immediately with the pest, kill or reduce pests to economically acceptable levels before dispersing. Inoculative augmentation describes the release of biocontrol agents with the expectation that they will establish, multiply and that the progeny will control the pest for an extended period of time (Eilenberg et al., 2001).

Augmentative biological control has been criticized on scientific foundation, efficacy, and cost effectiveness of its use in pest management (Collier et al., 2004). Several authors have called for development of predictive models to assist in implementation of augmentation biological control. In a modelling study conducted by (Wajnberg et al., 2018) the results indicated that some life history traits affect the overall outcome of the augmentative biological programme more than others. Biocontrol agents with a high fecundity rate were suggested to be more efficient from an economic point of view. The trade-off between reproduction rate and longevity reported to favour the quick production of eggs for effective augmentative biocontrol.

The relationships between parasitoids and their hosts in nature is remarkable considering the diversity of interactions that take place. Gregarious parasitoids follow a strategy of depositing multiple eggs into their host which usually results in a higher number of F1 offspring compared to the solitary parasitoids. The gregarious parasitoids are thus more tolerant to interspecific interactions. Fertility is based on body size which results in manipulation of the future fertiliy of offspring that depends on the pressure of external factors. (Samkova et al., 2022). Monitoring for and identifying various interactions is important to understanding augmentative biocontrol.

However great strides have been made recently with reported examples of the successful implementation of augmentation (Gurr et al., 2000).

1.3.4 Conservation biological control

Probably the most common pest management activity that negatively impacts beneficial organisms in agroecosystems is pesticide application. As a result, modifications of pesticide use practices are the most commonly implemented form of conservation biological control and have long been considered an important component of IPM programmes (Orr, 2009).

Conservation biological control seeks to understand human influences on resident natural enemies in a system, then manipulate those influences to enhance the ability of natural enemies to suppress pests. Conservation biological control is considered to be an environmental modification to protect and enhance natural enemies (Orr, 2009). These conservation practices include limited and selective use of pesticides but also active processes such as providing refuges adjacent to crops or within crops, facilitating transfer of modification of pesticide use practices to manipulation of beneficial insect habitat within an agroecosystem (Eilenberg et al., 2001). Decisions regarding pesticide use for insect pests in IPM programmes are typically based on monitoring of pest populations to determine if they have reached economic threshold levels (Orr, 2009), although some work has been done to incorporate natural enemy sampling into decisions on pesticide use.

1.3.5 Efficacy of biological control

The potential for using biocontrol in agriculture to supress arthropod pests has been recognised for many years. The main drive behind developing this technology has been to reduce the reliance on broad-spectrum pesticides. Often the results of application of biocontrol as part of an IPM programme fall short of the expectations of the user, which can be attributed to the following ecological limitations (Collier et al., 2004): unfavourable environment for the beneficial insect; dispersal of the beneficial insect away from the release site; predation; refuge for the pest; mutual interference; quality of the beneficial insects; incompatibility between the host and beneficial insect; immigration of pests; timing of releases and application method.

As a method for pest management today, biological control is environmentally safe and economically profitable (van Lenteren, 2012). Prospects for the future of effective biocontrol is looking very promising. More studies are underway which will lead to more novel approaches to habitat management and assist with improvement in efficacy of biocontrol agents (Kumar et al., 2019).

1.3.6 Pesticide use

The pesticides used in the early days of citriculture were relatively safe to man because of their very short residual action. Some of these insecticides included resin wash, lime sulphur, sulphur dust, nicotine sulphate, miscible petroleum oils, oil emulsions, cryolite and tartar emetic with sugar. The use of oil sprays was described as detrimental to the yield and quality of citrus at the time (Bedford et al., 1998). Some of the other treatments were a health risk due to toxicity to humans as well as the environment, including parathion, belonging to the organophosphates chemical group. With a single treatment per season, it solved many of the pest problems, initially being introduced as a control for red scale, *Aonidiella aurantii* (Maskel). However, through excessive use it soon caused repercussions of other citrus pests, and particularly citrus mealybug, *Planococcus citri*, resulting in up to five dedicated mealybug sprays required in a season.

Thrips, *Scirtothrips aurantii* Faure, also became difficult to control and saw the introduction of new chemicals, namely temephos (Abate) and dimethoate, with temephos causing even more outbreaks of mealybug (Bedford, 1971). Soon insects became resistant to the pesticides. Red scale resistance to parathion was one of the earliest cases of insect resistance in South Africa (Bedford et al., 1998). The need for effective pesticides led to the development of synthetic pyrethroids for thrips and insect growth regulators for red scale control. A preference for more chemical and often aerially applied sprays ensued. This too resulted in major pest repercussions. Hattingh et al. (1995) discovered that triflumuron (Alsystin), used in the control of false codling moth, significantly reduced the progeny of an important citrus mealybug predator, *Cryptolaemus montrouzieri* (Mulsant). The cycle of spraying insecticides was not sustainable.

A window of opportunity for inclusion of an IPM approach was opened, leading to the inclusion of narrow range mineral oils, which in many cases is still being used today. Various chemical control products remain an integral part of citrus pest control programmes and further became necessary as part of phytosanitory regulations imposed on South African citrus exports. These remain the source of pest repercussions in citrus orchards (Bedford et al., 1998) and development of IPM strategies and novel IPM-compatible products is ongoing, but limited. It has been known for many years that an integrated approach is vitally important for the control of citrus pests otherwise repercussions from other pests, particularly scale insects such as red scale and mealybug, will not be controlled sustainably.

1.4 Biology and control of *Planococcus citri*

1.4.1 Background

Mealybugs are economically important pests with a wide host range, including many crops and ornamentals. Populations of mealybug have the ability to reach economically damaging levels in a short period, when conditions are favourable (Bedford et al., 1998). There are many factors contributing to the pest status of mealybug on citrus, including a high reproduction rate (Mamoon-ur-Rasheed et al., 2014), cryptic behaviour, a clumped spatial distribution pattern and a waxy body covering, making it extremely difficult to control with the use of pesticides (Franco et al., 2009). Various mealybug species have attained conventional pest status on citrus, namely citrus mealybug, *Planococcus citri*, oleander mealybug*, Paracoccus burnerae* (Brain), *Pseudococcus longispinus* (Targioni-Tozetti) and the Karoo thorn mealybug, *Nipaecoccus viridis* (Newstead). Recently *Delottococcus aberiae* (De lotto) has become more abundant in some orchards in Limpopo Province, South Africa and has been recorded from orchards in Eastern Spain. The striped mealybug, *Ferrisia virgata* (Cockerell) and the citrophilus mealybug, *Pseudococcus calceolariae* (Maskell) are rarely encountered and are not of economic importance (Moore et al., 2022).

Mealybug overwinter in cracks and crevices in the trunk and main branches of the tree. In the spring and early summer, mealybug nymphs move outwards to the parts of the tree where more light penetrates the canopy and settle on new growth and fruitlets (Moore et al., 2022). Adult females of the different species are similar in size at approximately 4 mm in length, with oleander mealybug and long-tailed mealybug slightly smaller at 3 mm long. Eggs are laid in an ovisac, except for long-tailed mealybug which is viviparous and produces live crawlers. Of the citrus infesting species, citrus mealybug has the shortest life cycle from egg to egg in approximately 28 days in summer. The long-tailed mealybug, oleander mealybug and karoo thorn mealybug have a life cycles of 37, 45 and 68 days respectively (Bedford et al., 1998).

Mandarins, navel oranges and grapefruit are considered more susceptible to mealybug attack because they are early ripening (March to May), which does not allow natural enemies opportunity to bring mealybug populations under control before harvest (Bedford et al., 1998). Colonies will gather around the fruit calyx and between touching fruit and can also be found inside navel openings of navel fruit. However, karoo thorn mealybug aggregate on the stem above the calyx. Mealybug can cause direct damage to the fruit, causing yellowing and fruit drop in young fruit or malformation of the fruit rind, particularly caused by *N. viridis* and *D. aberiae* feeding around the calyx. Sooty mould developing on the honeydew produced by mealybug is often not removed in the packhouse. The dark markings on the fruit are unsightly and result in fruit being downgraded. Citrus is an important commodity worldwide and some countries have listed certain mealybug species as a phytosanitary concern and therefore the presence of live mealybug at the time of harvest has serious implications for fruit destined for these sensitive markets.

In general, mealybugs have a very efficient biocontrol complex, consisting of hymenopteran parasitoids, predatory fly larvae, lacewings and ladybird beetles. These biocontrol agents have the potential to control severe infestations of mealybug if not disrupted with pesticide sprays (Moore et al., 2022).

1.4.2 Taxonomy

Class: Insecta
    Order: Hemiptera
        Family: Pseudococcidae
            Genus: *Planococcus*
                Species: *citri*

1.4.3 Description

Planococcus citri females are soft bodied pseudococcids which have an elongated oval shape, a flattened body 3 mm long and 1.5 mm in width (Figure 1.4). There are 18 pairs of short, waxy filaments around the margin of the body. The last pair are called anal filaments and are only fractionally longer in citrus mealybug, unlike other mealybug species (Asiedu et al., 2014). The body is covered by a white, waxy layer forming distinguished transverse segments across the marginal filaments. Characteristic of citrus mealybug is a faint line, which runs dorsally along the length of body, due to a thinner wax layer which allows the body colour (yellowish brown) to become visible (Moore et al., 2022). A cottony, white egg-sac can be visible behind mature females containing up to 400 yellow coloured eggs. Tiny yellow crawlers emerge from eggs and disperse into the canopy of the tree to feed.

The male is a tiny gnat-like insect, approximately 1mm in length. They are fragile, yellowish-brown in colour with hyaline wings and two long, white anal filaments (Bedford et al., 1998).

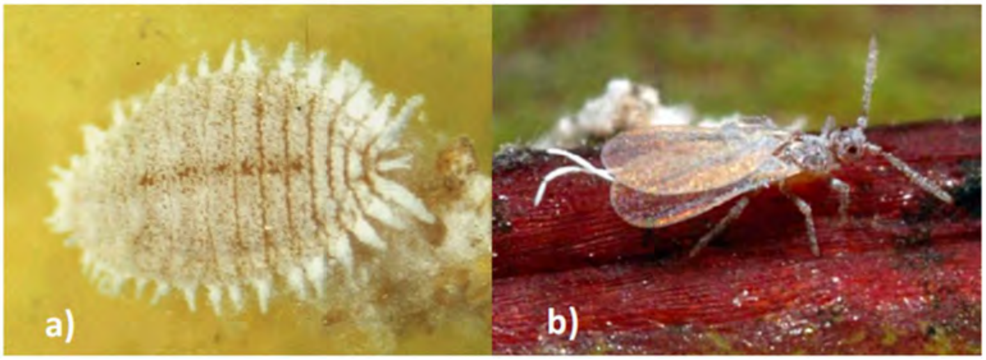


Figure 1.4 Morphology of *P. citri* female (a) and the male (b).
(Photos by Peter Stephen (a) and Cabi digital library (b).)

1.4.4 Distribution and host range

Citrus mealybug has a wide host range including many different crops (Özgökçe et al., 2018) including citrus, grapevine, ornamental plants, cocoa, bananas, tobacco, coffee, passion fruit, pineapples, figs, taro, date palms, pomegranates, potatoes and greenhouse plants. It occurs in many countries worldwide (Figure 1.5) and is economically damaging in citrus-pruducing countries (Bedford et al., 1998).

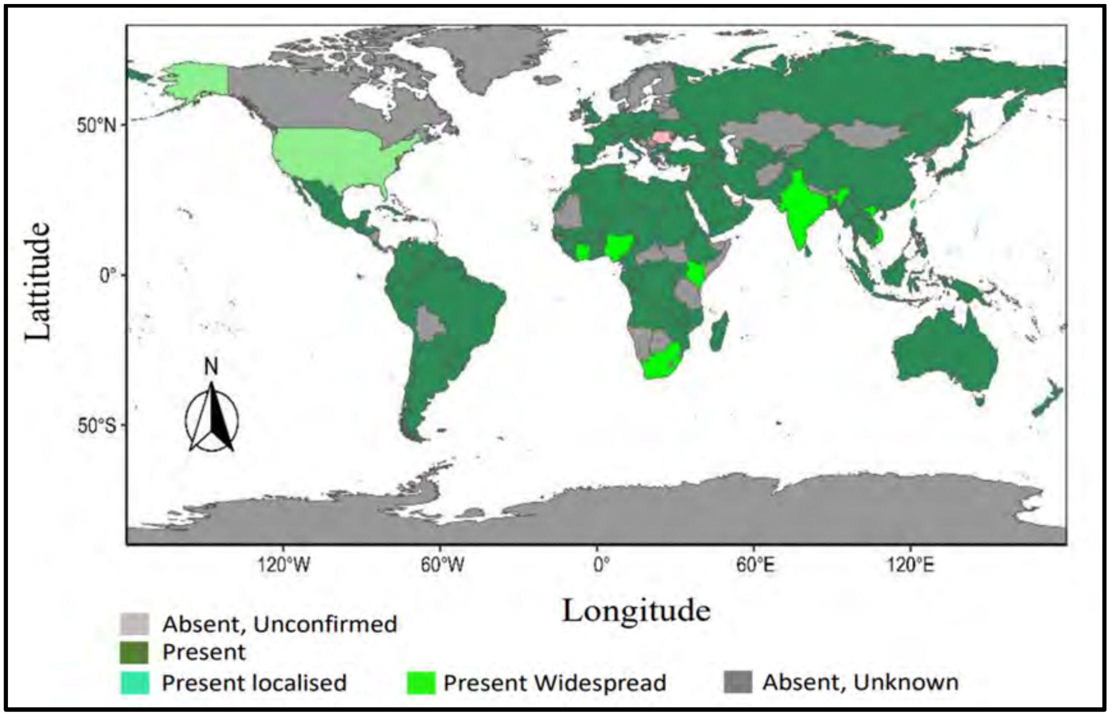


Figure 1.5 Worldwide distribution of *P. citri* (www.cabidigitallibrary.org).

1.4.5 Life-History parameters

Female *P. citri* can produce 300 to 587 eggs over a two-week period (Bedford et al., 1998). Incubation of the eggs takes approximately 6 to 11 days for females and males generally longer, between 11 and 12 days. The duration of the three female instars is 7 to 12 days, 6 to 11 days and 9 to 12 days, respectively (Asiedu et al., 2014). The duration of the life cycle of female *P. citri*, from egg to egg, at a constant temperature of 22°C varies between 37 to 44 days (Bedford et al., 1998). Longevity of adult females is between 32 and 38 days. There are two male instars and a prepupal and pupal stage with a combined duration of 21 to 32 days. Male longevity is 1.5 to 2.5 days (Asiedu et al., 2014).

1.4.6 Feeding habits, spatial and temporal distribution

On citrus, *P. citri* populations overwinter in the tree by hiding away in curled leaves, cracks in the bark or inside large pruning wounds. In spring, emerging crawlers will seek out new shoots, leaves and small fruit to feed on. Because of the cryptic behaviour of mealybug, crawlers prefer to feed between the cheeks of touching fruit, inside open navel ends and particularly under the fruit calyx (Bedford et al., 1998). It is characteristic of *P. citri* to form colonies on growing fruit during Summer, but can form these colonies anywhere on the plant, including girdle marks on the trunk or scaffold branches.

1.4.7 Chemical control

Pesticides are still the most common control tactic used for mealybug pests. However, many of the pesticide applications are largely ineffective. Partly because of the waxy protective layer, clumped spatial distribution and characteristic behaviour of mealybug hiding in cracks and crevices or under the fruit calyx (Franco et al., 2009). The level of control of pesticide sprays for mealybug is greatly influenced by application efficacy (Grout, 2022), which is hindered in grapefruit orchards, known for a dense tree canopy, as well as in navels that are open ended, providing a refuge for the mealybug. Repeated application of thripicides or other broad-spectrum insecticides are harmful to natural enemies of mealybug, resulting in high mortality rates, or otherwise may cause various non-lethal effects, which will negatively impact their contribution to control (Ndakidemi et al., 2016).

A mealybug population is most susceptible to chemical intervention in early spring when the first crawler movement takes place. A low population, susceptibility of immature life stages and the timing before the closure of calyxes increase the likelihood of successful control during this period (Bedford et al., 1998).

Both soil application and foliage cover sprays are commonly applied for mealybug control. Contact and systemic chemical actives are applied (Bedford et al., 1998; Franco et al., 2009). Historically the use of broad-spectrum insecticides, such as organophosphates and to a lesser extent, carbamates, were effectively used against mealybug infesting the tree canopy (Franco et al., 2009). The insect growth regulator, buprofezin, a chitin synthesis inhibitor, is an effective alternative with low human toxicity. Very few new molecules have been successfully commercialised for mealybug control and there is greater risk of the effective, registered options becoming redundant due to residue limits employed due to consumer preferences in export markets.

1.4.8 Biological control

Augmentative releases of biocontrol agents, mass reared in insectaries, have become a valuable tool in the implementation of control strategies. *Coccidoxenoides perminutus* is a tiny wasp, originating in Hawaii, with a preference for the first three instars of citrus mealybug (Mgocheki, 2008). *Anagyrus vladimiri* [previously *Anagyrus* sp nr *pseudococci*] is a solitary koinobiont endoparasitoid, which attacks the mature, third instar mealybug (Bedford et al., 1998; Wakgari et al., 2003). Studies have showed that *C. perminutus* can be effective at releases of 100 000 wasps per hectare, however, numbers released commercially appear to be lower than this (Moore et al., 2022). Augmentations of *A. vladimiri* were shown to be effective at rates between 2500 and 5000 individuals per hectare (Moore et al., 2014). Two species of ladybird beetles, *Cryptolaemus montrouzieri* and *Nephus* spp. are also present in citrus growing regions in southern Africa and both fed preferentially on mealybug. *Cryptolaemus montrouzieri*, is released at rates of 1000 to 2000 beetles per hectare, generally later in the season, when mealybug densities are high.

It is important to mention that there are a few major challenges for the implementation of biocontrol. There is a lack of understanding and familiarisation with biocontrol methods, the integration of biocontrol with the use of pesticides and commercialisation and availability of biocontrol products can be a limiting factor (Colmenarez et al., 2018)

1.4.9 Economic importance

Around the world, the financial implications of mealybug damage can be astronomical, for example, in the United States of America the estimated losses and cost of managing the pink hibiscis mealybug amounted to 700 million dollars (Subramanian et al., 2021).

All instars of female *P. citri* damage the host by sucking out plant sap and releasing droplets of honeydew as excrement, on which sooty mould can grow. Other than cosmetic blemishes on fruit from sooty mould, the honeydew can attract other damaging pests, such as carob moth, *Ectomyelois ceratoniae* (Zeller). Grapefruit and early maturing mandarins and navels do not allow much time for natural enemies to establish before harvest and therefore mealybug control may vary from season to season. It may also be important to note the challenge of mealybug control on lemons, due to multiple inflorescences. Pesticide residue restrictions on the early set often prevents application of necessary chemical controls on later sets. Impact on navel oranges, mandarins and grapefruit tends to be more severe. Direct damage caused by high populations includes young fruit turning yellow and dropping from the tree, malformed leaves and hyper-pigmentation on the fruit rind in the form of dark red pitting or blemishes. In some instances, dents and lumpy shoulders can form around the calyx where mealybug has been feeding (Moore et al., 2021).

Post-harvest removal of sooty mould on mandarins is challenging, improper colouring of grapefruit where mealybug feed on touching fruit and the Phytosanitary implications of live mealybug specimens on grapefruit and navels have escalated to those of serious economic concern (Moore et al., 2021).

1.5 Biology and ecology of *Anagyrus vladimiri*

1.5.1 Background

The genus *Anagyrus* (Howard) comprises over 350 described species of primary endoparasitoids of various mealybug hosts and a number of species have been reared in insectaries for releases within classical biocontrol programmes around the world (Andreason et al., 2019). *Anagyrus pseudococci* (Girault) (Hymenoptera: Encyrtidae) is a solitary koinobiont endoparasitoid of mealybug (Hemiptera: Pseudococcidae) and one of the most common parasitoids of the citrus mealybug and of the vine mealybug, *Planococcus ficus* (Signoret) (Suma et al., 2012a).

The term, "*Anagyrus pseudococci* complex" has been described, based on morphological similarity to *A. pseudococci.* In the Imperial Valley in California, where an unknown population from Nayarit region was studied alongside species that were informally classified namely, *A. pseudococci* (Girault), *A.* sp nr *pseudococci, A. kamali*, *A. dactylopii* (Howard)and *A. kivuensis* (Compere). Morphological and morphometric analysis, supported by mitochondrial and nuclear DNA analysis supported the definition of a new species. Phylogenetic analysis supported the monophyly of the *A. pseudococci* complex (Andreason et al., 2019). Only a colouration difference of the first funicular segment of the female antennae separates the two, morphologically. However, the life table parameters, when compared between *A. pseudococci* and *Anagyrus* sp nr *pseudococci* are very similar (Suma et al., 2012b). The new species was named *A. vladimiri* (Triapitsyn) after Vladimir Alexander Trjapitzin (Andreason et al., 2019).

1.5.2 Taxonomy

Kingdom: Animalia
  Phylum: Arthropoda
    Subphylum: Hexapoda
      Class: Insecta
        Order: Hymenoptera
          Family: Encyrtidae
            Genus: *Anagyrus*
              Species: *vladimiri*

1.5.3 Description

Female *A. vladimiri* are brownish-orange in colour with characteristically white antennae. The first antennal segment completely black in colour. Body length is approximately 1.1 to 1.75 mm in length. Wings are clear, hyaline with a marginal vein (Andreason et. al, 2019; Wohlfharter et al., 2014).

Figure 1.6 Dorsal view if *Anagyrus vladimiri* female (Andreason et al., 2019).

Males are smaller than females with a body length of approximately 0.76 mm to 0.92mm. Body colour is mostly black with clear wings and long filamentous antennae.

Figure 1.7 Lateral view of *Anagyrus vladimiri* male (Andreason et al., 2019).

1.5.4 Distribution and host range

Confirmed reports of *A. vladimiri* have been recorded in Israel, Italy, Russia, Spain, Turkmenistan, USA and Tunisia (Andreason et al., 2019). Other reports of *Anagyrus* sp. nr *pseudococci* in Brazil, Greece, Portugal and South Africa are proposed to be the same species (Bugila et al., 2015; Karamaouna, et al., 2011; Mgocheki et al., 2009, Triapitsyn et al., 2007). *Anagyrus vladimiri* is described as having a close evolutionary relationship with *P. ficus* but has been expanding its host range to include Planococcus, Pseudococcus and Phenacoccus genera (Bugila et al., 2015). Host suitability has been determined for *Pseudococcus calceloleriae*, *Pseudococcus viburni*, *Phenococcus peruvianis* (Bugila et al., 2015) and *Pseudococcus comstocki* (Ricciardi et al., 2021). Although not many studies have been carried out on the host range of *A. vladimiri*, early indications are that it has a much wider host range than other *Anagyrus* species.

1.5.5 Life-History parameters

The reproductive capacity of *A. vladimiri* is approximately 30 offspring per female wasps at 25 °C. The sex ratio was reported as 1:1 and can be expected to be slightly male bias (Suma et al., 2012b). The sex ratio depends on the size of the mealybug host. Early 3rd instars will produce predominantly male offspring, while mature adults will be female biased (Islam et al., 1997). Development time does not differ according to gender and takes 16.9 days from egg-laying to eclosion of adults (Suma et al., 2012b; Daane et al., 2004). The emergence rate for *A. vladimiri* was reported above 65% with *P. citri* as hosts. However, a direct correlation between emergence rate and size of the tibia of wasp females was established, which indicates the size of female *A. vladimiri* plays a role in optimal emergence (Bugila et al., 2015). The longevity of *A. vladimiri* has been estimated to be 21 days when exposed to citrus mealybug. Females live longer than males (Güleç et al., 2007), particularly when provided with a suitable sugar source for feeding, like honeydew produced by the mealybug host (Suma et al., 2012b).

1.5.6 Biology and host defences

Anagyrus species develop through four life stages: egg, larva, pupa, and adult. In general, host selection behaviour of parasitoid Hymenoptera includes habitat location, host location, host recognition and host acceptance, eventually resulting in oviposition (Bugila et al., 2015).

Anagyrus vladimiri are good at searching for a suitable host. Studies have indicated the females spend an average of 12 minutes searching and 5 minutes assessing the hosts suitability for egg deposition through antennation. For a successful host candidate, the female *A. vladimiri* will turn its body around sharply to deposit an egg into the body of the mealybug with its ovipositor. The egg will develop into a pupa within the "mummy" or outer layer of the mealybugs body. Although *A. vladimiri* is known as a solitary endoparasitoid, it often deposits more than one egg into a host, particularly in response to the hosts defence mechanisms (Suma et al., 2012a).

Mealybugs may respond to the attack of parasitoids by displaying defence behaviours that eventually may allow them escaping parasitism. Three types of defence behaviour, namely walking away, reflex bleeding and abdominal flipping, may be activated by a mealybug when attacked by a parasitoid. These three types of defensive behaviours can be further classified as: 1) evasive behaviours, in the case of walking away; or 2) aggressive behaviours, in the case of reflex bleeding and abdominal flipping (Bugila, 2014). Mealybugs also have an immune response to attack by parasitoids, called encapsulation. Encapsulation is an immune defence mechanism of insect hosts, triggered by eggs and larvae of parasitoids, which involves the production by haemocytes of a multi-layered capsule around the invader, usually associated with melanisation. The level of effective encapsulation of *P. citri* against *A. vladimiri* can be as high as 60% (Suma et al., 2012a).

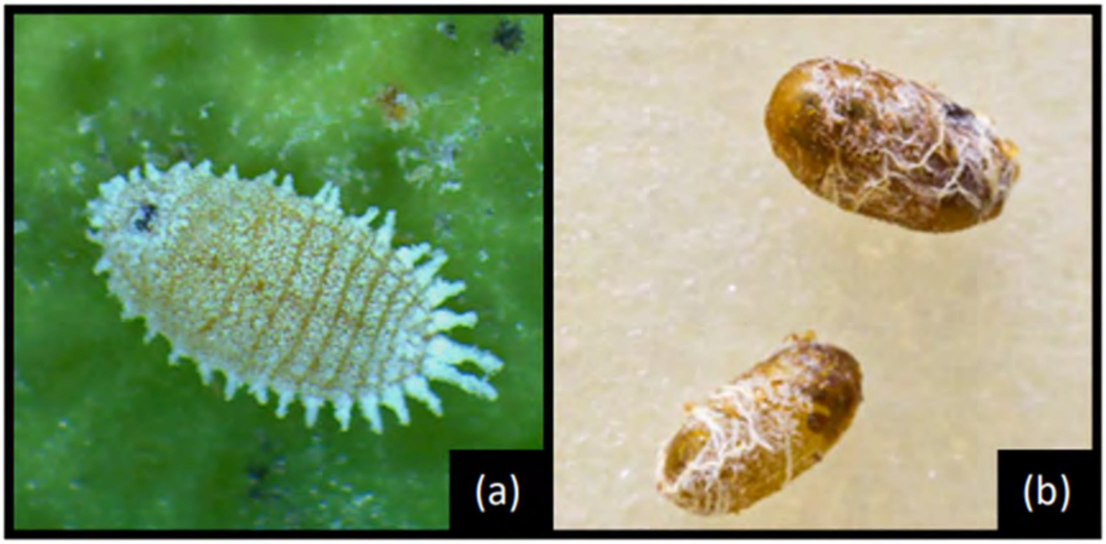


Figure 1.8 Egg and pupa stages of *Anagyrus vladimiri* showing the dorsal view of a parasitised citrus mealybug and the dark encapsulation of the egg (a) and fully developed pupae (b).

1.5.7 Quality control of beneficial insects for augmentation

The quality of natural enemies used in augmentation biocontrol is an important factor determining the success of pest control (Collier et al., 2004). Developments and collaboration between natural enemy producers and scientists in North America, Europe, Australia, New Zealand and South Africa have resulted in harmonisation of quality control guidelines. Historically, producers of beneficial insects have applied quality control in one or another form for more than 30 years. Today, stakeholders in the industry gather annually under the umbrella of the International Biocontrol Manufacturers Association (IBMA) and the Association of Natural Bio-control Producers (ANBP), which has helped facilitate the publishing of quality control guidelines written for 30 species of natural enemies that are most often used in commercial biological control (van Lenteren et al., 2018). Although this publication and other literature does not provide specific guidelines on *A. vladimiri* quality parameters, insectaries make use of quality guidelines for other species from the same insect order together with the most recent findings published in peer reviewed journals. For *A. vladimiri* the main quality parameters are longevity, reproductive capacity and sex ratio (Suma et al., 2012b).

1.6 Objectives of the study

The objectives of this thesis were to: determine non-target effects of various thripicides on *A. vladimiri* so that a compatible IPM programme can be followed to allow the establishment of the wasps for evaluation of the augmentative releases (Chapter 2); evaluate different timings of augmentative releases of *A. vladimiri* to determine the most effective strategy to control citrus mealybug (Chapter 3). This will answer a few important questions on the use of *A. vladimiri* as a biocontrol agent. For example, are early releases initiated as early as October more effective than later releases? Are releases initiated later than November still effective? And finally, investigate other ecological factors that impact augmentation of *A. vladimiri*, in particular, the impact of hyperparasites on the success of mealybug biocontrol, which is poorly understood (Chapter 4).

CHAPTER 2

NON-TARGET EFFECTS OF PESTICIDES ON *ANAGYRUS VLADIMIRI*

2.1 Introduction

Mealybugs are small, soft-bodied, sap-feeding Pseudococcids with their common name owing to the protective, waxy, layer produced on the outside of its body (Mamoon-ur-Rasheed et al., 2014). Mealybugs can have serious impacts on farms, economically, and on market access for the exporting country. Therefore, citrus growers turn to spraying a range of insecticides to keep threatening populations under control (Sakthivel et al., 2012). However, insecticides are not adequately effective on their own in controlling mealybug (Franco et al., 2009) and can undermine natural biocontrol, as well as the implemented IPM strategies employed by growers, which leads to repercussions such as increased numbers of mealybugs as well other pests (Hattingh et al., 2000).

Citrus thrips*, Scirtothrips aurantii,* is probably the most threatening of the other major pests on citrus. Growers apply insecticide sprays from early spring through to summer on young fruit to control thrips. These insecticides include a few broad-spectrum products originating from various chemical groups namely, carbamates, macrocyclic lactones, neonicotinoids, organophosphates, pyrethroids, phenyl pyrazoles and pyrroles. Because of the non-target effects of these thrips sprays, manufacturers of insecticides have considered the need for IPM in agriculture and have developed products from alternative chemical groups, with relatively lower impact on natural enemies namely: sulfoxamines (Closer ™240 SC), spinosyns (Delegate™250 WG), anthranilic diamide (Exirel® 100 SC) and ketoenoles (Tivoli 240 SC).

In the 1990s, the International Organisation for Biological Control (IOBC) developed a set of guidelines with testing procedures that can be used to screen pesticides for harmful effects on natural enemies used in classical biocontrol. In 2000, researchers from Outspan Citrus Centre used these procedures to develop a non-target evaluation system that tests five indicator species of importance to citrus in southern Africa (Hattingh et al., 2000). *Anagyrus vladimiri* was not included.

Consequently, little is known about non-target effects of insecticides on *A. vladimiri* in citrus. Therefore, the aim of this study was to investigate the harmful effect of a range of thripicides on *A. vladimiri* by following the same methodology developed by CRI to calculate the overall impact on this species. This will allow a direct comparison with the overall impact results on another important mealybug parasitoid, *C. perminutus*, which has previously been determined and which has already been implemented in the field. This will be a valuable guideline for growers when augmentative releases of *A. vladimiri* parasitoids are considered as part of IPM programmes in citrus. By simulating the application of pesticides in a citrus orchard prior to augmentative releases taking place, the withholding periods for augmentative releases of *A. vladimiri* can be determined.

2.2 Materials and methods

2.2.1 Setting up non-target bioassays

Chemical free nursery trees were obtained from Du Roi IPM in Letsitele in November 2020 and protected under white shade net until the bioassays commenced. The non-target assays were conducted in April, May and July of 2021. Parasitoids were obtained from the commercial insectary, Insectec, in Tzaneen just before each bioassay was conducted. Ten test cells (34 mm diameter polyethylene petri dish, 10 mm deep) were used per treatment group with each being mounted on treated citrus leaves (Figure 2.1).

Figure 2.1 Final assembly of test cells on top of a leaf containing an aged residue of insecticide and loaded with live *A. vladimiri* wasps.

The leaves were kept moist on top of a glass plate surfaced with wet filter paper and stabilised using aluminium clamps. A 6 mm diameter hole was made on one side of the test arena. A second 6 mm diameter hole was made on top of the test arena with a dual purpose, as an entrance hole for loading *A. vladimiri* wasps and was plugged with a short plastic tube (3mm internal diameter) wrapped with fine mesh material, preventing escape of the parasitoids and serving as an air escape vent. Test arenas were attached to the plenum via a longer plastic tube (150mm long), wrapped with fine mesh material at the end, attached to the test arena. A narrow streak of honey was provided as food on the inside wall of the arena. Approximately 12 adults were aspirated into a holding cell made from PVC tube and immediately driven out of the holding cell, through the top 6mm hole into the test arena, using a plunger made from 1.5mm PVC tubing. The hole was plugged before attaching the test arena to the pressurised plenum, so that air was passed through each cell at a rate of one volume exchange per minute (Figure 2.2). Test arenas were disconnected from the plenum after 24 hours of exposure and adult mortality was recorded for each of the 10 replicates (Grout et al., 2010).

Figure 2.2 A maximum of 10 replicate test cells connected to a plenum providing a constant air flow generated by an electric fan (attached to left wall of the plenum).

Mortality tests were conducted with progressively older residues of each of the pesticides on day 1, 7, 14, 28 and 42, or until the corrected mortality dropped below 25%. The following pesticides were tested within three trial-runs on different dates due to availability of space on the plenum. Agrimec® Gold (abamectin) Syngenta [Centurion, South Africa], Closer ™240 SC (sulfoxaflor) Corteva Agriscience [Centurion, South Africa], Exirel® 100 SC (cyantraniliprole) FMC Ag ZA [Centurion, South Africa], Imidan 50% WG (phosmet) Avima [Krugersdorp, South Africa] and Radiant® SC (spinetoram) Corteva Agriscience [Centurion, South Africa] were tested in April 2021. Dicarzol 500 SP (formetanate) Avima [Krugersdorp, South Africa], Klartan®240 EW (tau-fluvalinate) Adama South Africa [Johannesburg, South Africa], Savage 360 SC (chlorfenapyr), Tivoli 240 SC (spirotetramat) Villa Crop [Kempton Park, South Africa] and Tokuthion® 960 EC (prothiphos) Arysta Life Sciences [Durban, South Africa] were tested in May 2021. Maintain 200SP (acetamiprid Villa Crop [Kempton Park, South Africa]), Rossi 200 EC (fipronil) Villa Crop [Kempton Park, South Africa] and a 2X dose of Tokuthion® 960 EC, Arysta Life Sciences [Durban, South Africa] were tested in July 2021. For each of the trial runs, the mortalities of *A. vladimiri* from the various pesticides were compared to mortalities in the untreated control, which was treated with reverse-osmosis water. Methomex® 900 SP (methomyl) Adama South Africa [Johannesburg, South Africa] was included in the bioassays as a reference pesticide because of the very harmful impact rating it has on other parasitoids in citrus (Grout et al., 2011).

Treatment mortalities were corrected for the control mortalities (Abbott, 1925). The relevant persistence factor (P) for adult mortality was determined by the residue age at which the corrected adult mortality dropped below 25%. The factors used for the residue ages were categorised and the persistence factor was combined with the maximum corrected adult mortality (MA) as well as an additional factor of 0.5 to prevent the maximum possible combination of persistence factor and corrected mortality from exceeding 100%. Thus: IA = MA x 0.5P, where IA = maximum impact on adult mortality adjusted for persistence and the product's overall impact (I) is equivalent to IA (Hattingh et al., 2000).

2.2.2 Statistical analyses

The effect of 13 different pesticides on the mortality of *A. vladimiri* was analysed using a generalised linear mixed model (GLMM) (Bolker et al., 2009). The proportion of *A. vladimiri* that died, per replicate, was specified as the response variable. The pesticide residue treatment was specified as a categorical fixed effect variable, and the number of days since the start of the experiment was specified as numeric fixed effect. A random intercept term for 'rep' was included to account for repeated measurements taken from the same replicate over time (Bolker et al., 2009). The GLMM was specified with a binomial error distribution and a logit link function. A likelihood ratio test (LRT) was used to assess fixed effect parameter significance using the '*Anova*' function from the '*car*' R package ($P < 0.05$) (Fox et al., 2019). Post-hoc comparisons were performed to compare the treatment efficacy after 28 days (representing the final week of data collection) using the '*emmeans*' R package and Holm's adjustment for multiple comparisons (Lenth, 2022).

2.3 Results

2.3.1 Effect of aged residues on mortality of *Anagyrus vladimiri*

The mortalities of the different pesticide treatments (including the control and standard) were significantly different from each other (Chi-squared = 856.65, df = 4, $P < 0.001$). The proportion of *A. vladimiri* wasps that died in all the treatments, except two, was significantly different to the untreated control of "reverse osmosis" water (Figure 2.3). In the cyantraniliprole and prothiofos treatments, the proportion of *A. vladimiri* that died was not significantly different from the untreated control.

The results from the multiple comparisons analysis also presented a strong time effect (Chi Squared = 1036.60, df = 1, $P < 0.001$), as the mortalities at subsequent assay intervals, 7, 14 and 28 days, were significantly different from the initial mortalities at day 1 after treatment. Mortality decreased over time (Figure 2.3).

The highest *A. vladimiri* mortality after 24 hours on one day old residues was observed in treatments with methomyl, phosmet and spinetoram (90 -100% mortality) (Figure 2.4 A). Phosmet was the most harmful insecticide, with the highest mortality of *A. vladimiri* 14 days after treatment (70 – 80 % mortality) (Figure 2.4 C).

Tau-fluvalinate and chlorfenapyr also caused fairly high mortality in wasps exposed to 1-day old residues (75 – 85 % mortality) (Figure 2.5A). The mortality of wasps exposed to 7-day old residues of chlorfenapyr was 90%, which was the highest mortality in wasps on 7-day old residues. The mortalities caused by acetamiprid, fipronil and sulfoxaflor was 48%, 42% and 46% respectively (Figure. 2.4A & Figure. 2.6A).

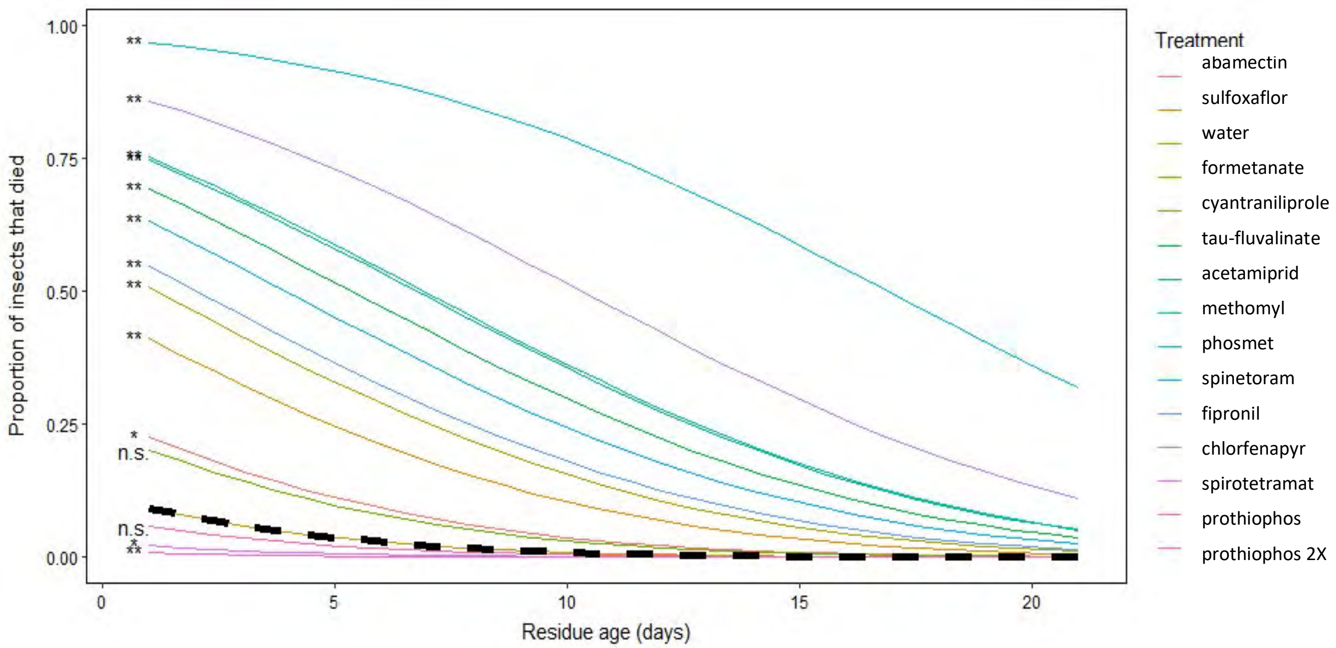


Figure 2.3 Summary of mortalities from a multiple comparisons analysis indicating the proportion of *A. vladimiri* that died (Y-axis) on treated versus the untreated leaf substrate over time (X-axis), where *A. vladimiri* was exposed to aged residues.

Abamectin and cyantraniliprole caused approximately the same mortality on day 1 after treatment, 28% and 26% respectively (Figure. 2.4A) and formetanate caused a slightly higher mortality of 31% (Figure. 2.5A). Spirotetramat and prothiofos were the only treatments resulting in zero mortality (Figure 2.5A).

A double (2X) dose of prothiofos was tested and also resulted in zero mortality on day 1 after treatment (Figure 2.6 C). Acetamiprid was the most persistent active ingredient in all tests and caused the highest mortality (21%) in wasps exposed to 28-day old residues (Figure 2.7).

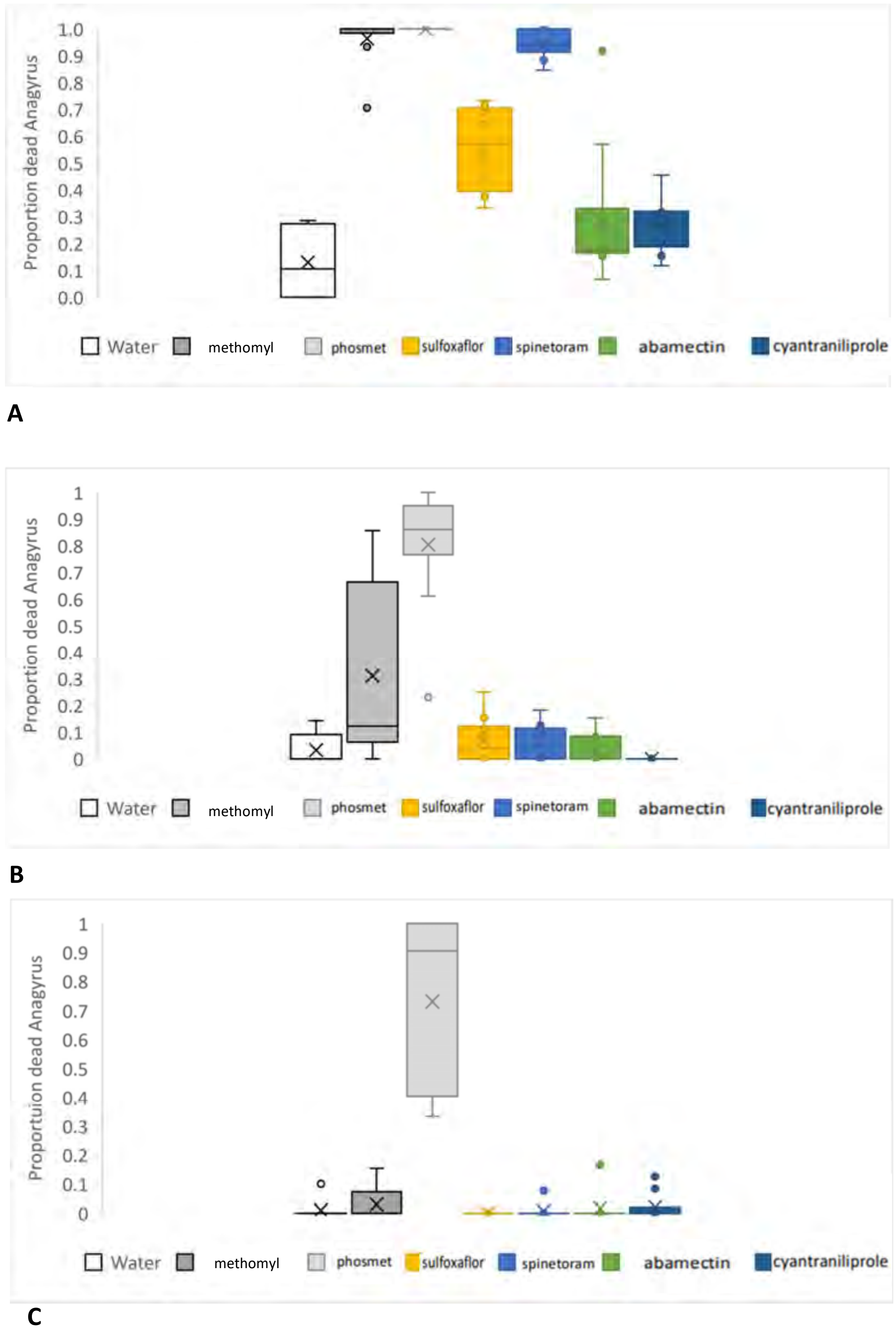


Figure 2.4 The proportion of *Anagyrus vladimiri* in test cells that died. Pesticide treatments in April 2021 compared to methomyl (standard) and water (control) at (A) 1day; (B) 7 days; and (C) 14 days after application.

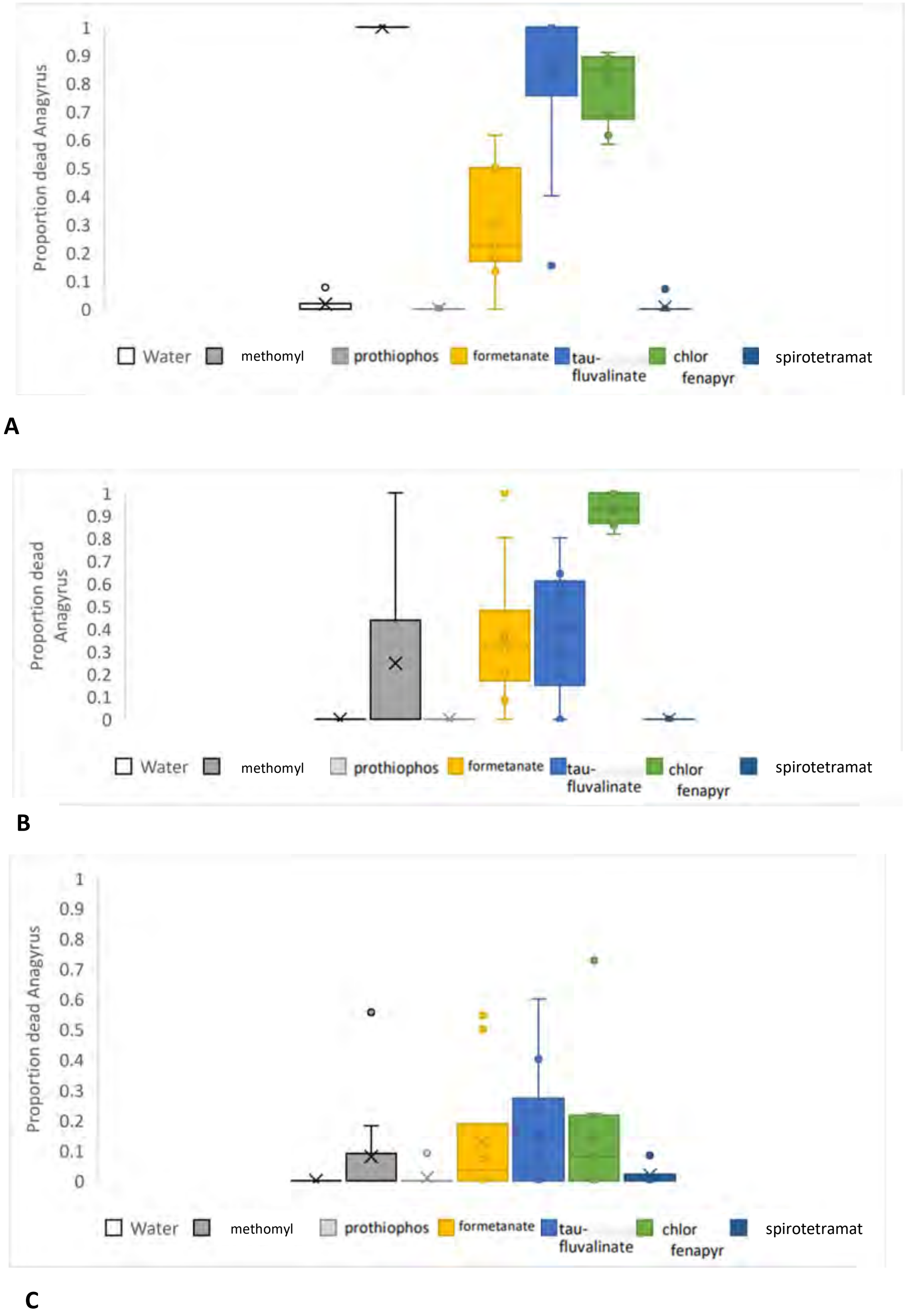


Figure 2.5 The proportion of *Anagyrus vladimiri* in test cells that died. Pesticide treatments in May 2021 compared to methomyl (standard) and water (control) at (A) 1 day; (B) 7 days; and (C) 14 days after application

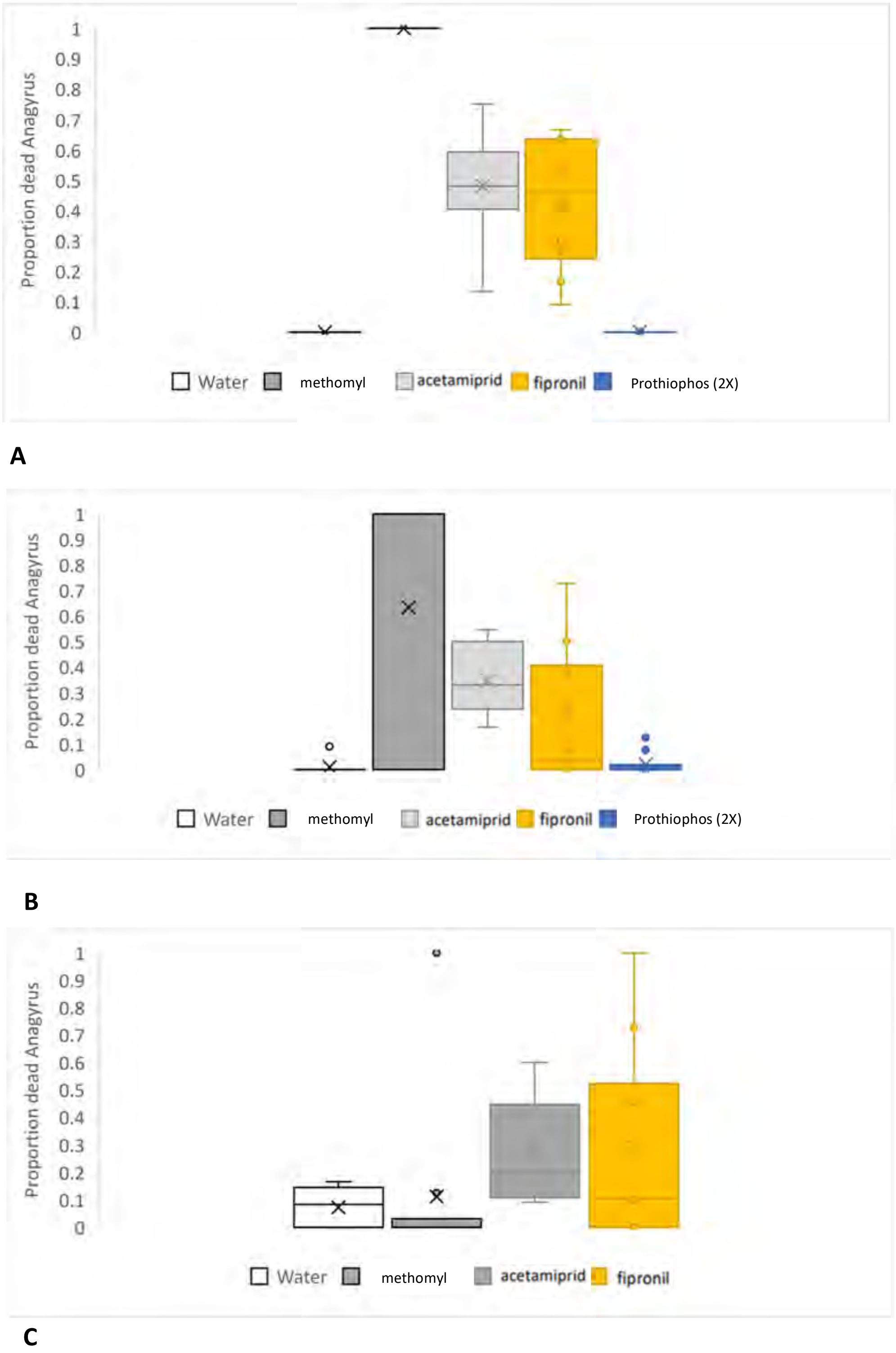


Figure 2.6 The proportion of *Anagyrus vladimiri* in test cells that died. Pesticide treatments in July 2021 compared to methomyl (standard) and water (control) at (A) 1 day; (B) 7 days; and (C) 14 days after application.

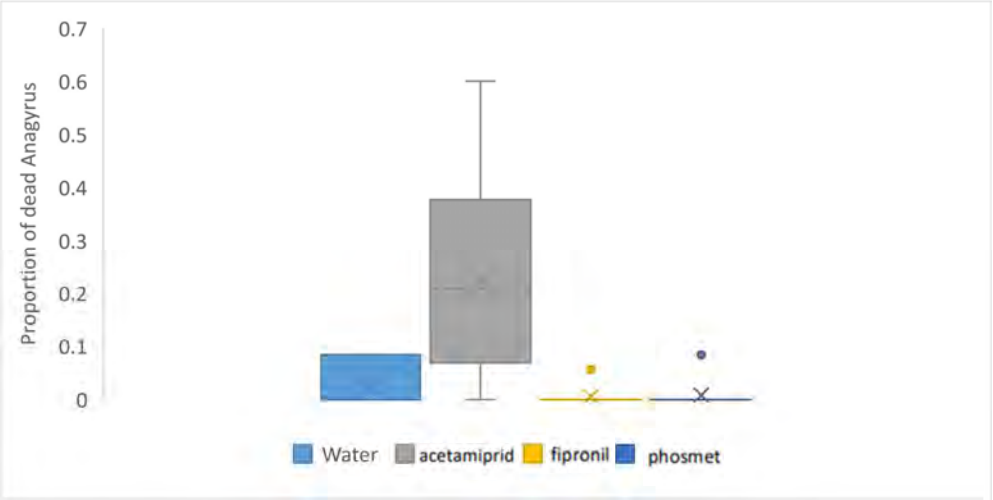


Figure 2.7 The proportion of *Anagyrus vladimiri* in test cells that died. Pesticide treatments in July 2021 compared to water (control) at 28 days after application.

2.3.2 Overall impact of pesticides

As described by Hattingh et al. (2000), the overall impact was calculated from a series of corrected mortalities for each individual pesticide (Table 2.1). Overall impact (I) categories were as follows: I≤25 = Harmless; I>25 to ≤50 = Slightly Harmful; I>50 to ≤75 = Harmful; and I>75 = Very Harmful. From the results in this non-target assay, methomyl, phosmet and chlofenapyr had corresponding overall impact values of 55, 61 and 51, which categorised them as harmful against *A. vladimiri*. Tau-fluvalinte and spinetoram were classified as slightly harmful, with overall impact values of 46 and 50. Abamectin (7), cyantraniliprole (5), fipronil (23), formetanate (20), prothiofos (0), spirotetramat (1) and sulfoxaflor (24) were each categorised as harmless against *A. Vladimiri*, based on the results of this study.

Table 2.1 Summary of non-target assays for a list of pesticides commonly used for thrips control on citrus, including corrected mortalities of *Anagyrus vladimiri* exposed to aged residues and overall impact ratings.

Date	**Active**	**Dosage per 100ℓ water**	**% Corrected Mortality**				**Overall Impact (I)**	**Rating**
			Day 1	**Day 7**	**Day 14**	**Day 28**		
April ‘21	methomyl	100g	95.6	28.7	2.0	-	53	**Harmful**
	phosmet	100g	100.0	79.7	72.8	0.8	60	**Harmful**
	sulfoxaflor	12ml	45.7	3.8	-	-	24	**Harmless**
	spinetoram (SC)	25ml	92,7	2,9	-	-	50	**Slightly Harmful**
	cyantraniliprole	100ml+300ml oil	10.3	0	1.1	-	5	**Harmless**
	abamectin	4.3ml	13.3	0	0,7	-	7	**Harmless**
May ’21	methomyl	100g	100.0	24.7	8.0	-	54	**Harmful**
	prothiofos	50ml	0	0	0.9	-	0	**Harmless**
	formetanate	25g + 200g sugar	29.9	36.7	12.8	-	20	**Harmless**
	spirotetramat	40ml+300ml oil	0	0	1.8	-	1	**Harmless**
	tau-fluvalinate	30ml	84.0	38.8	14.6	-	46	**Slightly Harmful**
	chlorfenapyr	30ml	75.5	92.6	14.1	-	51	**Harmful**
Jul ‘21	methomyl	100g	100	63.0	10.4	-	55	**Harmful**
	prothiofos 2X	100ml	0	1.1	3.2	-	2	**Harmless**
	acetamiprid	50g	48.2	34.2	22.2	21.0	27	**Slightly Harmful**
	fipronil	10ml	42.3	18.4	24.0	-	23	**Harmless**

Impact ratings of 11 of the insecticides on *A. vladimiri* were compared to impact ratings of the same insecticides or active ingredients on *C. perminutus* (Figure 2.8). Impact ratings for tau-fluvalinate and prothiophos on *C. perminutus* were not very different from the corresponding impact ratings for *A. vladimiri,* although lower for *A. vladimiri*. They were the same for spinetoram. The impact ratings of all the other pesticides on *A. vladimiri* were lower than the impact ratings on *C. perminutus* all the rest.

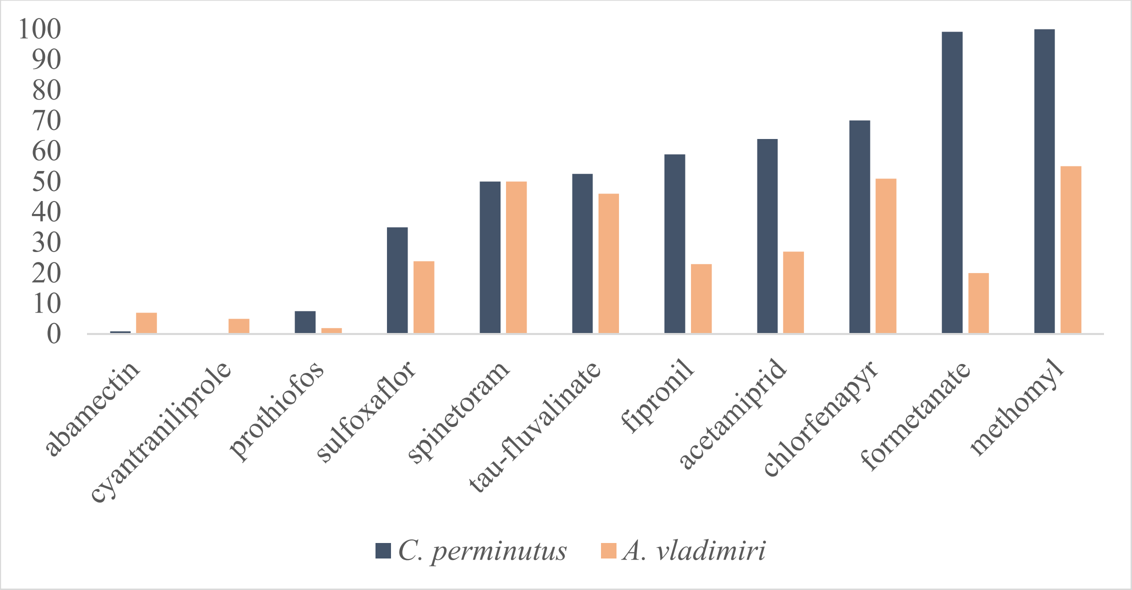


Figure 2.8 Impact ratings of insecticides on *Coccidoxenoides perminutus* (Grout et al., 2011) (dark) compared with *Anagyrus vladimiri* (light).

2.4 Discussion

From the list of insecticides included in this study, the majority were harmful towards *A. vladimiri* when exposed to 1-day old residues through contact with a treated citrus-leaf substrate in semi-field bioassays, when the field-dose of each pesticide product is expected to be at its highest. Differences in harmful effects of the various pesticides were clearly distinguished and persistence over time was also well established.

The impact on beneficial insects is not one dimensional. Mortality of parasitoids in a 24-hour period after treatment with a pesticide does not necessarily have a major effect on longevity, fertility and progeny sex ratio in many instances (Biondi et al., 2012). There are some important considerations to make when setting up non-target bioassays. Many publications present results from laboratory studies, which could be misleading. For example, laboratory studies do not consider degradation of active ingredients by abiotic factors such as sunlight and rainfall, which can reduce the harmful effects of a pesticide (Biondi et al., 2012). Laboratory studies also do not account for the possibility of synergism between multiple residues applied in the field under commercial farming conditions (Mgocheki et al., 2015). Therefore, field or semi-field bioassays are necessary for obtaining more reliable data for practical use.

The aim of this study essentially required that a practical, more accurate measure of the harmful effects of pesticides on *A. vladimiri* be investigated. By comparing results of our study with results from bioassays done on another mealybug parasitoid, *C. perminutus*, we can better envisage the expected harmful effects of certain pesticides on *A. vladimiri* augmentations, when these pesticides are sprayed in citrus orchards. In a similar study, *A. vladimiri* and *C. perminutus* were shown to be similarly susceptible to systemic residues of imidacloprid in vineyards (Mgocheki et al., 2015). In contrast, *A. vladimiri* was reported as being more robust than *C. perminutus,* possibly due to its larger body size, when subjected to contact residues of fipronil (Mgocheki et al., 2009). The current study confirms that fipronil, along with most of the other pesticides tested, has lower impact on *A. vladimiri* compared to the impact on *C. perminutus*. There is now more evidence to support that *A. vladimiri* is more robust than the smaller *C. perminutus* in withstanding effects from pesticides.

Consideration should also be given to the effect of direct spray contact of insecticides on adult wasps, which will almost certainly cause higher mortality. It is for this reason that releases of *A. vladimiri* must not coincide with spray treatments of insecticides or any other sprays and pesticides with high toxicity like phosmet and methomyl. Pesticides with longer residual activity like fipronil, chlorfenapyr and tau-fluvalinate should also be avoided where possible. Alternatively, these compounds could be included in the early part of the season, during spring, before parasitoids are released. Interestingly, the organophosphate prothiofos had nearly zero harmful impact on *A. vladimiri.* The reason for this is unknown. Under the same bioassay conditions, a similar study shows low impact of the closely related organophosphate, profenofos on *C. perminutus* (Grout et al., 2011). An observation was that *A. valdimiri* wasps had sufficient space to move that they could seek refuge on the walls and ceiling of the test cells. Should a possible repellent effect of prothiofos be present on the surface of the leaf, it could have reduced contact time of *A. vladimiri* with the residue on the leaf-substrate and explain the zero mortality. This is an aspect that could be investigated in future assays.

The most compatible insecticides for IPM from this study are abamectin, sulfoxaflor, cyantraniliprole, spirotetramat and tentatively, prothiofos. Augmentative releases could be considered approximately 7 - 14 days either before or after pesticide sprays are made. One can also estimate the expected timing of eclosion of the following generation of *A. vladimiri* and prevent sprays from taking place within acceptable intervals either before or after the expected eclosion date, thus protecting the parasitoids within the host. In general, enough time between the spray and the release date should be allowed, but not ignoring the life stage of mealybug hosts where successful parasitism takes place. In some instances, it may be necessary to withhold releases until the correct life stage is present. Following this study, a useful guideline can be drafted for developing a biological control strategy to support augmentative releases of *A. vladimiri,* in an IPM programme on citrus.

CHAPTER 3

EFFICACY OF AUGMENTATIVE RELEASES OF *ANAGYRUS VLADIMIRI* FOR CONTROL OF CITRUS MEALYBUG

3.1 Introduction

Over the past 100 years the use of augmentative biocontrol for controlling arthropod pests in agriculture has been explored in many commercial crops (Sharma et al., 2013). There is a need for augmentative biocontrol to be effective in supressing pest populations, it must be cost effective and there needs to be an understanding of the ecological factors that could inhibit the success of augmentative releases (Collier et al., 2004).

Biological control of mealybug is practiced in many countries around the world, employing three major tactics: classical biocontrol; augmentative biocontrol; and conservation biocontrol. Classical biocontrol has played an important part in the control of several invasive mealybug pests globally (Franco et al., 2009), but has not been very successful in the management of citrus mealybug, *P. citri* (Mendel et al., 1999). In an era where IPM has become important for sustainable agriculture, commercial augmentative biocontrol is widely practiced with mass production of many species of natural enemies made available as a viable alternative to repeated pesticide applications (van Lenteren, 2012).

There are seven important mealybug species on citrus in South Africa (Pieterse et al., 2010) (see Chapter 1), of which, the citrus mealybug is the major pest species. Infestations of citrus mealybug are responsible for the downgrading of fruit, due to the presence of cosmetic blemishes caused by sooty mould fungus growing on the honeydew excreted (Bedford et al., 1998; Mansour et al., 2018). Since the other species of mealybug are quarantine pests for sensitive markets such as South Korea, USA and China, the presence of mealybug on fruit is a risk for consignment rejections, at huge financial cost, if it cannot be identified to the correct species with certainty (Pieterse et al., 2010).

Spraying of insecticides for mealybug control is still common practice but it is not adequately effective and causes harm to their natural enemies (Franco et al., 2009). It is therefore important that IPM strategies include compatibility of chemical applications with releases of natural enemies, and it is only through innovative research that environmentally sound and effective options for mealybug management will be developed (Mansour et al., 2018).

The genus *Anagyrus* Howard (Hymenoptera: Encyrtidae) is well known in classical biological control programmes worldwide, contributing to sustainable mealybug control. *Anagyrus vladimiri* was previously known as *Anagyrus* sp. nr *pseudococci* (Andreason et al., 2019), and augmentative releases of this species have been previously trialled in citrus orchards and showed promise for the suppression of citrus mealybug (Moore et al., 2014). In this study different timings of augmentative releases of *A. vladimiri*, at different phenological stages of citrus development, were investigated. Early augmentative releases of *A. vladimiri*, coinciding with flowering, was compared to later releases during fruit development stages to determine where the efficacy of augmentation is highest.

3.2 Materials and methods

3.2.1 Selection of trial sites

Orchards with mealybug infestation levels of ≥ 20% were selected as suitable for the trial. Three trial sites were identified for the study in the 2019/20 season, two sites in the Burgersfort area on 2pH lemons (outside netting) and RHM mandarins respectively, and one site in Hoedspruit on Star Ruby grapefruit orchards (open orchards). At each site, four test orchards (T1 – T4) and an untreated control orchard was selected (C1). Trial plots of 1 hectare were measured out according to tree spacing and evaluations took place within the plot area. This allowed for buffer zones between trial plots and to allow at least 100 meters between orchards (Moore et al., 2014).

It was necessary to select two new sites in the 2020/21 season because of low mealybug infestation levels in those orchards. The RHM orchards (under netting) were replaced by Nadorcott mandarins (under netting) on a nearby farm in Burgersfort and a new farm in Hoedspruit was selected with suitable mealybug infestation in Grapefruit open orchards.

Another orchard was selected for addition to the trial in 2020/21 to evaluate the effects of a single chemical spray treatment early in October to serve as a positive control in comparison to the untreated control orchard.

Table 3.1 Details of trial sites where efficacy of *A. vladimiri* augmentations were evaluated.

Year	Region	Farm	Location: Latitude & Longitude	Orchard Identification	Cultivar
2019/20	Burgersfort	1	-24.849354 30.327538	M12, M14, M16, M18, M21	2PH lemons
2020/21	Burgersfort	1	-24.848245 30.331372	M1, M5, M12, M16, M21, M22	2PH lemons
2019/20	Burgersfort	2 (Under Net)	-24.782017 30.379997	V1, V2, V3, V4, V6	RHM mandarins
2019/20	Hoedspruit	3	-24.388418 30.839987	71a, 71c, 72b, 72d, 8a	Star Ruby grapefuit
2020/21	Burgersfort	4 (Under Net)	-24.87316 30.316675	W3, W4, W5, W6, W7, W8	Nadorcott mandarins
2020/21	Hoedspruit	5	-24.440959 30.820912	SR1a, SR1b, SR2a, SR2b, S1, S4	Star Ruby grapefruit

3.2.2 Spray programmes

An IPM strategy was discussed with farm management and agreement on the spray programme was reached where strictly pesticides with low impact values were used for the control of thrips, red scale, mealybug and other pests (Tables 3.2 and 3.3). Spray programmes were planned for compatibility with beneficial insects prior to the study and all trial orchards were treated the same, as far as possible, including the control orchard, where no *Anagyrus vladimiri* was released. In the event of mealybug numbers increasing to worrying levels, provision was made for treatment with IPM compatible insecticides, if necessary.

In the second season, the orchards added at each trial site were treated with a sulfoxaflor spray approximately two weeks after 100% petal fall.

Table 3.2 Registered insecticides sprayed and *A. vladimiri* released on each farm during the 2019/2020 season.

Treatment date	**Insecticide**	**Dose per 100L**	**Spray Cover**
Farm 1			
01-09-2019	Methomyl	100g	Full
01-09-2019	Bio-Insek	25ml	Full
03-10-2019	Closer	12ml	Full
03-10-2019	Envidor	10ml	Full
03-10-2019	Bio-Insek	35ml	Full
11-10-2019	Abamectin	4.3ml	Full (CBS Spray)
30-10-2019	Abamectin	4.3	Full (CBS Spray)
30-10-2019	*Anagyrus*	2500	T1
8-11-2019	Spinetoram	10g	Light
18-11-2019	*Anagyrus*		T1&T2
13-12-2019	Abamectin	4.3	Full (CBS Spray)
19-12-2019	*Anagyrus*	2500	T2 &T3
31-12-2019	Tartar emetic	400g	Bait
5-01-2020	Abamectin	4.3ml	Full (CBS Spray)
19-01-2020	*Anagyrus*	2500	T3 & T4
30-01-2020	Abamectin	4.3ml	Full
19-02-2020	*Anagyrus*	2500	T4
23-03-2020	Bio-Insek	55ml	Full
Farm 2			
14-09-2019	Spinetoram	12g	Light
18-09-2019	Buprofezin	30g	Full
18-09-2019	Spirotetramat	30ml	Full
18-09-2019	Bio-Insek	17ml	Full
1-10-2019	Bio-Insek	17ml	Full
7-10-2019	Buprofezin	30g	Full (CBS Spray)
7-10-2019	Fenpropathrin	35ml	Full
25-10-2019	Abamectin	4.3ml	Full (CBS Spray)
31-10-2019	Methomyl	100ml	Full

31-10-2019	Bio-Insek	12ml	Full
18-11-2019	*Anagyrus*	2500	T1
28-11-2019	Abamectin	6ml	Full (CBS Spray)
9-12-2019	*Anagyrus*	2500	T1&T2
7-01-2020	Abamectin	4.3ml	Full (CBS Spray)
19-01-2020	*Anagyrus*	2500	T2&T3
17-02-2020	*Anagyrus*	2500	T3&T4
22-03-2020	Exirel	75ml	Light
22-03-2020	*Anagyrus*	2500	T4
Farm 3			
23-09-2019	Buprofezin	30g	Full
11-10-2019	Buprofezin	30g	Full (CBS Spray)
23-10-2019	Methomyl	25g	Light/Medium
23-10-2019	Spinetoram	10g	Light/Medium
28-10-2019	*Anagyrus*	2500	T1
20-11-2019	*Anagyrus*	2500	T1&T2
12-12-2019	Abamectin	30ml	Full (CBS Spray)
17-12-2019	*Anagyrus*	2500	T2&T3
14-01-2020	Spinetoram	10ml	Light/Medium
14-01-2020	Methomyl	25g	Light/Medium
20-01-2020	Abamectin	30ml	Full (CBS Spray)
20-01-2020	*Anagyrus*	2500	T3&T4
20-02-2020	*Anagyrus*	2500	T4

Table 3.3 List of registered Insecticides sprayed on each farm for the 2020/2021 season.

Location	**Insecticide**	**Dose per 100L**	**Spray Cover**
Farm 1			
09-09-2020	Spirotetramat	20ml	Medium
09-09-2020	Bio-Insek	17ml	Medium
09-09-2020	Dipel	13.3g	Medium

24-09-2020	Spinetoram	12g	Medium
13-10-2020	Spirotetramat	40ml	Full (CBS Spray)
13-10-2020	Abamectin	6ml	Full
15-10-2020	Closer	12ml	Full (Control2)
20-10-2020	*Anagyrus*	2500	T1
31-10-2020	Abamectin	4.3ml	Full (CBS Spray)
17-11-2020	*Anagyrus*	2500	T1&T2
7-12-2020	Abamectin	4.3ml	Full (CBS Spray)
18-12-2020	*Anagyrus*	2500	T2&T3
21-12-2020	Broadband	20ml	Light
30-12-2020	Exirel	750ml	Bait
4-01-2021	Abamectin	4.3ml	Full (CBS Spray)
9-01-2021	Abamectin	4.3ml	Full (CBS Spray)
21-01-2021	*Anagyrus*	2500	T3&T4
21-02-2021	*Anagyrus*	2500	T4
Farm 4			
10-09-2020	Spirotetramat	20ml	Full
10-09-2020	Buprofezin	30g	Full
10-09-2020	Bio-Insek	13ml	Full
24-09-2020	Spinetoram	12g	Full
10-10-2020	Spirotetramat	20ml	Full (CBS Spray)
10-10-2020	Abamectin	6ml	Full
15-10-2020	Closer	12ml	Full
20-10-2020	*Anagyrus*	2500	T1
01-11-2020	Abamectin	4.3ml	Full (CBS Spray)
17-11-2020	*Anagyrus*	2500	T1&T2
05-12-2020	Abamectin	4.3ml	Full (CBS Spray)
18-12-2021	*Anagyrus*	2500	T2&T3
12-01-2021	Abamectin	4.3ml	Full (CBS Spray)
21-01-2021	*Anagyrus*	2500	T3&T4
03-02-2021	Methomyl	100g	Full
10-02-2021	Spirotetramat	40ml	Full Block 3,5,7,8

16-02-2021	Spirotetramat	40ml	Full Block 3 (CBS Spray)
21-02-2021	*Anagyrus*	2500	T4
24-02-2021	Methomyl	90g	Full Block 4,5,6
Farm 5			
02-09-2020	Methomyl	10g	Full
02-09-2020	Methidathion	150ml	Full
18-09-2020	Methomyl	20g	Medium (Microelements)
3-10-2020	Tau-Fluvalinate	50ml	Full (CBS Spray)
17-10-2020	Closer	12ml	Full (Control2)
22-10-2020	*Anagyrus*	2500	T1
27-10-2020	Methomyl	20g	Full (CBS Spray)
27-10-2020	Abamectin	6.5ml	Full
13-11-2020	Tau-Fluvalinate	50ml	Medium
19-11-2020	*Anagyrus*	2500	T1&T2
02-12-2020	Methomyl	20g	Full (CBS Spray)
12-12-2020	Spinetoram	10g	Medium
12-12-2020	Methomyl	20g	Medium
23-12-2020	*Anagyrus*	2500	T2&T3
27-01-2021	*Anagyrus*	2500	T3&T4
18-02-2021	Methoxyfenocide	60ml	Full
26-02-2021	*Anagyrus*	2500	T4

3.2.3 Augmentative releases of *Anagyrus vladimiri*

Anagyrus vladimiri parasitoids were supplied by both BioBee Integrated Crop Solutions and Insectec in 2020 due to availability. All parasitoids were supplied by Insectec in 2021. The four release orchards, T1 – T4, were each dosed with a treatment of 5000 *A. vladimiri* for the season, released in two equal tranches spaced one month apart (Table 3.4). Initiation of releases in trial plots occurred at different timings which distinguished the treatments from each other. 12 Insectec containers with 200 *A. vladimiri* mummies were hung on tree branches inside the canopy as shown in Figure 3.1(a). When releases of Biobee containers were made, 10 containers of 250 *A. vladimiri* mummies were attached to tree branches by the sticky ant repellent band as shown in Figure 3.1(b).

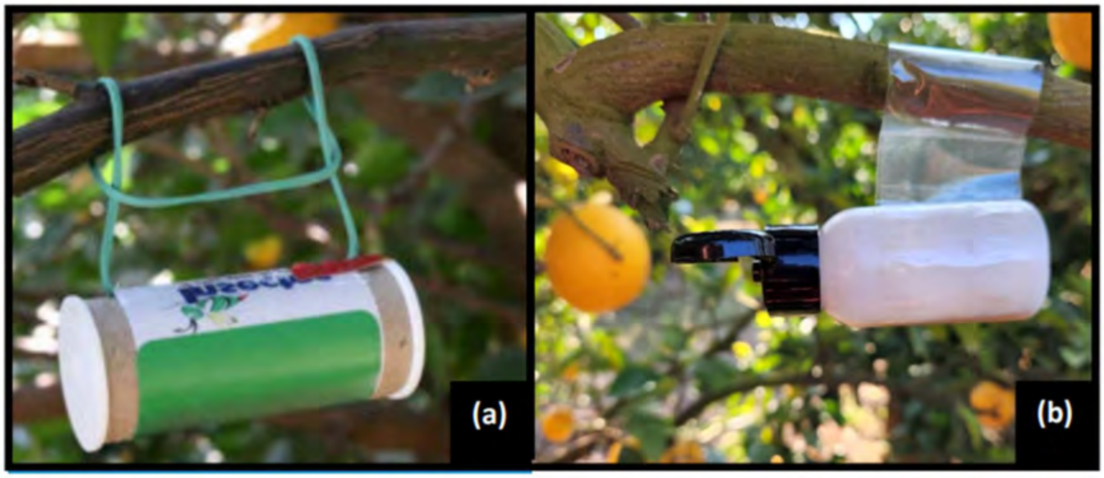


Figure 3.1 Placement of containers containing *A. vladimiri* mummies inside the tree canopy. Mummies were supplied from two insectaries, (a) Insectec and (b) BioBee.

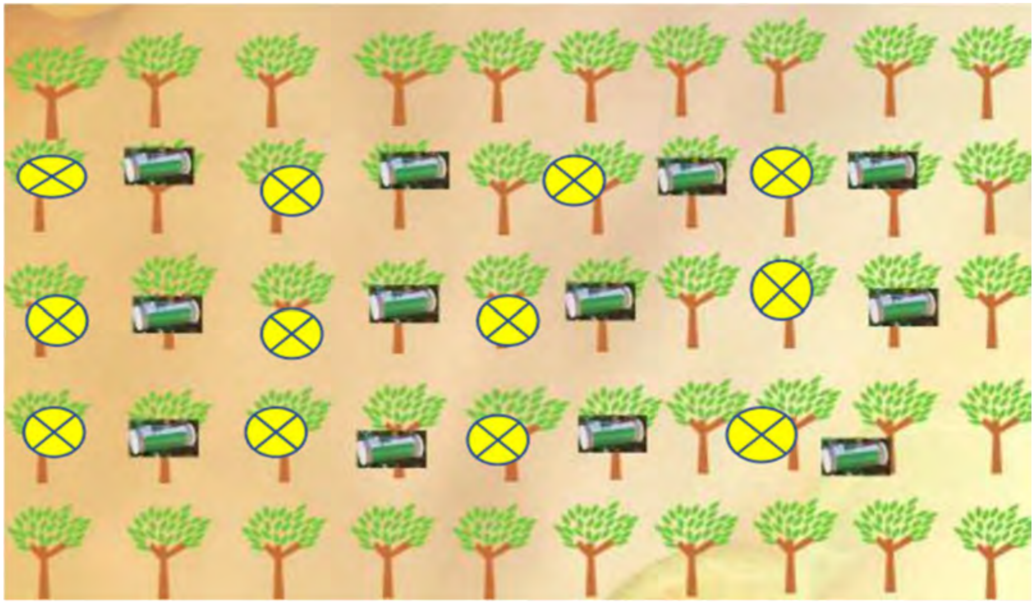


Figure 3.2 Releases of *A. vladimiri* in a single 1-hectare trial plot. Containers (green) were distributed evenly in the plot. One month later, containers for the second release were placed in an alternative position (yellow).

Table 3.4 Treatments of *Anagyrus vladimiri* releases at different phenological stages of citrus and mealybug development on one trial site.

Treatment (Orchard)	October	November	December	January	February
T1	2500	2500			
T2		2500	2500		
T3			2500	2500	
T4				2500	2500
C1	UNTREATED CONTROL				
C2*	POSITIVE CONTROL				

*Only in 2021, Closer ™240 SC

3.2.4 Quality control

An extra container of *A. vladimiri* pupae was supplied with each consignment and kept in the laboratory, inside ventilated 4 litre plastic eclosion chambers until adult eclosion was complete. Adults were counted and the ratio of males to females determined for each individual container. The number of eclosed and non-eclosed mummies as well as the total number of mummies was counted and recorded.

To asses quality of *A. vladimiri* released in the field, an additional 20 containers were collected randomly across all trial sites, about 1 month after releases were made, and taken back to the laboratory. Approximately 50 mummies from each container was inspected for eclosion and the number of eclosed and non-eclosed mummies was recorded.

3.2.5 Mealybug infestation monitoring

Monitoring of mealybug infestation took place in all trial plots concurrently at a single trial site. The start of monitoring was as early as possible in the spring. Ten randomly selected data trees in each orchard were monitored for the presence of mealybug on fruit, every two weeks until harvest. Ten random fruit were inspected from different inspection points in each tree and the presence of mealybug adults, crawlers and egg-sacs on fruit were recorded.

3.2.6 Statistical analysis

Fruit infestation was analysed by using a generalised linear mixed model (GLMM) (Bolker et al., 2009). The proportion of fruit infested by mealybug was specified as the response variable. Treatment was specified as a categorical fixed effect variable to assess the effect of timing of *A. vladimiri* releases on efficacy, while weeks since the start of the experiment was specified as a categorical fixed effect variable to assess how mealybug infestation varied over time. A nested random intercept term of 'orchard' nested within 'farm' was included to account for repeated measurements taken from the same plot over time (Bolker et al., 2009). The GLMMs were specified with a binomial error distribution and a logit link function. A likelihood ratio test (LRT) was used to assess fixed effect parameter significance using the '*Anova*' function from the '*car*' R package ($P < 0.05$) (Fox et al., 2019). Post-hoc comparisons were performed to compare the treatment efficacy during the last week of sampling for each year (week 28 in 2020 and week 34 in 2021) using the '*emmeans*' R package and Bonferroni adjustment for multiple comparisons (Lenth, 2022). All statistical analyses were performed using *R* ver. 4.3.0 (R Core Team, 2023). Separate GLMMs were run for the data from 2020 and 2021 due to singular model fits when combined in the same model.

3.3 Results

3.3.1 Quality control

For *A. vladimiri* that were allowed to eclose in the laboratory, the number of pupae counted in each container adequately represented the numbers indicated on the label. In the 2019/20 season containers from BioBee and Insectec were inspected. BioBee containers were labelled to contain 250 mummies and Insectec containers 200 mummies. Insectec was the sole supplier in 2020/2. The average percentage pupae that eclosed from quality samples from the insectary was 86.8% in the 2019/20 and 83.2% the following season. However, in both seasons, lower eclosion percentages of 71%, 75.8%, 76.5% and 75% respectively was noted in some samples. The average ratio of males to females that eclosed in sample bottles was 50:50 in 2019/20 and 56:44 the following season (Table 3.5)

Table 3.5 Percentage eclosion and sex ratio of *A. vladimiri* inspected in samples received directly from the insectary in both seasons

					Quality Inspection Data			
Year	**Males**	**Females**	**M-ratio**	**F-ratio**	**Mummies Total**	**Inspected**	**Eclosed**	**% Eclosion**
2019/20	56	71	0.44	0.56	302	102	88	86.3%
	-	-			-	161	144	89.4%
Sample	79	54	0.59	0.41	210	210	149	71.0%
n= 10	-	-	-	-	-	100	95	95.0%
	-	-	-	-	-	101	94	93.1%
	88	100	0.47	0.53	240	240	194	80.8%
	-	-	-	-	-	143	137	95.8%
	90	87	0.51	0.49	231	231	175	75.8%
	-	-	-	-	-	44	39	88.6%
	-	-	-	-	-	53	49	92.5%
Average	78.25	78	0.50	0.50	245.75		AVERAGE	86.8%
							STDEV	8.4%

					Quality Inspection Data			
Year	**Males**	**Females**	**M-ratio**	**F-ratio**	**Mummies Total**	**Inspected**	**Eclosed**	**Eclosion**
2020/21	102	70	0.59	0.41	225	193	164	85.0%
	91	72	0.56	0.44	189	189	165	87.3%
Sample	37	49	0.43	0.57	151	151	128	84.8%
n= 8	79	73	0.52	0.48	241	100	80	80.0%
	59	57	0.51	0.49	153	153	117	76.5%
	125	79	0.61	0.39	231	231	216	93.5%
	77	46	0.63	0.37	188	188	141	75.0%
	89	60	0.60	0.40	197	197	165	83.8%
Average	82.38	63.25	0.56	0.44	196.875		AVERAGE	83.2%
							STDEV	6.0%

Possible reasons for the lower eclosion percentages could be from cold storage or sub-lethal effects from mealybug that have ingested low doses of a harmful pesticide at the insectary.

It is common practice to check containers in the field after releases. One reason is to confirm a high eclosion percentage and the other reason is to make sure ants have not gained access to mummies and carried them out the containers. In 2019/20 the average percentage eclosion was 86.7 % (Std.dev 5%; n=19) and 89.7 % (Std.dev 6.5%; n=20) the following season (Table 3.6).

Table 3.6 Percentage eclosion of *A. vladimiri* inspected in sample containers from orchards after releases in both seasons

	Field Quality Inspection				Field Quality Inspection		
Year	**Inspected**	**Eclosed**	**Eclosion%**	**Year**	**Inspected**	**Eclosed**	**Eclosion %**
2019/20	28	20	71%	2020/21	50	44	88.0%
	50	33	66%		50	43	86.0%
Sample	55	43	78%	Sample	50	48	96.0%
n= 19	51	40	78%	n= 20	50	41	82.0%
	53	35	66%		50	42	84.0%
	51	39	76%		50	42	84.0%
	57	46	81%		50	37	74.0%
	53	44	83%		50	44	88.0%
	56	39	70%		50	43	86.0%
	53	46	87%		50	43	86.0%
	53	49	92%		50	39	78.0%
	45	39	87%		50	43	86.0%
	60	55	92%		50	43	86.0%
	52	45	87%		50	42	84.0%
	52	47	90%		50	43	86.0%
	53	46	87%		50	43	86.0%
	54	47	87%		50	49	98.0%
	51	46	90%		41	36	87.8%
	51	38	75%		50	45	90.0%
					50	50	100.0%
		AVERAGE	86.7%			AVERAGE	89.7%
		STDEV	5.0%			STDEV	6.5%

3.3.2 Mealybug infestation

During the 2019/20 citrus season in Hoedspruit, citrus mealybug infestation in Star Ruby grapefruit open orchards peaked between 7 and 20% for the three orchards where initial releases of *A. vladimiri* were made in November, December and January respectively. The infestation in the grapefruit orchard where *A. vladimiri* releases was made early in October peaked at 3%, while mealybug infestation in the control orchard, where no releases were made, peaked at 6 %.

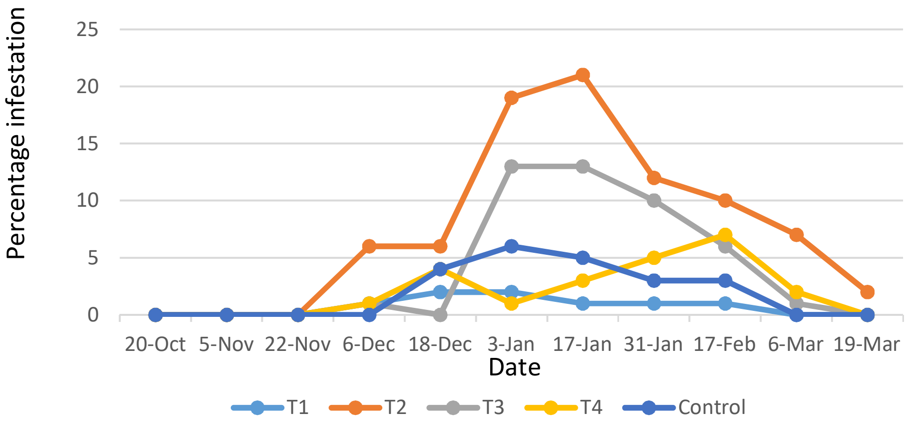


Figure 3.3 Mealybug infested fruit in Star Ruby grapefruit trial orchards in the 2019/20 season on Farm 3, Hoedspruit. Comparison between early release of *A. vladimiri* in October (T1) and later releases (T2 - T4) and the untreated control.

The distribution curve for mealybug infestation in the Grapefruit orchards was as expected, with the peak of the curve in January (Figure 3.3). Before harvest in March, the mealybug infestation declined to zero for three of the test orchards and the control orchard. Notably, the mealybug infestation peak in the T2 orchard, where November releases were made in grapefruit, was highest in January and infestation was also highest before harvest, at 3%.

In Burgersfort RHM mandarin orchards under net, the mealybug infestation did not reach a peak as expected between January and February (Figure 3.4). Infestation peaks in respective RHM orchards peaked at different times, much later in March, compared to infestation in lemon orchards outside netting in Burgersfort and the Star Ruby grapefruit orchards in Hoedspruit. Peak infestations were similar, between 6% and 20%, for the test orchards but unfortunately fruit infestation in the control orchard did not increase at any point in the season and remained at < 3%. Prior to harvest in April there was a resurgence of mealybug in the October and November release orchards, which is not what is expected within an IPM programme. In the remaining orchards, infestation was reduced to less than 5%.

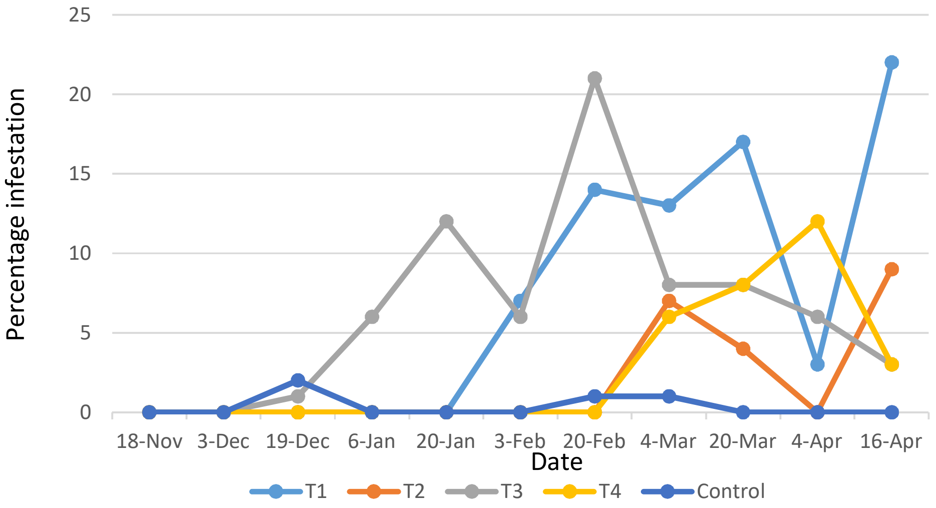


Figure 3.4 Mealybug infested fruit in the RHM mandarin trial orchards under net in the 2019/20 season on Farm 2, Burgersfort. Comparison between early releases of *A. vladimiri* in November (T1) and later releases (T2 - T4) and the untreated control.

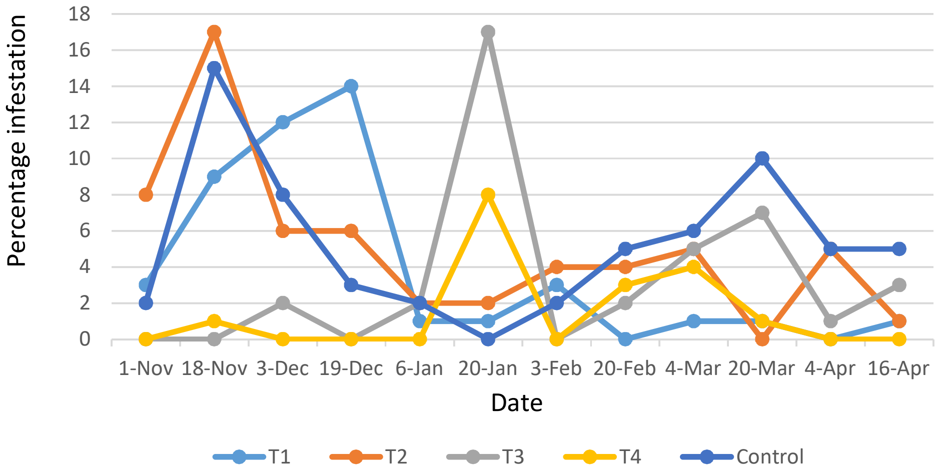


Figure 3.5 Mealybug infested fruit in 2PH lemon trial orchards in the 2019/20 season on Farm 1, Burgersfort. Comparison between early release of *A. vladimiri* in October (T1) and later releases (T2 - T4) and the untreated control.

As with the RHM mandarin orchards, the 2PH lemon open orchards, mealybug infestation did not peak as expected. In these trial orchards two mealybug infestation peaks can be seen, each representing a different fruit-set as lemons produce on average 3 fruit sets per year (Figure 3.5). A third peak representing only two orchards in January was observed. It was later learned that two of the test orchards had received an imidacloprid drench application, which resulted in a month delay of the first mealybug peak, compared to other orchards. Again, mealybug infestation on fruit in the October release orchard was lower than other orchards, including the control orchard in the weeks leading up to harvest. The mealybug infestation on the main set of lemons, monitored in test orchards for harvest in April, remained below 5% for 3 of the orchards and 7% in the December release orchard. The infestation in the control orchard increased to 10%. Mealybug infestation on fruit in trial orchards, in general, was reduced from 14 -18% in November to below 5% before harvest.

In the following 2020/21 season, mealybug infestations for respective trial sites peaked at an average of 14% (January) in Hoedspruit grapefruit orchards, 55% (February) in Burgersfort Nadorcott orchards under net and 18% (February) in the Burgersfort 2H lemon orchards outside net. Normal IPM mealybug distribution curves were again observed in the grapefruit orchards in Hoedspruit as well as the Nadorcott mandarin orchards under net in Burgersfort. Mealybug infestation was much higher during the second season in Burgersfort, particularly in the Nadorcott orchards under net and peak infestation occurred later in February. The infestation in Hoedspruit Star Ruby grapefruit orchards was similar to the previous season, with peak fruit infestation not exceeding 20% in any orchard. It was observed that mealybug infestation levels in the T1 orchard, the October releases, were again lower than the mealybug infestation levels of the other treatments (Figure 3.6). By harvest the mealybug infestation in all orchards, including the control, was reduced to zero. Observations indicate a positive impact of biological control in Hoedspruit grapefruit orchards.

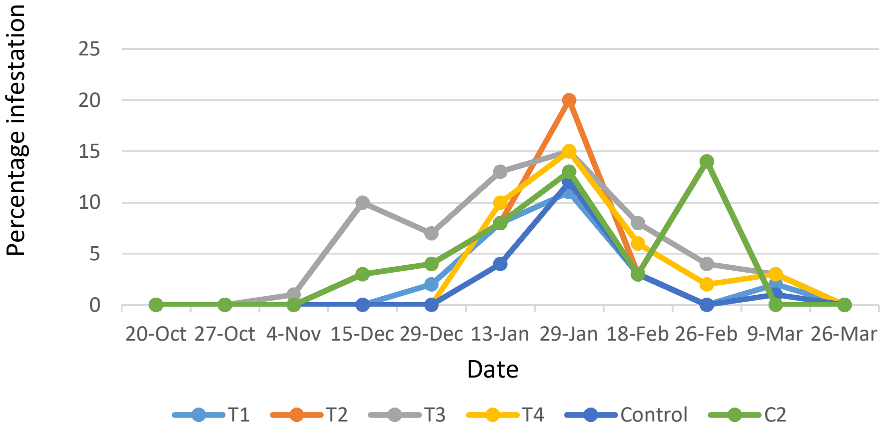


Figure 3.6 Mealybug infested fruit in Star Ruby grapefruit trial orchards in the 2020/21 season on Farm 5, Hoedspruit. Comparing releases of *A. vladimiri* in October (T1), later releases (T2 - T4), a positive (C2) and untreated control.

In Burgersfort poor biocontrol activity was observed in the early part of the season and mealybug infestation of fruit increased to alarming levels, reaching up to 85% in the Nadorcott orchards under net. Mealybug infestation increased sharply in January for all orchards including the control. Mealybug was deemed by the farmer to be uncontrollable and management opted to spray a corrective treatment in February after which mealybug infestation continued to increase. Interestingly, the mealybug infestation in the Nadorcott mandarin orchards declined to below 10% before harvest at the end of May, which was not much higher than infestation in the RHM orchards under net in the previous season (Figure 3.7).

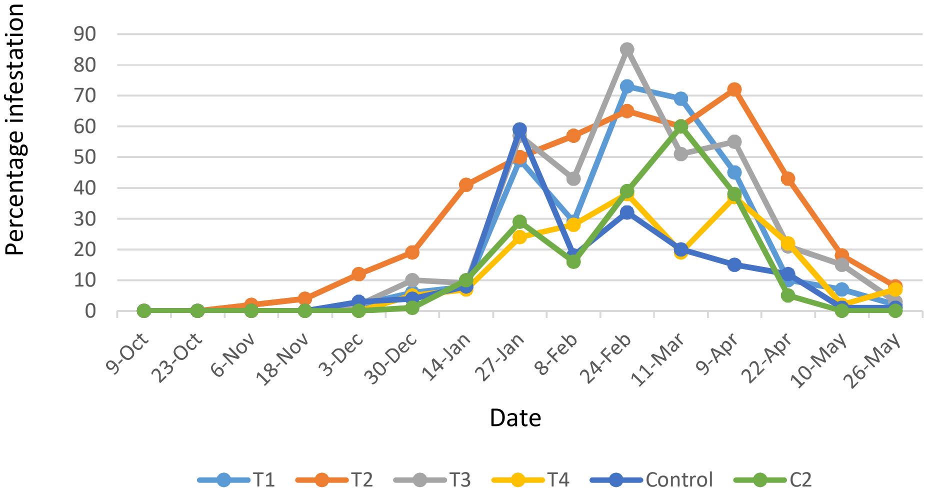


Figure 3.7 Mealybug infested fruit in Nadorcott mandarin trial orchards in the 2021/22 net on Farm 4, Burgersfort. Comparing releases of *A. vladimiri* in October (T1), later releases (T2 - T4), a positive (C2) and untreated control.

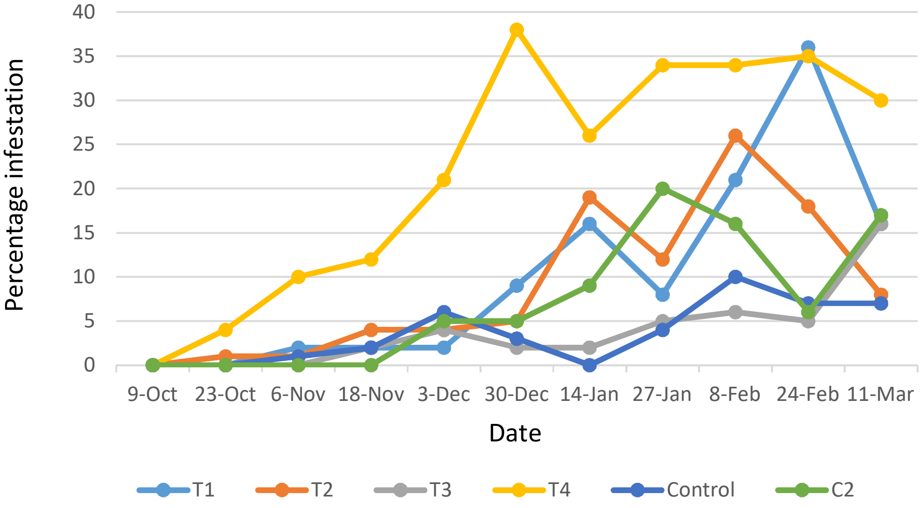


Figure 3.8 Mealybug infested fruit in 2PH lemon trial orchards in the 2020/21 season on Farm 1, Burgersfort. Comparing releases of *A. vladimiri* in October (T1), later releases (T2 - T4), a positive (C2) and untreated control.

Mealybug infestation levels in the 2PH lemons increased steadily from November until March when the main set of fruit (spring set) was harvested (Figure 3.8). Strangely the mealybug infestation in the control orchards in Burgersfort, where no releases of *A. vladimiri* took place, was far lower than all other orchards throughout the season.

3.3.3 Efficacy of augmentative releases of *Anagyrus vladimiri*

Results from a multiple comparison analysis of fruit infestation data across all the trial sites in the 2019/20 season indicated no significant difference in efficacy between treatment groups and the control, where no releases of *A. vladimiri* were made. There was evidence for a statistically significant interaction term between treatment and week (X2 = 15.93, df = 5, $P < 0.001$). This result indicates that fruit infestation rates varied between treatments over time (Figure 3.9), which was largely driven by a slight (2-5%) increase in fruit infestation rates over the growing season for treatments1, 3 and 4, while infestation rates remained relatively constant over the growing season for T2 and the control treatment (< 1% change in infestation rate). By the end of the season, all four parasitoid release treatments produced comparable fruit infestation rates, ranging from 4 to 8%, and while the control treatment was not statistically significantly lower, fruit infestation rates were between 2 and 6% lower in the control compared to the release-treatments (Figure 3.9; Table 3.7).

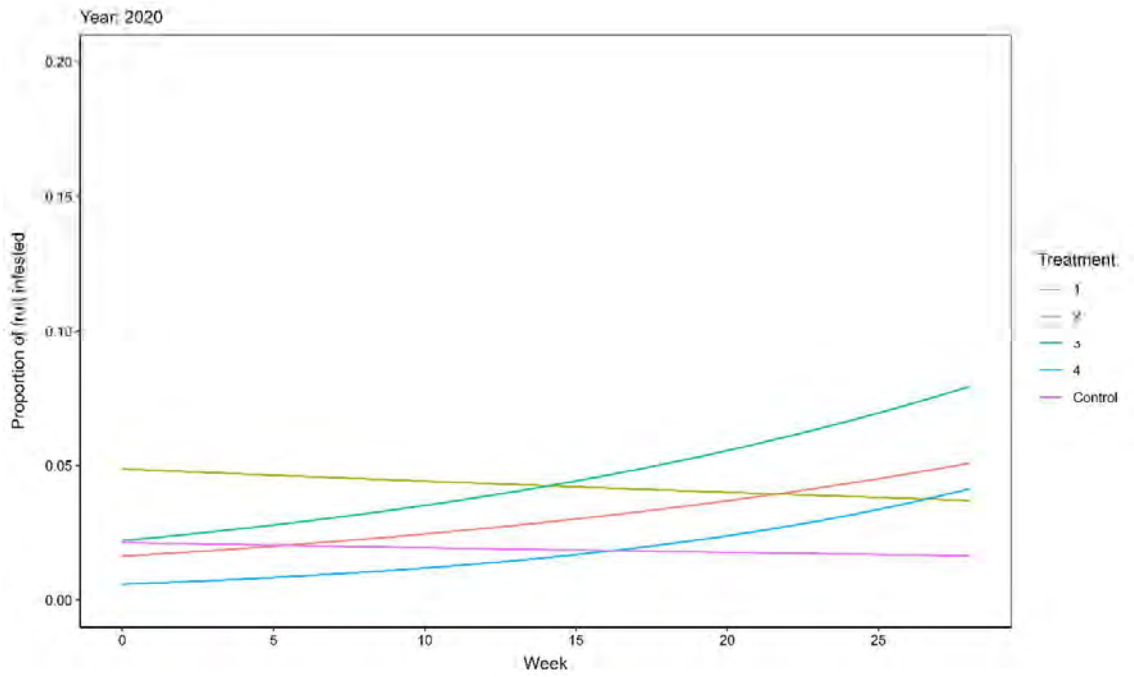


Figure 3.9 Mealybug infestation for all trial sites in the 2019/20 season, representing the statistical correlation between treatments and the untreated control (P< 0.05); (x-axis, time after first treatment; y-axis, proportion infested fruit)

Table 3.7 Post-hoc comparisons for fruit infestation rates in 2019/20 following post-hoc comparisons adjusted using Bonferronni correction for multiple comparisons

Contrast	**Estimate**	**s.e.**	***z*-ratio**	***P***
1-2	0.014	0.024	0.564	1.000
1-3	-0.028	0.035	-0.815	1.000
1-4	0.010	0.026	0.367	1.000
1-Control	0.034	0.021	1.638	1.000
2-3	-0.042	0.032	-1.310	1.000
2-4	-0.004	0.022	-0.192	1.000
2-Control	0.021	0.016	1.275	1.000
3-4	0.038	0.033	1.138	1.000
3-Control	0.063	0.030	2.113	0.346
4-Control	0.025	0.018	1.356	1.000

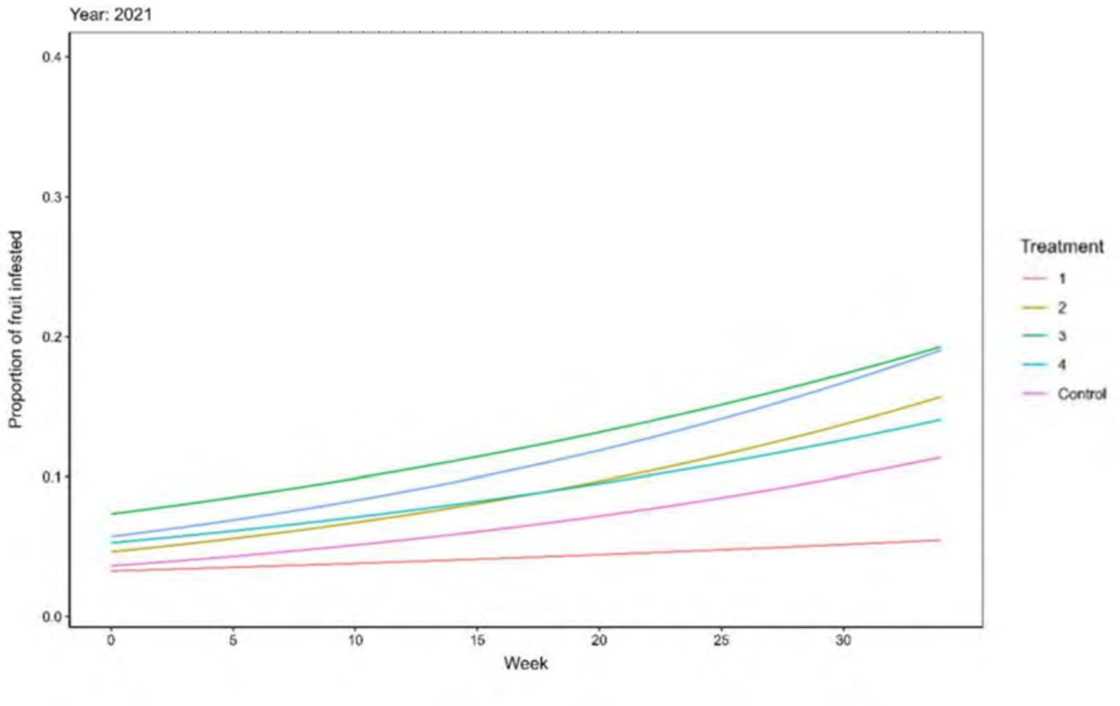


Figure 3.10 Mealybug infestation for all trial sites in the 2020/21 season showing the average of all treatments and the untreated control (x-axis, time after first treatment; y-axis, proportion infested fruit)

In the following season, analysis of fruit infestation data across all the trial sites in 2020/21 indicted no evidence for a statistically significant interaction term between treatment and week (X2 = 8.39, df = 5, P = 0.135). In contrast, there was evidence for a statistically significant effect of *A. vladimiri* release treatments on fruit infestation (X2 = 10.84, df = 5, P = 0.05), and a significant difference in fruit infestation rates over the growing season (X2 = 189.78, df = 1, P < 0.001). Taken together, these results indicate that fruit infestation rates differed between the parasitoid release treatments and changed over the growing season, however, the rate at which fruit infestation changed over the growing season did not vary significantly between release treatments (Figure 3.10; Table 3.8). Although not statistically significantly different, all the parasitoid release treatments produced approximately 6-15% higher infestation rates than the control treatment.

Table 3.8 Post-hoc comparisons for fruit infestation rates in 2020/21 following post-hoc comparisons adjusted using Bonferronni correction for multiple comparisons

Contrast	Estimate	s.e.	*z*-ratio	*P*
Control-1	-0.102	0.054	-1.887	0.888
Control-2	-0.138	0.065	-2.123	0.506
Control-3	-0.086	0.049	-1.772	1.000
Control-4	-0.136	0.065	-2.089	0.551
Control-5	-0.059	0.040	-1.479	1.000
1-2	-0.036	0.059	-0.612	1.000
1-3	0.016	0.051	0.317	1.000
1-4	-0.033	0.059	-0.568	1.000
1-5	0.043	0.050	0.863	1.000
2-3	0.052	0.058	0.906	1.000
2-4	0.002	0.062	0.038	1.000
2-5	0.079	0.058	1.364	1.000
3-4	-0.050	0.058	-0.861	1.000
3-5	0.027	0.046	0.582	1.000
4-5	0.077	0.058	1.320	1.000

3.4 Discussion

3.4.1 Quality control

Results from quality inspections of *Anagyrus vladimiri* emergence and sex ratio were satisfactory in the samples collected from the insectary. Small percentages of *A. vladimiri* that eclosed and did not exit containers were rarely found and there were no signs of deformities in any of the parasitoids inspected. Most importantly the average percentage eclosion of *A. vladimiri* in samples collected from the field was 86.7% in the 2019/20 season and was 89.7% the following season. This percentage eclosion was slightly better than for laboratory kept samples which could be as a result of stress of being kept in containers in the laboratory.

3.4.2 Efficacy of augmentative releases

Overall commercial control of mealybug in all trial orchards was attained in the 2019/20 season and in Hoedspruit Star Ruby orchards in 2020/21. Observations of early releases of *A. vladimiri* (T1) showed a lower mealybug infestation during the season in the Star Ruby grapefruit orchards over two seasons in Hoedspruit. The same was seen in two other trials in the 2PH lemon orchards in 2019/20 and the Nadorcott mandarin orchards under nets in 2020/21. Lower levels of mealybug during the season would be advantageous, by reducing the incidence of sooty mould, damage to fruit and lowering the risk of finding live mealybug on fruit at harvest. A study in the San Joaquin Valley indicated a sharp increase in parasitism of vine mealybug by *A. pseudococci* in late spring, which coincides with the initial overwintering mealybug emergence of mealybug from protected locations (Daane et al., 2004). Multiple generations of the parasitoids, rather than improved fecundity of parasitoids compared to the mealybug was noted. This supports the theory of early releases being more effective, provided no other limitations are in effect. Previous studies describe the importance of timing of releases and the impact of incorrect timing on efficacy of augmentations (Collier et al., 2004). In this study, the Star Ruby grapefruit orchards presented the most reliable infestation trends of mealybug over two seasons. This was true on two different farms which eliminates the possibility of a “Farm effect”. The reason is likely a constant, uniform fruit set and possibly the microclimate under the dense grapefruit foliage, which is characteristic of the variety. The early releases in these orchards were consistently more effective, which gives merit to at least the strategy of early augmentations in Star Ruby orchards. Experimentally, in the 2019/20 season, no significant differences in efficacy of treatments across all trial sites could be established. Relatively low infestation levels of mealybug on fruit might have had an impact. However, the differences in infestation over time between treatment groups is encouraging. The early releases treatment group and the untreated control group followed a decreasing trend in mealybug infestation, which was statistically different from the other treatment groups, which followed a trend of increasing mealybug infestation. One of the limitations in our trial design was an abundance of orchards with similar mealybug infestation on one farm within one particular citrus variety. The early releases of *A. vladimiri* were, by default, released into orchards with slightly higher mealybug infestation compared to other trial plots. This may have assisted with early parasitism and possibly a more effective establishment of *A. vladimiri*. It provides an alternative explanation for lower levels of mealybug observed in the early release orchards. Nonetheless, peak infestation levels in these orchards were very much the same as all the other orchards, yet mealybug infestation was reduced at a faster rate in the orchards where early releases took place. For this reason, we can propose that initiation of releases be slightly flexible and can take place as early as possible, in October or November, since a slightly higher mealybug population may support better or more rapid establishment of *A. vladimiri*. Slightly delayed releases in November may also avoid harmful sprays, which are less common later in the season. There was no indication that later releases of *A. vladimiri* in December and January would be more effective than early releases. It could be argued that releases are too late in December and January because of a lack of the benefit of reduced peak infestations. However, this study did not look at the impact of release quantities. This should be investigated to assess the potential of different timing of releases at various release quantities to see if parasitism and efficacy of augmentations can further reduce mealybug infestation on fruit.

During the 2020/21 season in Burgersfort the mealybug infestation levels could not be explained. This was unexpected. No signs of parasitism of first and second instar mealybug were observed in early summer. *Anagyrus vladimiri* was initially found in orchards, but subsequently declined to zero with further sampling. The high infestation trend in Burgersfort orchards had a definite impact on the multiple-comparisons analysis. An increasing trend in mealybug infestation was calculated for all treatment groups where *A. vladimiri* was released but not for the untreated control group. This was a very clear indication of influences from other factors in the field. Treatment 5 was included as a positive control where a preventative early season spray of sulfoxaflor was made to treat for mealybug. Our results show an initially significantly lower level of mealybug infestation in that orchard, but no difference in the rate of increase in mealybug infestation over time compared to the other treatments. However, it can be used as a strategy, similar to early releases of *A. vladimiri* to reduce the peak levels of mealybug during the season. Maybe a combination of a preventative spray and early releases would be synergistic and is something that could be investigated in future research.

At the outset of the project there was sufficient information describing the general efficacy of *A. vladimiri* augmentations to control citrus mealybug (Moore et al., 2014). The challenge was to design a study that excludes other major contributing factors so that the effect of "timing of releases" would be the major contributing variable for analysis of mealybug control in trial plots. Orchard selection and the IPM programme were generally well managed. However, we underestimated the potential influence of the naturally occurring parasitoids and hyperparasitoids on the potential of *A. vladimiri* to control mealybug on a commercial scale (Pitan et al., 2000; Petersen et al. 2000; Agricola et al., 1991; Beltrà et al., 2013; Kaushalya et al., 2010; Collier et al., 2004). A number of factors could have played a role in undermining mealybug biocontrol. Pitan et al. (2000) describes the potential for hyperparasitoids to create a stabilising mechanism in the host/natural enemy interaction, while on the other hand, high levels of hyperparasitism could suggest undesirability of the affected parasitoid. The question is whether hyperparasitism is the only reason for poor mealybug control? Wajnberg et al. (2015) have provided us with a more complicated explanation, yet it makes more sense when looking at some of our observations. The article explains four factors in behavioural ecology, which influences efficacy of parasitoid-based pest control, namely, residence time in a host patch; number of eggs deposited in the host; sex ratios; and host or patch marking. From observations in this study a number of potential factors were observed that could explain a potential tip in the balance of mealybug biocontrol in Burgersfort. In the first instance, the quality control of parasitoids received from the insectaries indicated a small change in sex ratio of *A. vladimiri*, released into orchards. In 2021 a 60:40 ratio of males to females was collected from sample bottles inspected, compared to a 50:50 ratio the previous season. On the other hand, interspecific competition through superparasitism or hyperparasitism related to the presence of a variety of different parasitoids and hyperparasitoids (Chapter 4 - Table 4.4) is suspected. The latter seems very likely.

It is apparent that the influence of shade-net and the multiple fruit-sets on the 2PH lemons impacted mealybug infestation levels differently in orchards. Lower mealybug infestation related to early releases was also clearly observed in the 2PH lemon orchards during 2020. We are now more aware of other factors, which could influence both pest and natural enemy populations. A number of observations were made with regard to this. For instance, the phenology of mealybug under net differs with a population peaking a month later and for longer into April or May. In the RHM mandarin orchard under net, a white plastic mulch was introduced in the early release orchard without our prior knowledge. This seemed to cause an increase in mealybug infestation at the end of the season before harvest. It is possible that more ants colonised under the plastic, increasing activity in surrounding orchards. The effect of ants is an important factor to consider. In many of the orchards it was a struggle to control ants, particularly in the lemon orchards. Ants tend to mealybug patches to harvest the honeydew and in return protect the mealybug by chasing away natural enemies. One study showed that *A. vladimiri* spent less time in patches of citrus mealybug and deposited less eggs where ants had visited previously and left a contact infochemical scent behind (Mouratidis et al., 2020). The above are examples of cultural practices in citrus production that negatively influence pest management and biological control in particular. There are a number of factors or interactions that could explain why the biocontrol was compromised (Wajnberg et al., 2015), but more data must be collected to quantify any of the other interactions. The focus of the study was on the interaction of *A. vladimiri* with citrus mealybug.

The findings in this report have provided a new perspective on augmentations of parasitoids for mealybug biocontrol in citrus. However, further research is needed to investigate the influence of these factors on the success of *A. vladimiri* augmentations.

CHAPTER 4

EFFECTS OF NATURALLY OCCURRING PARASITOIDS AND HYPERPARASITOIDS ON THE EFFICACY OF *ANAGYRUS VLADIMIRI*

4.1 Introduction

The potential for augmentative biological control has been driven by the need to reduce the reliance on agricultural pesticides (van Lenteren et al., 2018). Regulations on pesticide use is escalating, and some pesticides have already been withdrawn by governmental agencies with many more being reviewed (European Commission, 2022). Augmentative biocontrol refers to natural enemies that are mass reared in commercial insectaries and released into crops in order to assist with the control of specific pests. This includes areas of agriculture, such as fruit orchards, maize, cotton, sugarcane, soybean, vineyards, and greenhouses, and is commonly described as an environmentally and economically smart alternative to chemical pest control (Colmenarez et al., 2018), and could be implemented as a substitute for pesticides, if pest populations are sufficiently suppressed by the released natural enemies (Collier et al., 2004).

Control of the target pest is not always achieved and can be seen as a failure of the biological control agent to control the pest-host (Schooler et. al, 2011). The three main reasons for failure of control include climatic conditions, predation or parasitism of the primary biological control agent (hyperparasitism), and lack of alternative hosts or food for the biocontrol agent. Hyperparasitism is a highly evolved behaviour among Hymenopterans and in a few species of Diptera and Coleoptera, in which an adult hyperparasite (or secondary parasitoid) oviposits on or in a primary parasitoid host that has attacked another insect species (Sullivan, 2009). There is evidence from some biological control programmes that hyperparasitism can impact the parasitoid biological control agent but it has not been clear whether it can result in control failure. The lack of control could also be as a result of intrinsic and extrinsic factors (Boosalis, 1964).

Many of the successful biocontrol programmes involving releases of natural enemies have been associated with mealybugs. The most abundant natural enemies of mealybugs belong to the family Encyrtidae, which often establish host specific interactions (Beltrà et al., 2013). One example is *Leptomastix dactylopii*, originally described to be a specific parasitoid of *Plannococcus citri*. However, many studies have since reported *L. dactylopii* attacking more than 20 mealybug species (Muştu et al., 2015). Another example is from the genus *Anagyrus*, with at least 350 described species as primary endoparasitoids of various mealybug hosts (Andreason et al., 2019).

Species of *Anagyrus* have been reared and released in many biological control programmes worldwide, as a sustainable solution for control of various mealybug pests (Andreason et al., 2019), but only a few studies have been conducted on *A. vladimiri* in South Africa (Mgocheki et al., 2009 & 2015; Moore et al., 2014). The parasitoids have since gained commercial popularity in all citrus growing regions. The aim of this chapter was to monitor parasitism levels of *A. vladimiri*, confirm establishment on the mealybug population in citrus orchards and determine the correlation between parasitism levels and mealybug infestation. A further objective was to see if natural enemies are conserved in the trial locations where an IPM strategy was followed.

4.2 Materials and methods

4.2.1 Field survey sites

Surveys for parasitic wasps were conducted in 33 citrus orchards over a two-year period between October 2019 and April 2021 in Hoedspruit and Burgersfort, a warm subtropical climate and a cool and dry region respectively. Orchards in these two regions were selected for the abundance of citrus mealybug, *Planococcus citri*, as the predominant species in commercial citrus orchards.

Survey orchards were selected for the elevated mealybug levels, based on historical scouting records and because a strict IPM program was followed, which would promote the establishment of natural enemies. Augmentative releases of 5000 *Anagyrus vladimiri* were made in four survey plots at each of the sites. Releases were initiated at different phenological stages of citrus and mealybug development in October, November, December and January for respective plots as described in Chapter 3. At each site in 2019/20, one of the plots did not receive treatment with releases of *A. vladimiri* and two plots in 2010/21.

In the 2019/20 season, five orchards at each of 3 survey sites were under IPM management and were surveyed. The following season six orchards per site were surveyed. A total area of approximately 60 hectares of commercial citrus orchards were surveyed on 5 different farms (Table 4.1). Eleven of the orchards surveyed were covered by net-house structures and the remaining 22 orchards were open field orchards.

Table 4.1 Field survey - details of citrus orchards surveyed for mealybug parasitoid populations

Province	Region	Farm Name (Year)	Location latitude & longitude	Orchard Names	Cultivar
Mpumalanga	Burgersfort	Motsepula (2020)	-24.849354 30.327538	M12, M14, M16, M18, M21	2PH lemons
		Viljoen (2020)	-24.782017 30.379997	V1, V2, V3, V4, V6	RHM mandarins (Under Net)
		Motsepula (2021)	-24.848245 30.331372	M1, M5, M12 M16, M21, M22	2PH lemons
		Winterbach (2021)	-24.87316 30.316675	W3, W4, W5, W6, W7, W8	Nadorcotte mandarins (Under Net)
Limpopo	Hoedspruit	Venren (2020)	-24.388418 30.839987	71a, 71c, 72b, 72d, 8a	Star Ruby grapefruit
		Moria Citrus Estate (2021)	-24.440959 30.820912	SR1a, SR1b, SR2a, SR2b, S1, S4	Star Ruby grapefruit

4.2.2 Sampling

As soon as mealybug infested fruit became available in survey plots, 10 citrus fruit infested with mealybug were sampled at random from different points inside each of the designated plots. The fruit were carefully picked from the tree and placed into brown paper bags and sealed for transport to the laboratory for microscopic inspection. Sampling continued regularly at two-week intervals until harvest. During the 2019/20 season, a total of 50 citrus fruit, infested with *P. citri*, at each survey site was sampled every two weeks and 60 citrus fruit at each survey site, every two weeks in the 2020/21 season.

4.2.3 Inspections for mealybug parasitism

No more than 10 third-instar mealybug on each of the 10 fruit were inspected under the microscope for signs of parasitism. Numbers of mealybug on fruit ranged from 1 to over 100 during the duration of the trial depending on the level of mealybug infestation in the orchard. Third-instar mealybugs were inspected randomly and signs of parasitism included encapsulation of a parasitoid egg or a developing pupa. The number of parasitized and unparasitized mealybug was recorded. If there were less than 10 third-instars on a fruit then all of them were inspected. After inspection, the 10 fruit from each orchard were placed together in a 4-litre plastic eclosion chamber with a large ventilation window, made from material voile (Figure 4.1).

Figure 4.1 Eclosion chamber with voile material ventilation window.

4.2.4 Diversity and seasonal abundance of parasitoids and hyperparasitoids

Containers were kept at room temperature (Ceballo et al., 1998), inspecting the containers daily for the emergence of parasitoids (Wakgari et al., 2003). Containers were kept for approximately 21 days and then emptied, inspecting each fruit carefully under a microscope again to look for the presence of small parasitoids or hyperparasitoids hiding among the mealybug and waxy residue. All the individual parasitoids and hyperparasitoids that eclosed were placed into petri dishes and inspected under the microscope. After visual inspection the unknown species were aspirated into Eppendorf tubes containing 70% ethanol, counted and labelled for transport to the ARC Biosystematics Division for identification. The proportion of all parasitoids and hyperparasitoids that eclosed from samples was recorded. This was repeated for two consecutive seasons, recording the abundance of parasitoids and hyperparasitoids that eclosed from samples collected every two weeks.

4.2.5 Statistical analysis

Mealybug parasitism was modelled using a generalised linear mixed model (GLMM) (Bolker et al., 2009). The proportion of third instar mealybugs parasitised per replicate was specified as the response variable. *Anagyrus vladimiri* release timing (Treatment) was specified as a categorical fixed effect variable to assess the effect of timing of releases of *A. vladimiri* on parasitism rates, while weeks since the start of the experiment was specified as a numeric fixed effect variable to assess how parasitism rates varied over time. An interaction term between week and treatment was included to allow mealybug parasitism rates to vary between release treatments over time. A nested random intercept term of 'orchard' nested within 'farm' was included to account for repeated measurements taken from the same orchard over time (Bolker et al., 2009). The GLMMs were specified with a binomial error distribution and a logit link function. A likelihood ratio test (LRT) was used to assess fixed effect parameter significance using the '*Anova*' function from the '*car*' R package ($P < 0.05$) (Fox et al., 2019). Separate GLMMs were run for the parasitism data from 2020 and 2021 due to singular model fits when combined in the same model.

4.3 Results

4.3.1 In field parasitism of 3rd instar mealybug

The trend in parasitism rates varied between orchards and between farms. In the 2019/20 season the parasitism rates early in the season, between October and January, ranged between 0% and 25%. Increased parasitism rates corresponded with elevated mealybug infestation. After January, percentage parasitism ranged between 30% and up to100% later in the season. Parasitism of 3rd instar mealybug increased to above 30% in February, which was common to all trial locations in 2019/20. In the 2PH lemons, relatively low percentages of parasitism were consistently recorded from November to February in the 2019/20 season (Figure 4.2).

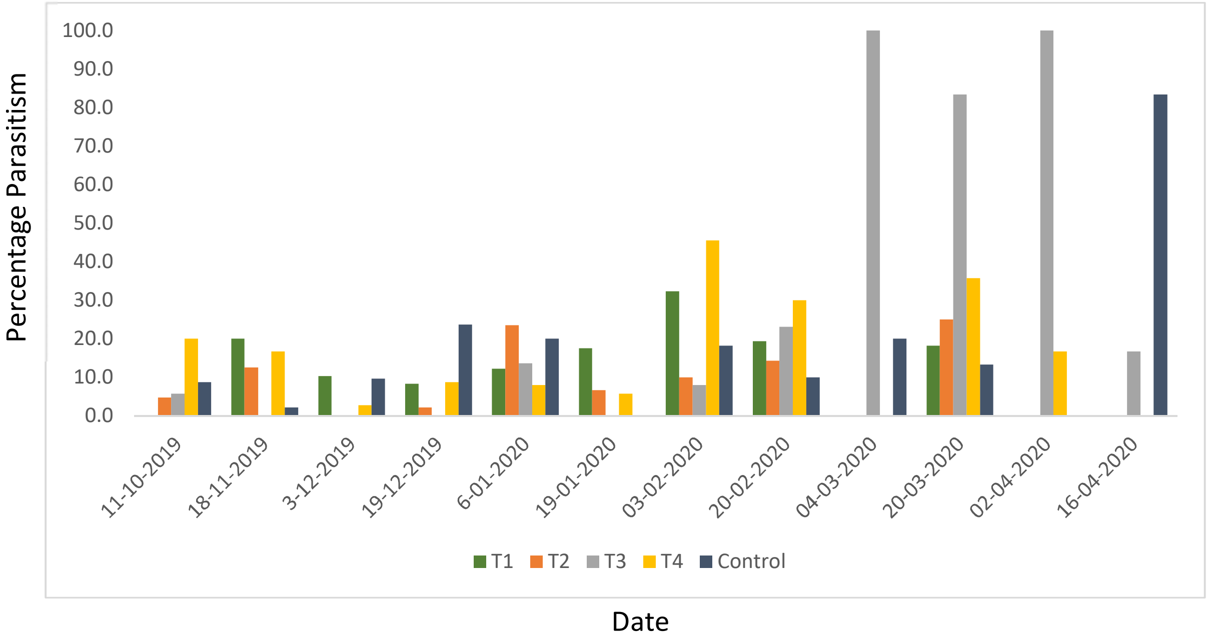


Fig 4.2 Percentage parasitism of 3^{rd} instar mealybug inspected on 10 fruit from each of the 2PH lemon trial orchards in Burgersfort for the 2019/20 season.

It is common in lemons to produce three flower inflorencences in a year. Young fruit are a fresh source of food for mealybug crawlers. Therefore, multiple sets of fruit on the lemons ensured a persistent level of mealybug as a result of carry-over from earlier sets. From November to March, a comparitively and consistently higher percentage parasitism was observed in the orchard where early releases of *A. vladimiri* were made in October. Parasitism rates in the December and January release orchards only peaked later in March and April. A much sharper increase in percentage parasitism was observed in the treatment 3 orchard where *A. vladimiri* releases were made in December and parasitism in the control orchard only peaked in April.

There was an initial increase in percentage parasitism in all the RHM mandarin orchards, under net, in February of 2019/20. However, a peak occurred a month later in March (Figure 4.3). Compared to parasitism in the 2PH lemons, percentage parasitism above 60% and higher was observed in all the RHM mandarin orchards. Although at different stages, 100% parasitism of 3rd instar mealybug was observed on fruit in four out of the five trial orchards. The highest percentage parasitism reached in the January release orchard (T3) was 85%.

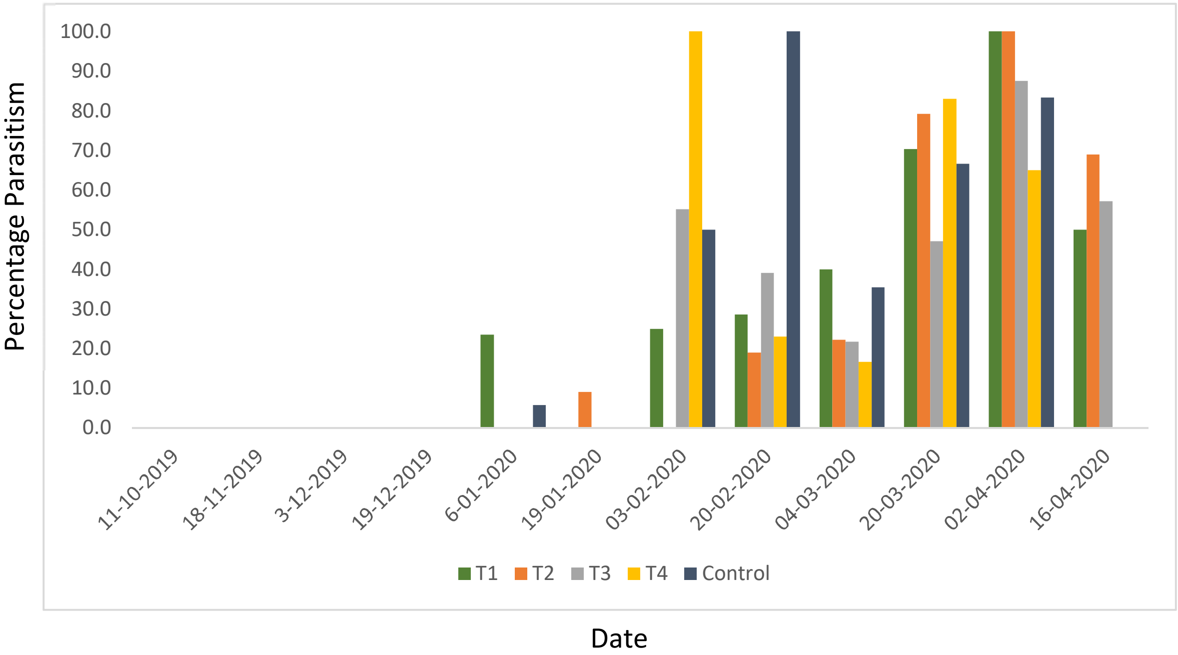


Fig 4.3 Percentage parasitism of 3rd instar mealybug inspected on 10 fruit from each of the RHM mandarin trial orchards in Burgersfort during the 2019/20 season.

In Star Ruby grapefruit orchards in Hoedspruit, a sharp increase in percentage parasitism in February coincided with the peak in mealybug infestation on fruit (Figure 4.4). Percentage parasitism peaked above 40% in all orchards at this point. In the orchard where *A. vladimiri* was released in October (T1), parasitism was comparatively higher and reached 100% before harvest

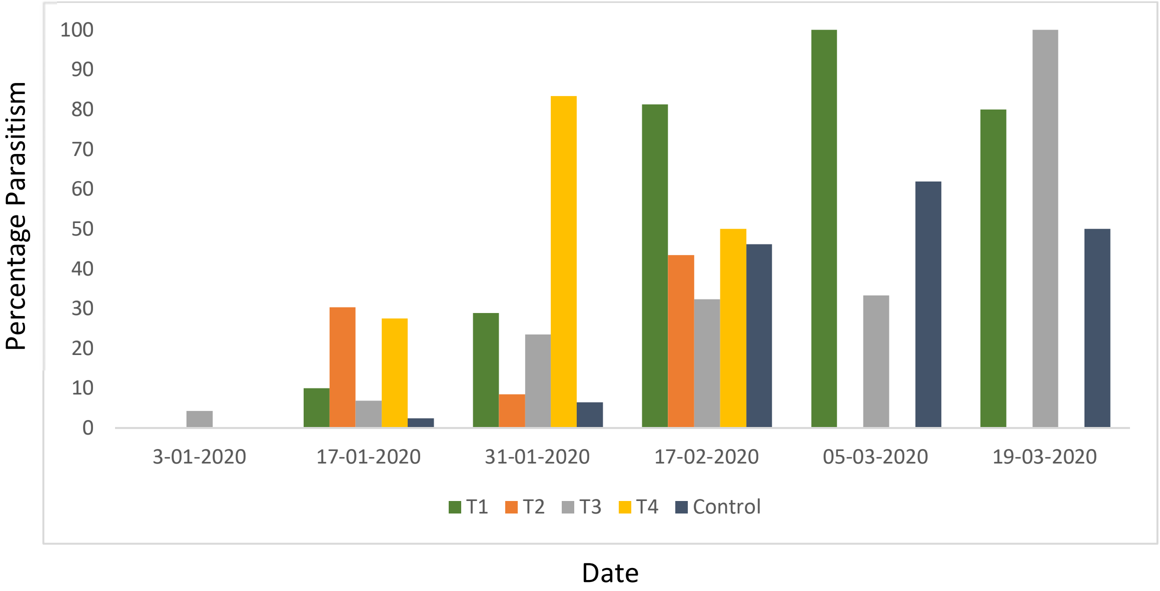


Fig 4.4 Percentage parasitism of 3rd instar mealybug inspected on 10 fruit from each of the Star Ruby grapefruit trial orchards in Hoedspruit (Venren) during the 2019/20 season.

In the following 2020/21 season, the trend in parasitism observed in the Star Ruby trial orchards was similar to the previous season, but peaked at a slightly higher percentage parasitism. However, the parasitism rate in the 2PH lemons did not increase above 32% in any of the trial orchards before harvest. Varying parasitism rates, between 0% and 100 %, were observed in the Nadorcott orchards under net in December and then declined dramatically to lower than 5% in all orchards in January. The Nadorcott trial orchards required a late corrective treatment (methomyl) in February and an additional release of 2000 *A. vladimiri*, after which the parasitism rate gradually increased to above 50% in all orchards in June 2021.

The percentage parasitism in the 2PH lemon orchards in the 2020/21 season was notably lower than the previous season. Parasitism rates increased in February (Figure 4.5). Parasitism of 3rd instar mealybug in the 2PH lemons control orchard was comparatively and consistently higher than the parasitism in other orchards and surprisingly associated with a lower level of mealybug infestation.

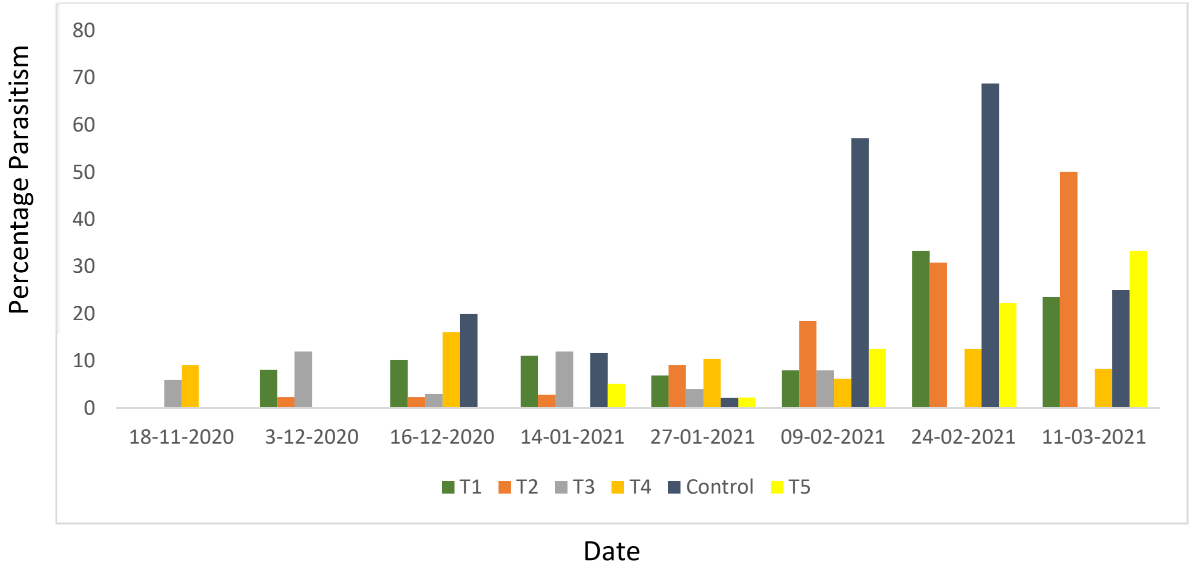


Fig 4.5 Percentage parasitism of 3rd instar mealybug inspected on 10 fruit from each of the 2PH lemon trial orchards in Hoedspruit during the 2020/21 season.

Parasitism rates in the Nadorcott orchard under net during 2020/21 were relatively low for the period between December and March (Figure 4.6). This is in contrast with parasitism observed in the open field orchards where parasitism peaked in February. Percentage parasitism continued to increase up to June, which corresponded with the elevated levels of mealybug infestation. It was noted for the second time on two different farms that an increase in parasitism occurred later in the season compared to open field orchards.

Percentage parasitism in Star ruby orchards on Moria Citrus Estate (Figure 4.7) was very much the same as percentage parasitism on Venren Farm the previous year.

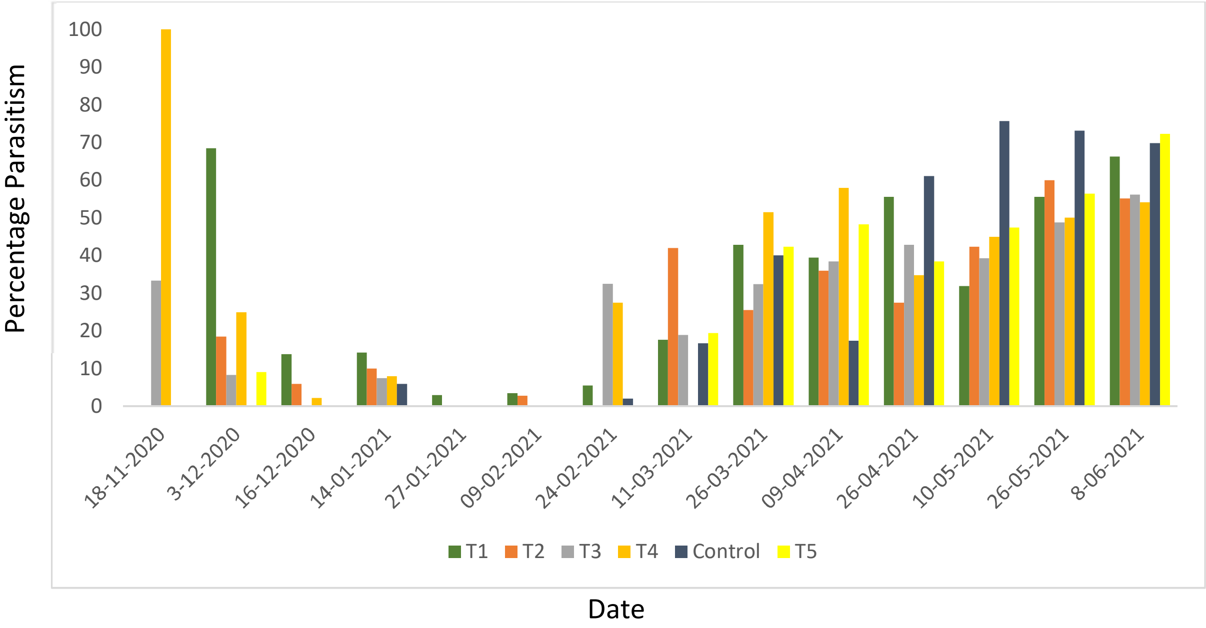


Fig 4.6 Percentage parasitism of 3rd instar mealybug inspected on 10 fruit from each of the Nadorcott mandarin trial orchards in Burgersfort during the 2020/21 season.

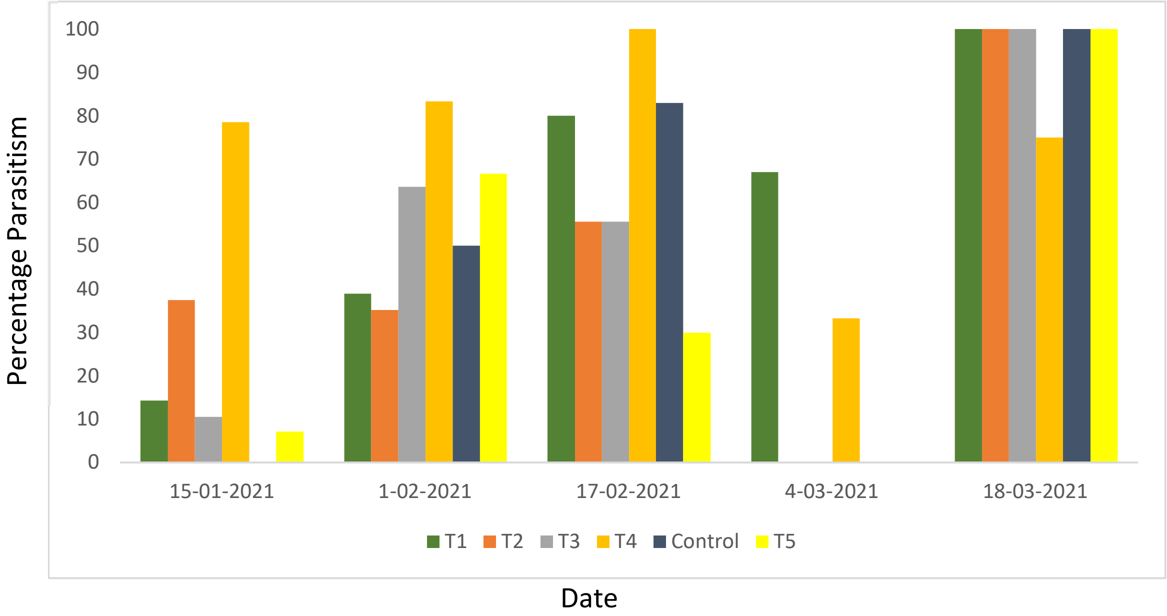


Fig 4.7 Percentage parasitism of 3rd instar mealybug inspected on 10 fruit from each of the Star Ruby grapefruit trial orchards in Hoedspruit (Moria Citrus) during the 2020/21 season.

4.3.2 Comparing percentage parasitism between treatments

Treatments comprised different timings of releases of *A. vladimiri*, across all trial sites. During the 2019/2020 season, there was evidence for a statistically significant interaction term between treatment and week (X2 = 31.53, df = 4, P < 0.001). This result indicates that mealybug parasitism rates varied between parasitoid treatments over time. Initially, the calculated mealybug parasitism rates in T1, T2 and T3 were comparable at 12%, 14% and 17% respectively. The late-season release treatment of 5% parasitism was significantly different at week 10. Surprisingly, the control treatment and treatment 3 produced comparable parasitism levels to the two early-season treatments at week 10 (Figure 4.8). It can be noted that *A. vladimiri* had not yet been released into the T3 orchard (and of course not the control orchard) at this time.

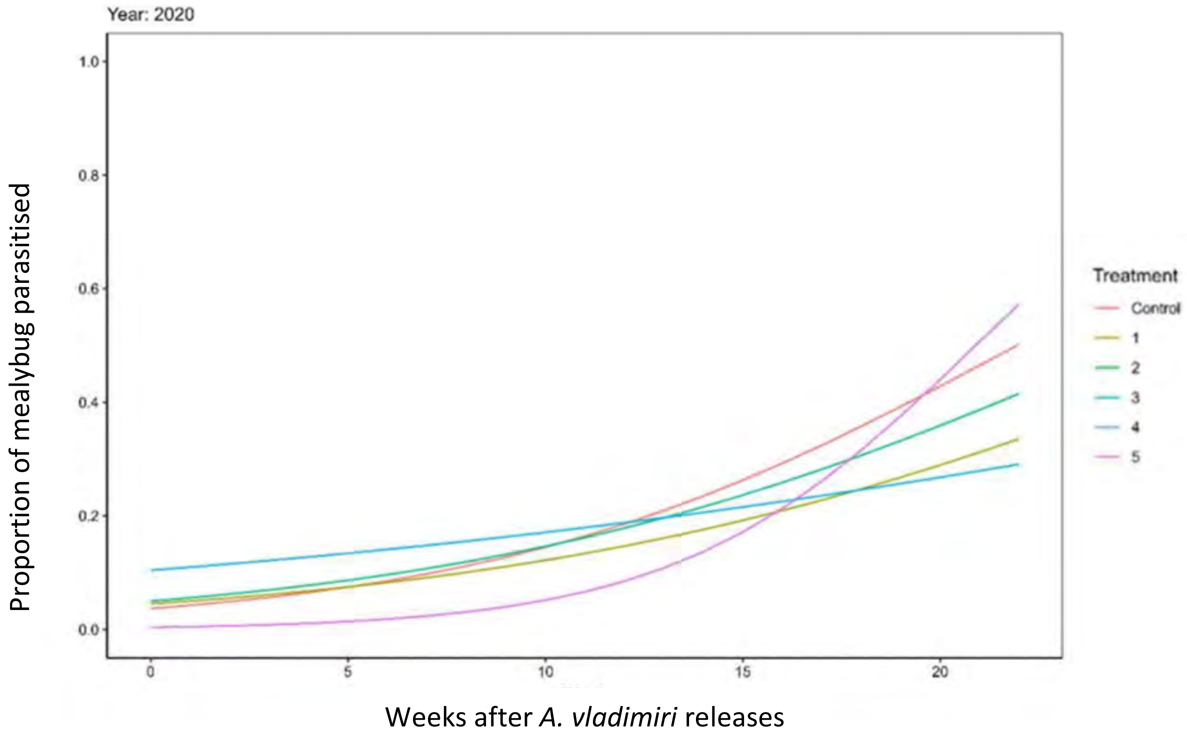


Figure 4.8 Variation in parasitism of third instar mealybug for each treatment as a categorical fixed affect over time during 2019/20.

In contrast, by the end of the sampling period (week 22 = 19/03/2021), mealybug parasitism rates were significantly higher in the control and treatment 4 group of orchards (Control = 50%, treatment4 = 57%) versus the two early-season treatments (treatment1 = 34%, treatment2 = 41%), and the remaining late-season treatment (treatment3 = 29%) (Figure 4.8). P-values in bold indicate statistical significance (Table 4.2).

Table 4.2 Post-hoc comparisons for mealybug parasitism rates in 2019/20 following post-hoc comparisons, adjusted using Bonferronni correction for multiple comparisons.

Contrast	**Estimate**	**s.e.**	***z*-ratio**	***P***
Control-1	0.166	0.071	2.358	0.184
Control-2	0.087	0.074	1.170	1.000
Control-3	0.211	0.071	2.979	**0.029**
Control-4	-0.071	0.077	-0.926	1.000
1-2	-0.079	0.067	-1.182	1.000
1-3	0.045	0.062	0.723	1.000
1-4	-0.237	0.069	-3.418	**0.006**
2-3	0.124	0.067	1.843	0.653
2-4	-0.158	0.074	-2.145	0.319
3-4	-0.282	0.069	-4.059	**0.001**

In the following season, the parasitism rates of the early season releases, treatment 1 and 2, were higher than the other treatments and the control, up to 17 weeks after the first parasitoid releases. There was evidence for a statistically significant interaction term between treatment and week (X2 = 15.93, df = 5, $P < 0.001$). This result indicates that mealybug parasitism rates varied between parasitoid treatments over time. Mealybug parasitism rates were initially higher in the early-season release orchards (treatment1 = 22% parasitism rate in week 10, treatment 2 = 13% parasitism in week 10) versus the late-season release orchards, particularly in the latest release orchard (treatment 5 = 1% parasitism rate) and in the control orchard (control = 5% parasitism rate). One exception is the 18% parasitism rate in treatment 4, which was unexpected because parasitoid releases had not been done in these orchards at this time. In contrast, by the end of the sampling period, mealybug parasitism rates were qualitatively comparable across the release treatments, with no significant differences observed between treatments at week 21 after initial releases (Table 4.3). Parasitism rates ranged from 43-48% across treatments, albeit treatment 3 had slightly lower parasitism rates (36%), and surprisingly, the control treatment had the highest parasitism rates (56%) (Figure 4.9)

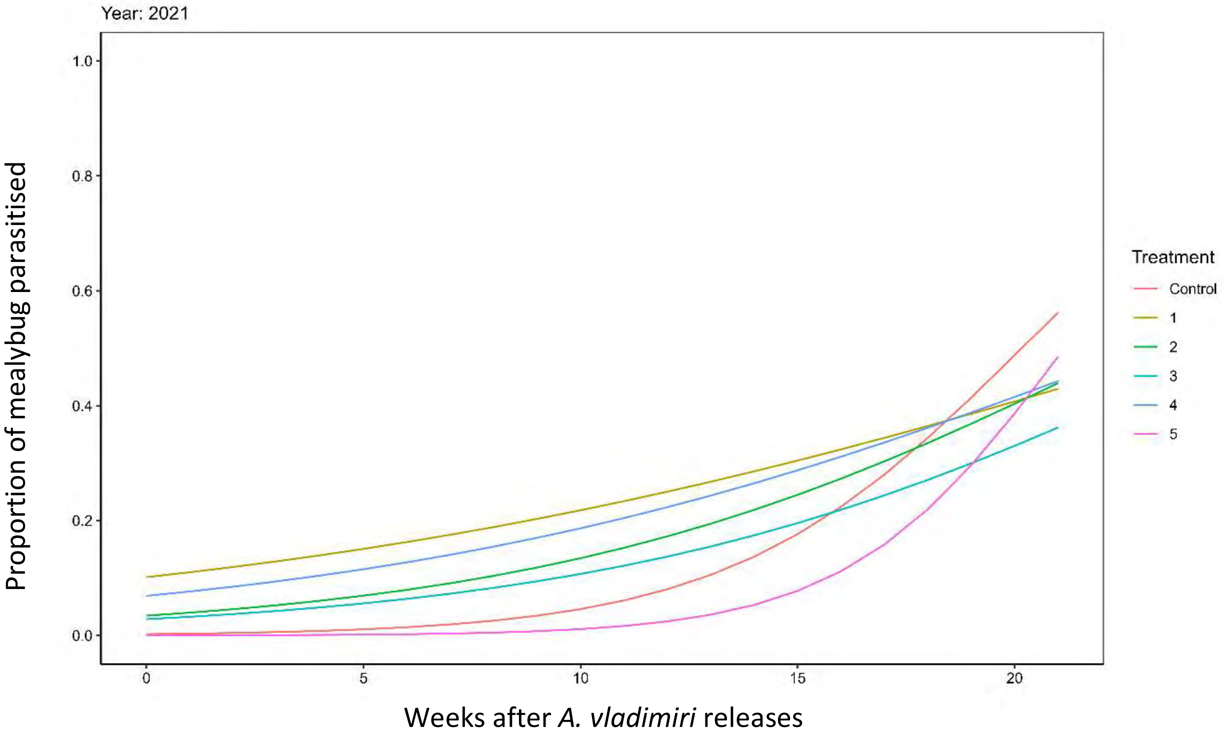


Figure 4.9 Variation in parasitism of third instar mealybug for each treatment as a categorical fixed affect over time during 2021.

Table 4.3 Post-hoc comparisons for mealybug parasitism rates in 2021, following post-hoc comparisons adjusted using Bonferronni correction for multiple comparisons.

Contrast	**Estimate**	**s.e.**	***z*-ratio**	***P***
1-2	-0.010	0.081	-0.122	1.000
1-3	0.067	0.082	0.824	1.000
1-4	-0.014	0.080	-0.169	1.000
1-5	-0.056	0.083	-0.674	1.000
1-Control	-0.133	0.089	-1.495	1.000
2-3	0.077	0.082	0.942	1.000
2-4	-0.004	0.080	-0.046	1.000
2-5	-0.046	0.083	-0.554	1.000
2-Control	-0.123	0.089	-1.381	1.000
3-4	-0.081	0.081	-0.994	1.000
3-5	-0.123	0.085	-1.458	1.000
3-Control	-0.200	0.090	-2.219	0.397
4-5	-0.042	0.082	-0.514	1.000
4-Control	-0.119	0.089	-1.349	1.000
5-Control	-0.077	0.091	-0.847	1.000

4.3.2 Identification of parasitoids

The parasitoid species complex was determined with more than 1030 specimens collected over the two-year study. Five primary parasitoids, four hyperparasitoids and one other parasitoid associated with mealybug were collected. Three parasitoids of other pests on citrus were collected from samples (Table 4.4).

Table 4.4 Identity of parasitoids, hyperparasitoids and other species that eclosed from mealybug samples that were collected every two weeks in trial orchards.

Parasitoid species	**Family**	**Number (n)**
Primary parasitoids		
Anagyrus sp.	Encyrtidae	1
Anagyrus vladimiri	Encyrtidae	398
Cheiloneurus sp.	Encyrtidae	6
Coccidoxenoides perminutus (Girault)	Encyrtidae	75
Leptomastix dactylopii (Howard*)*	Encyrtidae	20
Hyperparasitoids		
Chartocerus sp.	Signiphoridae	92
*Coccophagus rusti (*Compere)	Aphelinidae	1
Pachyneuron sp.	Pteromalidae	114
Procheiloneurus sp.	Encyrtidae	5
Other		
Homalotylus africanus (Timberlake)	Encyrtidae	1
Marietta connecta (Compere)	Aphelinidae	1
Oencyrtus sp.	Encyrtidae	1
Pseudaphycus sp.	Encyrtidae	315

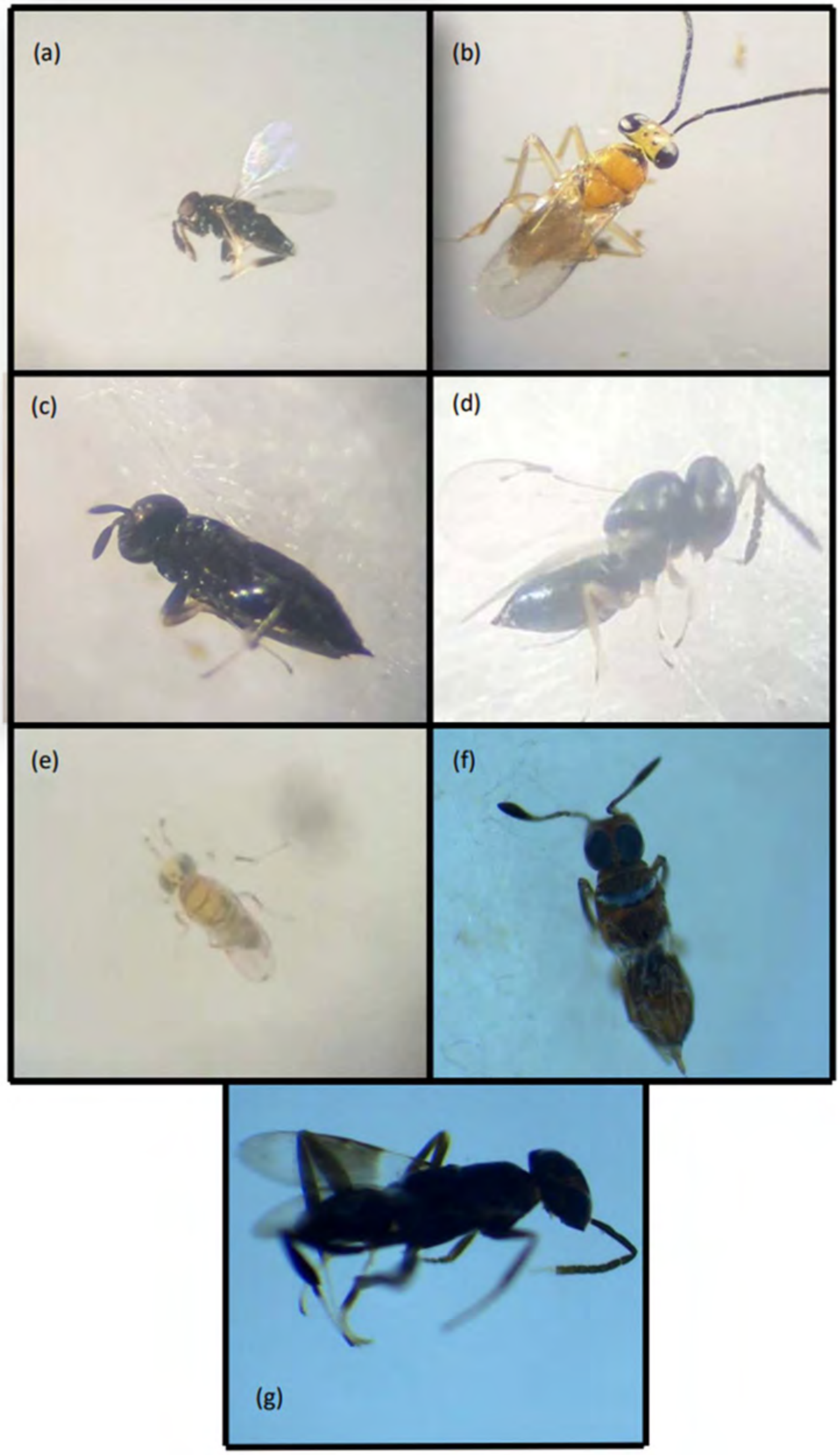


Figure 4.10 Parasitoids collected from mealybug infested citrus between October 2019 and June 2021: a. *C. perminutus*, b. *L. dactylopii*, c. *Chartocerus* sp., d. *Pachyneuron* sp., e. *Pseudaphycus* sp., f. *Procheiloneurus* sp., g. *Homalotylus africanus*.

Anagyrus vladimiri comprised the highest proportion of parasitoids collected. Parasitoids and hyperparasitoids, other than *A. vladimiri*, that were collected in notable proportions were *Coccidoxenoides perminutus*, *Chartocerus* sp., *Leptomastix dactylopii*, *Pachyneuron* sp. and the encyrtid *Pseudaphycus* sp. (Figure 4.10e), which was discovered for the first time on citrus. Interestingly, the second highest proportion of parasitoids collected comprised of *Pseudaphycus* sp. (Table 4.4). Samples were often damaged during collection. Therefore, a photograph of each specimen was taken, when possible, for future reference and some of the photos can be viewed in Figure 4.10 above.

4.3.3 Seasonal abundance of parasitoids and hyperparasitoids

Observations of successful mealybug parasitism in the 2PH lemons were made between November and April in the 2019/20 season (Figure 4.11 a), as live parasitoids and hyperparasitoids were aspirated from samples of mealybug infested fruit.

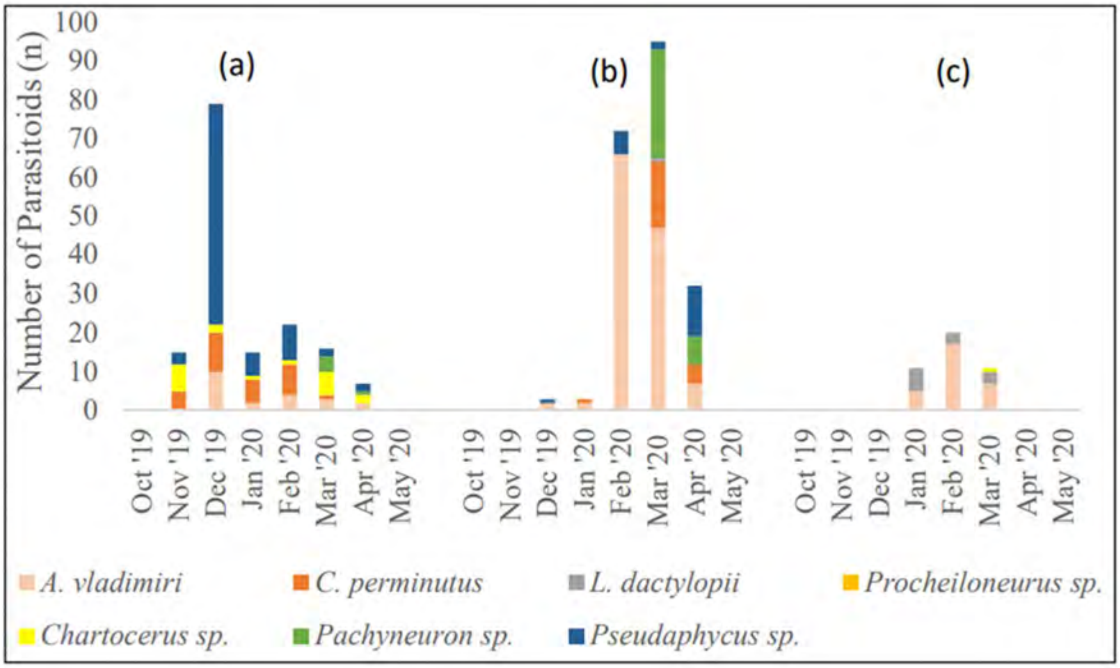


Figure 4.11 The seasonal abundance of primary parasitoids and hyperparasitoids eclosed from samples of mealybug from citrus orchards during the 2019/20 season. (a) 2PH lemons; (b) RHM mandarins under net; (c) Star Ruby Grapefruit.

In RHM mandarin orchards under net (Figure 4.11 b), parasitoids and hyperparasitoids were collected from December to April 2019/20. In Star Ruby orchards, monitoring of successful parasitism was possible for only a short period between January and March because mealybug infestation was very low and there was hardly any fruit with suitable infestation on until January (Figure 4.11 c)

Higher numbers of parasitoids were collected in the 2020/21 season which coincided with elevated levels of mealybug infestation, particularly in Burgersfort 2PH lemon and Nadorcotte orchards (Figure 4.12). Parasitoids were collected between November and March in the lemons and collections continued for longer, until June, in the Nadorcott orchards under net (Figure 4.12 a and b). Parasitoid numbers collected from mealybug infested grapefruit in Hoedspruit were much lower than in the numbers on lemon and mandarins (under net) in Burgersfort.

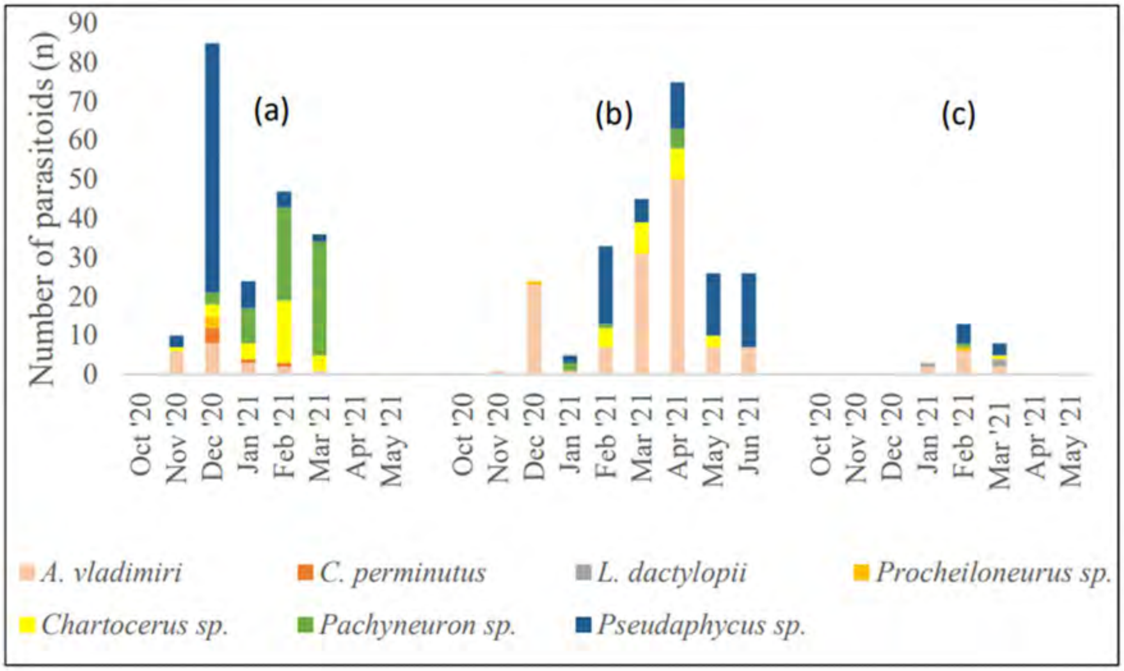


Figure 4.12 The seasonal abundance of primary parasitoids and hyperparasitoids eclosed from samples of mealybug from citrus orchards during the 2020/21 season. (a) 2PH lemons; (b) Nadorcott mandarins under net; (c) Star Ruby Grapefruit.

The highest proportions of *A. vladimiri* were collected in mandarin and grapefruit orchards. The highest proportions of the hyperparasitoid *Pachyneuron* sp., the unknown *Pseudaphycus* sp., as well as *C. perminutus* and *Chartocerus sp.* were collected in the 2PH lemon and the mandarin orchards in the Burgersfort region. *Leptomastix dactylopii* was abundant primarily in mealybug samples from grapefruit orchards in Hoedspruit (Figure 4.12 c).

There was a major shift in balance between the overall abundance of primary parasitoids and hyperparasitoids between the two seasons, with a reduction in numbers of primary parasitoids sampled in 2021 and the increase in numbers of particularly *Pseudaphycus* sp. and *Pachyneuron* sp. (Figure 4.13).

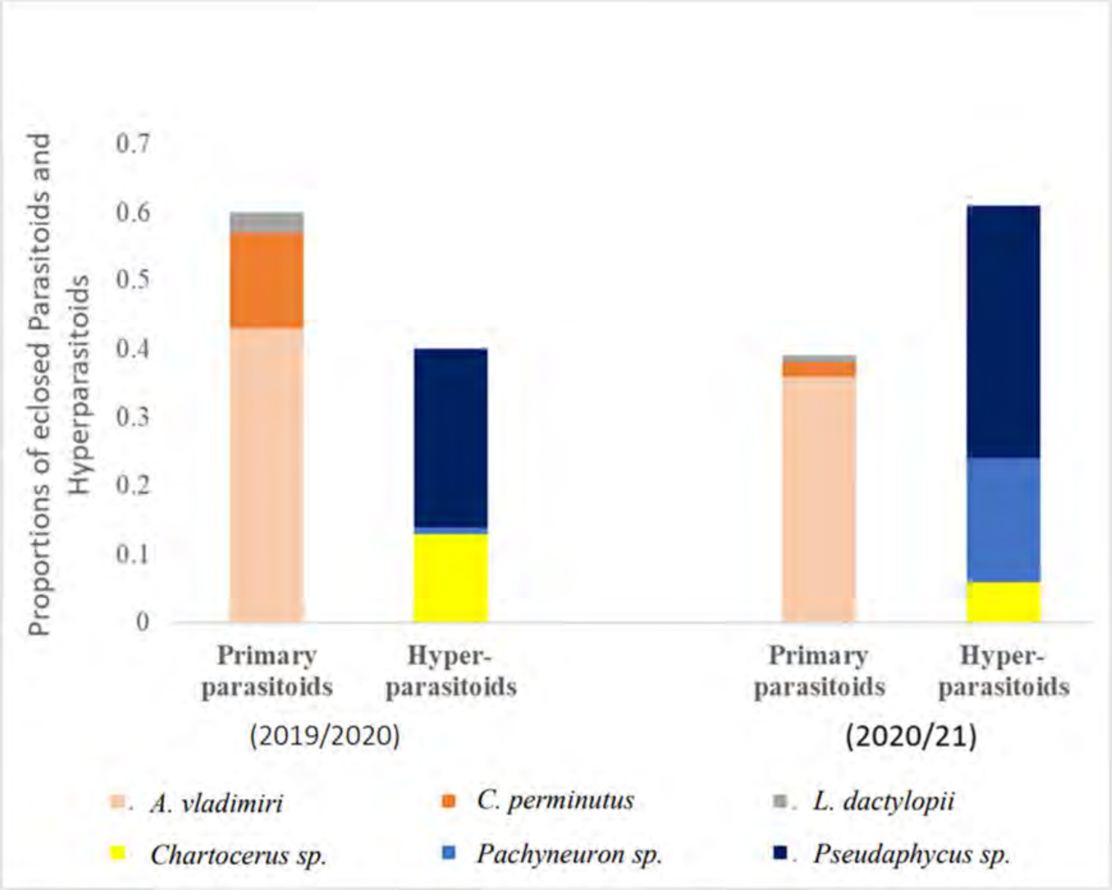


Figure 4.13 The accumulated proportion of primary parasitoids vs. hyperparasitoids eclosed from samples of mealybug infested fruit over two seasons.

4.4 Discussion

Initially, the investigation of parasitism data was to determine the abundance of natural and released parasitoids in the trial plots with the expectation that we could correlate catches of *A. vladimiri* with the successful parasitism of citrus mealybug. However, the presence of various hyperparasitoids in samples, often in greater abundance than primary parasitoids, was unexpected. In the 2019/20 season there was little cause for concern, considering mealybug populations were well managed at relatively low levels in trial orchards. The following season, concerns were raised about the efficacy of *A. vladimiri* augmentations when mealybug infestation in Burgersfort trial orchards escalated to uncontrollable levels, particularly in the Nadorcott mandarin orchards under net. With the IPM programme that was applied during the season, there was little explanation as to why mealybug control was failing. The analysis of parasitoid abundance, post-season in 2020/21, alerted to a higher proportion of hyperparasitoids compared to primary parasitoids. In Hoedspruit, hyperparasitoids were also present in grapefruit orchards in 2021, but not at the same levels. The increased abundance of hyperparasitoids coincided with the severe mealybug infestation recorded in Burgersfort trial orchards. In the field, observations of 3rd instar mummies hosting multiple parasitoids, up to seven, was a clear indication of the presence of a gregarious parasitoid. *Pseudaphycus* sp. was suspected. In other studies, *Pseudaphycus maculipennis* has been described as a host specific, primary parasitoid of the obscure mealybug, attacking third instar females as a facultative, endoparasitoid (Charles et al., 2004). *Anagyrus vladimiri* was abundant in the early-treatment orchards in November and December in Burgersfort. However, the numbers of *A. vladimiri* suddenly declined into January, correlating with an increase in collections of *Pseudaphycus* sp. in December. Another observation was the decline in the abundance of *A. valdimiri* in 2PH lemon orchards, which coincided with a sharp increase in *Pachyneuron* sp. A study on the biocontrol of the invasive mealybug *Phenacoccus peruvianus* in Europe describes a higher mealybug infestation in 2008 where *Pachyneuron* was abundant (23.0%) and parasitism by the primary *L. dactylopii* was low. In the following season, *L. dactylopii* was displaced by the gregarious *Acerophagus* sp. and the abundance of *Pachyneuron* sp. dropped to 2.1% in 2009 and 1.0% in 2009 (Beltrà et al., 2013).

In orchards under net, captures of *A. vladimiri* increased in March. *Pseudaphycus* sp. was more abundant when mealybug infestation was increasing, which suggests a positive correlation with mealybug density, as described to be the norm in other studies (Beltrà et al., 2013). However, not knowing the species makes it difficult to comment on the type of interaction between *A. vladimiri* and *Pseudaphycus* sp. In 2021, in the Nadorcott orchards, there was no sign of early instar parasitism, including zero captures of *C. perminutus*. This was unexpected and in contrast to the previous season. Parasitism of younger instars is expected to contribute positively to biocontrol. The failure in biocontrol of younger instars could explain the escalation of mealybug infestation in the Nadorcott orchards in 2021. One might expect this to be a result of a disruptive chemical treatment. Analysis of spray records showed that only applications of abamectin between 10 October and 12 December were made, which would not have caused any repercussions because it is a soft option. Another argument is that the *Anagyrus* releases, possibly at an opportune stage in the establishment of the biocontrol complex, could themselves contribute to a destabilising effect. The displacement of *Leptomastix epona* by releases of *Acerophagus* sp. has been previously described, with the latter becoming the main primary parasitoid in the biocontrol of *Phenococcus peruivianus* (Beltrà et al., 2013). It is possible that *Pseudaphycus* sp. could have contributed in a similar manner in trial orchards in Burgersfort in 2020/21.

Irrespective of timing of releases our results show an abundance of *A. vladimiri* in February. A study of the abundance of parasitoids in the control orchards indicated the presence of *A. vladimiri* in comparible proportions to test blocks, later in the season. Over time the dispersal of *A. valdimiri* to other orchards is expected.

Statistical analysis of partasitism between treatment groups indicated a positive correlation between early releases and increased parasitism levels at an earlier stage of the season. This supports a strategy of early releases. However, the same orchards had lower parasitism later in the season. Competition between two parasitoids sharing the same target has been described where one has a suppression effect on the other (Muştu et al., 2015). The interactions between all naturally occurring species and the species we released are far more complex, as studies have shown that the difference between a synergistic effect and a suppression effect is the timing, and which of the two parasitoids arrive at the host first (Aguirre et al., 2021). One could argue that earlier control of mealybug leads to a reduction in mealybug numbers and impacts on the probability of finding sufficient sample fruit that are infested with mealybug to accurately assess parasitism. Observations were that increased parasitism coincided with slightly elevated mealybug infestation for a longer period, compared to other orchards where mealybug infestation declined more rapidly due to biocontrol. Only in one orchard, the control orchard in the 2PH lemons during the 2020/21 season, did the high parasitism correlate with lower infestation.

Statistical analysis showed no significant difference in parasitism rates, at the end of the season before harvest, that correlated to timing of releases of *A. vladimiri.* Parasitism of 3rd instar mealybug was not only from *A. vladimiri*, but also from the naturally occurring parasitoids. This skews interpretation of the parasitism data and can be noted as a limitation in the methodology followed in this study. For future studies it is therefore important to isolate individual, parasitized mealybug where possible and follow up with corresponding eclosion studies.

Maximum parasitism rates in individual orchards were in excess of 40% in season one, when adequate mealybug control was achieved. In season two, the maximum parasitism never went above 32% in lemon orchards and poor control of mealybug was recorded. In the Nadorcott orchards, the early parasitism was below 20% and there was no notable control of mealybug in December and January. However, when parasitism increased to above 50% later in the season, after a corrective chemical treatment had been applied and additional *A. vladimiri* releases had been conducted, good control was achieved. In Hoedspruit, the maximum parasitism ranged consistently between 40% and 100% and mealybug control was consistently attained over two seasons. I refer to a study which investigated the maximum parasitism rates of 58 pest species. A weighted threshold average for parasitism rates, as a measure of successful biocontrol, was proposed (Hawkins et al., 1994). The higher of two thresholds was reported as a range between 33 - 36% parasitism. The lower threshold was reported as 32%. It was hypothesised that biocontrol has a high probability of being successful if parasitism levels reached the threshold or surpassed it.

The abundance of natural enemies in citrus orchards is indeed similar to previous studies conducted in other crops in South Africa, for example, in vineyards (Wakgari et al., 2003). During a survey of the natural enemies of *P. ficus* in three vineyards in the Western Cape Province, South Africa, parasitoids were caught from January to May, with a peak in February (Faure, 2015). Peak activity of the natural enemies in citrus occurred in February in the 2PH lemons and Star Ruby open orchards. Peak activity occurred later in the RHM mandarin orchards under net, in March, and even later in the Nadorcott orchards, in April, when high mealybug levels persisted in 2020/21. Conservation practices should be employed to prevent harmful residues in any orchard so that the natural biocontrol is not undermined. Future qualitative studies looking at interactions between the *A. vladimiri* and the local natural enemies in citrus orchards will assist with the identification of the different interaction mechanisms between the different species of natural enemies and lead to an understanding of how it impacts on augmentative releases.

Chapter 5

General Discussion

5.1 Introduction

It was the objective of this study to investigate strategies within a strict IPM context, which included augmentative releases of *A. vladimiri* to effectively supress mealybug population numbers to lower levels than is currently being achieved through conventional sprays and conservation biological control. This is important to the citrus industry, because any live mealybug on citrus fruit imposes serious risk to market access. The study considered the holistic management of all pests, including other major economic pests. This was done firstly, by considering the impact of pesticide treatments on parasitoids, using *A. vladimir*i as an indicator species. This was to assist with planning of spray strategies that ensure a safe environment for augmentative releases to take place. The overall benefit of an environment free from harmful pesticide residues extends to the naturally occurring biocontrol agents. Interactions between established natural enemies of mealybug (other than *A. vladimiri*) and released *A. vladimiri* have not been well studied, so the aim was to determine if improving conservation biocontrol would contribute to better control of mealybug. The other important focus of the study was to investigate the influence of timing of augmentative releases. The aim was to identify the most appropriate timing for initiation of augmentative releases, which could lead to improved parasitism rates and ultimately improved mealybug control.

5.2 Scope of research

Crop protection is essential in commercial agriculture in order to grow fresh produce economically, for food security and to meet the demands of consumers around the world. Conventional crop protection has relied on the application of pesticides to control insect pests for many years, with success, but an increasingly discerning consumer market has driven the need to reduce chemical pesticide usage and develop alternative technologies for pest control (Beckman et al., 2022) that address human health and the reduction in carbon emissions (Heimpel et al., 2013). The increase in the number of pest species that have become resistant to pesticides and the number of pesticide chemistries that some species have become resistant to (Hawkins et al., 2019) has also increased emphasis on biological control. I further, international markets have enforced restrictions on maximum allowable residues on fresh fruit for consumption (MacLachlan et al., 2010), practically reducing the chemistries that can be used. Also, the rate at which new active ingredients have been produced has been far below the requirements of fruit production industries around the world. On the scale at which crop production takes place, pest populations are increasing and challenges with effective management are inevitable. This leads to poor decision making and poor pest control. The general sentiment is thus that current crop protection strategies are not sustainable.

IPM has been a concept that has gained traction over the last 20 years by introducing biological control into pest management programmes with the aim of using less chemicals (Ehler, 2006). This has been accepted by industries across various crops as an environmentally responsible means to control pests. The progression of IPM has been well documented by the citrus industry in southern Africa (Grout, 2015). In a growing export industry environment, with multiple logistics events and long shipping times to export destinations, the need for quality fruit production has been extremely important. Fruit with good taste, without blemishes and free from infestation of pests is the primary standard. Insect pest management is a major part of citrus production and fruit quality and a large proportion of efforts go into controlling bollworm, thrips, red scale and mealybug economically. These are the springtime pests that can cause major damage if populations are allowed to flourish. Pesticide use is commonly associated with outbreaks of these pests and in particular, outbreaks of the different mealybug species are well known to be repercussions from disturbances caused to their natural enemies.

The ability of mealybugs to seek refuge in alternate hosts is both good and bad in that it persists in the environment and eradication is not possible. The recent escalation of mealybug to phytosanitary pest status has brought about the need to develop alternatives to conventional pesticide application. *Planococcus citri* has however co-evolved with many diverse species of natural enemies, constituting a very effective biological control complex. Many of the species have been identified and well documented over years of research in South Africa (Prinsloo, 1984). Because of the close relation between citrus mealybug and the vine mealybug, *P. ficus,* much of the research has been conducted on these two species and they are commonly referenced with regard to biological control. Because of a good base of understanding of these species and reliability in supply of commercial biocontrol agents for augmentative releases in the field, there is merit in further research in the field of biological control, which can answer conceptual questions that are relevant for other species.

Large scale production of *A. vladimiri* in South Africa has been established since 2017 and has resulted in it becoming a popular agent for augmentative biocontrol in citrus orchards. However, there are still many unknowns with regards to effective use of *A. vladimiri* as a biocontrol agent on citrus and should be addressed through future research.

5.3 Managing non-target effects

Biocontrol representatives have been promoting the sales of beneficial insects for a little over two decades in South Africa. Growers rely on technical information that is provided by the representatives, very often, with little reference to information on efficacy that is based on sound data, including application and limitations. There is a very general understanding of IPM and the need for incorporating biocontrols into such a programme, but this is often undermined by an innate fear that beneficial insects will be killed by pesticide applications and therefore, ironically, are not compatible with an IPM strategy.

The methodologies developed for conducting non-target assays have only been applied to a few commonly used parasitoid species namely *Aphytis* sp., *Coccidoxenoides perminutus*, *Euseius* sp. and *Trichogrammatoidea cryptophlebiae,* and the results have been used to develop an IPM compatibility rating system that growers can use to make informed decisions (Hattingh et al., 2000). It was the aim of this study to continue this initiative with a contribution towards information on a new parasitoid species relevant to the industry.

There are three important findings in this non-target effects study that are relevant for the use of augmentative biocontrol in general and the specific releases of *A. vladimiri*. The first is is that each pesticide needs to be evaluated on merit and it cannot simply be assumed that a product is incompatible with an IPM programme. This is highlighted by the low mortality of *A. vladimiri* when treated with prothiofos. The efficacy of organophosphates as broad-spectrum pesticides is known to the industry. In this study, under semi-field conditions, the residue of the active ingredient was surprisingly harmless to adult *A. vladimiri*. It can be debated that there might have been some reduced effect due to the translaminar-systemic ability of the chemical, or the possibility of a repellent effect.

The second important finding is that there is a potential for shorter withholding periods for augmentative releases with a better understanding of the effects of pesticides on non-target organisms. For comparative purposes, it is important that the same method of testing is used. For example, the laboratory assays where prothiofos was sprayed on petri dishes resulted in 98% mortality of *C. perminutus* after 24 hours (Wakgari et al., 2003). This is very different from results obtained in the semi-field bioassays conducted by Grout et al. (2011), where the mortality recorded was 7.5%. Results from the current study show that impact values for the majority of the selected pesticides on *A. vladimiri*, except spinetoram, were lower than the impact value for *C. perminutus*. This supports findings in previous studies that described *Anagyrus vladimiri* as being more robust than *C. perminutus* and can better withstand the impact of certain pesticide residues (Mgocheki et al., 2015). This information could add value in the decision-making process on what products to incorporate in an IPM programme on citrus. Shorter withholding periods for releases of *A. vladimiri* after a pesticide sprays will assist in targeting the correct life stage of mealybug for parasitism to be successful.

The third important finding is alluded to by the differences in impact of pesticides between species of the same family. Consideration for the impact of a pesticide on natural enemies other than the ones being released is very important. For example, the study where the direct contact effects of acetamiprid and clothianidin at label rates differ between the ladybird beetle, *Cryptolaemus montrouzieri* (Mulsant) and the parasitoid, *Leptomastix dactylopii* Howard (Cloyd et al., 2006).

A few questions have been answered about effects of pesticides on non-target parasitoids, but importantly it provides additional information on when and where *A. vladimiri* augmentative releases should be made, specifically in citrus in South Africa. Growers and IPM consultants will now have an additional reference point on what decisions can be made, and can use the information as a guideline for implementing IPM in their pest control strategies. Consequently, confidence to manage the augmentative releases around a historically rigorous thrips spray programme will grow.

5.4 Impact of ecological factors

Supplementary to the monitoring of mealybug infestation in orchards, where the focus is on the efficacy of augmentative releases to reduce the mealybug population, it is just as important to keep an eye on the mechanism of control. In this instance it is the percentage parasitism of mealybug by *A. vladimiri*. It is also an opportunity to survey for other parasitoids and predators, which can be identified, to determine the abundance of different species in the the crop environment. Such studies can provide a snapshot in time as to the ecological activity within the crop. Various researchers have reported on the abundance of parasitoids associated with mealybug in vineyards and other crops, including citrus (Mgocheki, 2008; Faure, 2015; Wakgari et al., 2003).

For the improvement of IPM strategies in citrus it is vital that observations be converted into practical solutions. This study has drawn attention to some fundamental intricacies in biological control that will lead to future studies and a better understanding of augmentative releases of *A. vladimiri*. Only once a better understanding is achieved, will the practical solutions follow. In this instance, the aim was to investigate parasitism of *A. vladimiri*, and local parasitoid species, to see if the different timings of augmentative releases would have a positive effect on parasitism and ultimately efficacy of augmentative releases.

Parasitism from *A. vladimiri* was recorded in all trial orchards. This shows that *A. vladimiri* is well-adapted in citrus orchards, which is positive, and supports the use of *A. vladimir*i in citrus. Parasitism percentages were higher in the Star Ruby grapefruit and the mandarin orchards under net. Also, the proportion of *A. vladimiri* collected in Star Ruby orchards was consistently the highest over two seasons. The resulting decrease in mealybug peak infestation in these orchards, when *A. vladimiri* was released early in October, is encouraging for the citrus industry. Practically this is a step in the right direction for reducing risk of mealybug on this variety, which is harvested early in the season.

In the mandarin orchards, it has been reported that elevated mealybug levels are to be expected (Marsberg et al., 2023). Parasitism under net is also expected to be good. However, it takes longer for biocontrol to take place and bring mealybug under control (Grout, 2022). Outbreaks are likely to occur, in which case biocontrol may not be able to suppress mealybug to low enough levels in time before harvesting takes place. It is therefore an opportunity for augmentative releases of *A. vladimiri* to make a difference. In this study a mealybug outbreak event was experienced in the Nadorcott mandarin orchards under net. This is not unusual. Mealybug is deemed uncontrollable and investigators have sometimes not been able to produce a good explanation. In this study an investigation into the abundance of parasitoids of mealybug highlighted a very important observation in this study. This was the presence of hyperparasitoids, including a new parasitoid discovered for the first time on citrus in South Africa. Further investigation into proportions of parasitoids and hyperparasitoids, indicated elevated levels of hyperparasitoids in proportion to the primary parasitoids, which occurred at a 40:60 (hyperparasitoids to primary parasitoids) ratio in the first season and in the second season, the ratio shifted in favour of the hyperparasitoids, which was an eye-opening discovery. This may have answered the previously inexplicable question of historical, uncontrollable mealybug outbreaks, which seem to be of a cyclical nature. This will open the door to a deeper understanding of the management of biocontrol and augmentative releases of *A. vladimiri* for mealybug control. However, in order to acquire full understanding on the role and cyclical nature of hyperparasitism, further research is strongly justified.

Comparing parasitism of third-instar *P. citri* between different treatments, across all trial sites, indicated a significant correlation between early season releases and increased parasitism. However, later in the season, before harvest, this difference had generally disappeared. The parasitism in the other treatment groups indicted higher parasitism than the early release treatment group, later in the season. One explanation is the correlation between elevated mealybug levels and parasitism, indicating a previously unsuspected density dependent performance of the parasitoids. It therefore stands to reason that early season control of mealybug, which lowers the peak infestation levels, reducing the availability of mealybug hosts, may indeed have a negative impact on of the extent of parasitism. This may lead to differences in parasitism levels, but does not necessarily mean that biocontrol of the mealybug pest has failed. These differences may in part be because observations on parasitism have been assessed in a similar manner to the assessment of efficacy (mealybug infestation), which may not be the most appropriate method. Analysis of percentage parasitism has been problematic over the years because of the challenge of obtaining accurate estimates of mortality from parasitism (van Driesche et al., 1991). Other methods for improved monitoring of parasitism, where augmentative releases of *A. vladimiri* are made for mealybug control, should be investigated.

Early releases and possibly additional follow up releases might be a solution in orchards under net when elevated mealybug levels are present in orchards. This is something that needs to be researched.

5.5 Efficacy of *A. vladimiri* as an augmentative biocontrol agent

Since the start of the millennium, a number of opportunities have arisen for the implementation of biocontrol and bio-rational methods for controlling important citrus pests in South Africa. Successes where efficacy has been statistically verified include the application of the sterile insect technique in the Western Cape (Hofmeyr et al., 2015) and the spray application of a novel granulovirus, which kills the larval stages of the false codling moth, *Thaumatotibia leucotreta* (Moore, 2002). Efforts to control red scale with a few different species of the aphelinid parasitoid *Aphytis* spp., have not been as successful (Daneel et al., 2000; De Beer, 2023). A common frustration is the inability to show a significant difference between a treated area and a control.

The aim of biocontrol is to reduce the populations of the pest, which is citrus mealybug in this instance. There have been many studies conducted on the biocontrol of mealybugs with parasitoids from the family Encyrtidae being most successful (Moore, 1988). Augmentations of *Coccidoxenoides perminutus* for control of citrus mealybug is mentioned in literature (Bedford et al.,1998) and its association with citrus mealybug in South Africa is described by various authors (Prinsloo, 1984; Grout, 2015). However, some studies have described the sensitivity of C. perminutus in a dry climate which raises some questions on the effectivity of augmentations in the field. (Gol'Berg, 1982; Ceballo et al., 2005). In 2012, *A. vladimiri* was introduced as a biocontrol agent for *P. citri* in organic orchards in the Eastern and Western Cape with a high level of efficacy (Moore et al., 2014)

The citrus industry maintains a high standard in management of the crop and has a very strong technical support base from self-funded research. The industry has faced many challenges with regard to fruit quality and shelf life, even though these are well managed in general. The new era in citrus exports of high volumes of fresh fruit around the world has added a few more challenges in the phytosantiary status of some of the major citrus pests. Science has been the backbone for motivating and maintaining market access. The challenge we face with control of the phytosanitary pests is the global drive to reduce chemical applications on fresh citrus, which reduces the tools for controlling pests. There is therefore a need to focus attention on alternative, more biological approaches.

The efficacy of different timing of *A. vladimiri* augmentations on the reduction of *P. citri* infestation levels was not significantly different between treatments. Similar results were reported in another study where no difference in efficacy between early and later treatments was determined (Mendel et al., 1999). A number of observations were extremely valuable in the current study. Early releases lowered the peak percentage of mealybug during the season. This is important because it reduces the presence of sooty mould on fruit, reducing the phytosanitary risk. It also reduces the risk of high numbers of crawlers closer to harvest. Growers are advised to employ strict monitoring and quality control measures two weeks before harvest in orchards and when fruit arrive at packhouses to ensure fruit are free from any mealybug and sooty mould before sending fruit to markets sensitive to mealybug (Hattingh et al. 2020). It is also important to note that time is needed for successful generations of *A. vladimiri* to multiply before the peak in mealybug infestation, which was generally at the end of January.

In this study, the augmentative releases in the Star Ruby grapefruit trial sites are considered more effective compared to augmentative releases in the other trial sites over the two seasons of the trial. This was particularly so with early releases of *A. vladimiri* in October in the Star Ruby trial orchards. In lemon orchards, inoculative augmentation of *A. vladimiri* may not be as effective as in other cultivars, because of the regular re-infestation by mealybug crawlers from an earlier fruit set onto new young fruit observed in this study. Lemons can set flowers repetitively in suitable climatic conditions and depending on water and nutrition management (Krogscheepers, 2020). From our observations it is evident that differences in effectivity of augmentations exist between different regions or cultivars which was also suggested in the study by Mendel et al. (1999).

It was also observed that efficacy of augmentative releases in control orchards was in some instances better than the release orchards. The abundance of natural enemies in orchards plays an important role. Not mentioned in this study was the abundance of predators. Various ladybird species were observed at low levels. In Hoedspruit, a large number of lacewings were abundant during the summer. It is believed that the high abundance of lacewing made a positive contribution to mealybug control in the Star Ruby orchards. Messelink et al. (2016) showed that the green lacewing, *Chrysoperla lucasina*, reduced mealybug infestations to low levels in all treatments during trials done in greenhouses.

The management of pesticide sprays, including their scheduling, were vital to maximise the abundance of parasitoid species in this study. It is also the reason for comparatively good biocontrol in the orchards, where augmentative releases did not take place. It is recommended to growers to follow this example and to create a safe environment for beneficial insects where augmentative releases have a better chance of succeeding. From observations in this study, it will be advisable to apply preventative controls for mealybug early in the season on citrus to reduce the risk of disturbing the biocontrol agents in the summer, when the pest populations start to increase. This is a vital contribution for progression in the efforts to improve biological control in citrus.

5.5 Future research

From this study a number research proposals can be made, which could be explored in future, such as: studies on repellent effects of pesticides on natural enemies; the effect and cyclical nature of hyperparasitism on conservation and augmentation biocontrol of mealybug; the influence of different release rates of *A. vladimiri* on the efficacy of augmentations; identification of *Pseudaphycus* sp in citrus orchards and the mode of parasitism of *P. citri;* investigation of new and more accurate methods to asses parasitism of *P. citri*, as a measure of biocontrol, which can aid in developing a parasitism-related infestation threshold for this pest.

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