Integrated management of false codling moth, *Thaumatotibia leucotreta*, on stone fruit and table grapes

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Summary

Integrated pest management (IPM) strives to control insect pests with a multifaceted approach that mirrors that of the ecology and endeavours to restore balance to the agricultural environment. To ensure the success of an IPM programme it is vital to understand the biology and the life cycle of the target insect. False codling moth (FCM), *Thaumatotibia leucotreta* is a tortricid moth with an extensive host range of more than 70 plants and is a key pest on citrus, stone fruit and table grapes. Insecticides are still relied upon for the control of FCM however, due to stricter regulations and their cascading environmental effects, there is a drive to reduce the chemical load in our agricultural environments. This encourages the need for a more sustainable approach such as IPM, which targets every stage in the life cycle of FCM. In this study, entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) were isolated from agricultural soils. The susceptibility of eggs and pupae to EPNs was investigated, and both entomopathogen types were screened against larvae. The EPNs proved to be the more potent pathogen and the larval stage was the least resilient against the pathogen. The EPN species that showed the most promise in laboratory trials were used as an *in vitro* cultured product and were tested in the field against FCM. The EPNs caused mortality of FCM larvae within 48 h and remained effective four weeks after application. Mating disruption is a non-chemical control technique that interferes with the mate-finding ability of insects in order to reduce mating events and is therefore an ideal candidate for the control of FCM. However, the effect of mating disruption has not been quantified against FCM in stone fruit and table grapes. Using multiple mark-release-recapture experiments, this is the first study to reliably quantify mating disruption in stone fruit and table grapes. Mating disruption proved to be a highly successful technique causing up to 99% disorientation of male FCM, but how the method alters FCM's mate-finding ability was unclear. To determine FCM's behavioural response to the addition of the female pheromone, quantitative tools and dosage-response profiles were used. The study proved that at low dosages the success of mating disruption is dependent on FCM density, however at high dosages there is a shift to non-competitive disruption causing mating disruption to remain effective even at high pest densities with few pheromone point sources. The integration of the techniques investigated here will ensure the effective control of FCM at each stage of the life cycle, ensuring low population growth that may lead to the successful suppression of this problematic pest.

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Opsomming

Geïntegreerde plaagbestuur (GPB) streef daarna om insekplae te beheer met 'n veelvlakkige benadering wat die ekologie weerspieël en poog om biologiese balans in die landbouomgewing te herstel. Om die sukses van 'n IPM-program te verseker, is dit noodsaaklik om die biologie en die lewensiklus van die teikeninsek te verstaan. Valskodlingmot (VKM), *Thaumatotibia leucotreta* is 'n tortricid mot met 'n wye gasheerreeks van meer as 70 plante en is 'n belangrike plaag op sitrus, steenvrugte en tafeldruiwe. Insekdoders word steeds aangewend vir die beheer van VKM maar, as gevolg van strenger regulasies en hul negatiewe omgewings-effekte, is daar 'n beweging om die hoeveelheid chemiese produkte in ons landbou-omgewings te probeer verminder. Dit moedig die behoefte aan om 'n meer volhoubare benadering soos GPB te volg, wat elke stadium van VKM se lewensiklus teiken. In hierdie studie, is entomopatogeniese nematodes (EPNs) en entomopatogeniese swamme (EPF) vanaf landbougrond geïsoleer. Die vatbaarheid van eiers en papies vir EPNs is ondersoek, terwyl beide entomopatogene teen laat-instar larwes getoets was. Die EPNs was die sterker patogeen en die larwale stadium was die mees vatbaar. Die EPN-spesies wat die mees belowende resultate in laboratoriumproewe getoon het, is as 'n *in vitro* gekweekte produk gebruik en in die veld teen VKM getoets. Die EPNs het die mortaliteit van VKM-larwes binne 48 uur veroorsaak en was na vier weke na toediening nogsteeds effektief. Paringsontwrigting is 'n nie-chemiese beheerstegniek wat inmeng met die vermoë van insekte om mekaar opstespoor, wat paringsgeleenthede verminder en is dus 'n ideale kandidaat vir die beheer van VKM. Paringsontwrigting teen VKM is egter nog nie in steenvrugte en tafeldruiwe gekwantifiseer nie. Met behulp van meervoudige-merk-hervang eksperimente, is hierdie die eerste studie om paringsontwrigting betroubaar te kwantifiseer in steenvrugte en tafeldruiwe. Paringsontwrigting het tot 99% van VKM se paringsgeleenthede verminder en is dus 'n hoogs suksesvolle tegniek, maar hoe die beheer VKM se vermoë om mekaar op te spoor verander het, was steeds onduidelik. Om VKM se gedragsreaksie na die toevoeging van die vroulike feromoon te bepaal, is kwantitatiewe modele en doses-reaksieprofiele gebruik. Die studie het bewys dat by lae dosisse is die sukses van paringsontwrigting afhanklik van die grootte van die VKM bevolking, maar by hoë dosisse is daar 'n verskuiwing na nie-kompeterende ontwrigting wat veroorsaak dat paringsontwrigting doeltreffend bly, selfs met groot VKM bevolkings en min feromoonbronne. Die integrasie van die tegnieke wat hier ondersoek word sal verseker dat VKM effektief beheer word tydens elke stadium van die lewensiklus. Dit sal lae bevolkingsgroei verseker wat kan lei tot die suksesvolle onderdrukking van hierdie problematiese plaag.

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Dedication

To Dr Kate Mitchell,

Your balance between sound scientific advice, reassurance and passion for your work played a “significant” role in the researcher I am today.

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Chapter 1:

General Introduction

The Tortricidae is a lepidopteran family, with more than 10 000 species (Horak, 1998; Brown, 2005), which is well-researched with 687 of these listed as important economic pests worldwide (van der Geest and Evenhuis, 1991; Zhang, 1994). In South Africa, four of these are regarded as the most economically important lepidopteran agricultural pests with research directed at their identification, control and biology (Blomefield, 1989; Timm et al., 2007; Prinsloo and Uys, 2015). These include codling moth, *Cydia pomonella* (Linnaeus), false codling moth (FCM), *Thaumatotibia* (= *Cryptophlebia*) *leucotreta* (Meyrick), oriental fruit moth, *Grapholita molesta* (Busck) and the macadamia nut borer, *T. batrachopa* (Meyrick).

FCM (Fig. 1.1) is a particular problem as it is a polyphagous pest with an extensive host range (Schwartz, 1981; Newton, 1998; Timm et al., 2010) and is a pest of many cultivated South African crops; including citrus, stone fruit, vegetable crops and recently table grapes (Blomefield, 1989; Prinsloo and Uys, 2015). FCM and its control has been researched for several decades in citrus (Brain, 1929; Catling and Aschenborn, 1974; Hofmeyr et al., 1991; Grout and Moore, 2015), however, limited work has been conducted on FCM in stone fruit and table grapes, and of these most were focused on crop suitability for FCM (Blomefield, 1989). The limited applied research regarding FCM in stone fruit and table grapes is of concern, especially as the annual value of the industry is over R 10 billion (stone fruit ~R 2 billion; table grapes ~R 9 billion) and supply almost 80 000 jobs to the South African economy (Hortgro, 2017; SATI, 2017). Economic losses may arise due to FCM's direct damage to the stone fruit or table grapes as well as its status as a phytosanitary pest (since 2013, https://www.eppo.int/ACTIVITIES/plant_quarantine/A2_list) that may cause closed markets or cancellation of consignments (Newton, 1998; Bloem et al., 2003).

Fig. 1.1 *Thaumatotibia leucotreta* adult, (left) (photo taken by V.M. Steyn) and (right) animated image created and supplied by R.F. Jacobs.

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In this introduction, different aspects, such as the life cycle, reproduction, population dynamics, ecology and current control of FCM are shortly revisited with the aim of finding solutions for the control of this pestiferous lepidopteran in an integrated pest management (IPM) programme.

1.1 Integrated Pest Management

In managed crops 10 - 50% may be lost pre-harvest to insects (Oerke, 2006). The first use of insecticides (in the form of sulphur) was recorded 4 500 years ago in Mesopotamia (modern day Iraq and Kuwait; Oerke, 2006) and are still relied upon today to reduce insect damage to food crops (Ghimire and Woodward, 2013; Schreinemachers and Tipraqsa, 2012; Guedes et al., 2016). However, due to stricter regulations, cases of resistance, damaging and long-lasting effects of broad-spectrum chemicals (e.g. DDT), there is a drive to reduce the chemical load in our agricultural environments and use more environmentally friendly products (Guedes et al., 2016). IPM aspires to achieve this by using an ecological approach to control insect pests (Koul et al., 2004). For instance, in a natural environment when sufficient resources are available, the pest population should increase. However, their natural enemies (predators and pathogens) will likely also increase to maintain the balance. In an agricultural system the natural enemies may control up to 90% of the pest population (Pimentel, 2005; Martin et al., 2013; Milligan et al., 2016). However, this may not be the case, due to the absence of natural enemies in the system (Meisner et al., 2014) or the pest population being too high for natural enemies to be effective (Tscharntke et al., 2016). To address this, natural enemies may need to be augmented to reduce the pest population effectively (Rabb, 1978; Botrell and Smith, 1982). The philosophy of IPM therefore encourages simultaneous use of biological, cultural, mechanical and chemical (species-specific) approaches to reduce pest damage (Sandler, 2008). In order for IPM to be effective it is important to understand the pest's biology and specifically its life cycle so that the IPM programme can be tailored towards a specific species (Bottrell and Smith, 1982).

1.2 IPM for false codling moth

1.2.1 Life cycle

FCM has multiple (at least six) generations per year and completes its life cycle in 42 - 46 days at temperatures of 25°C (Opoku-Debrah et al., 2014) and does not undergo diapause (Terblanche et al., 2014). The eggs are laid singly on the outside of the fruit and these take ~5 days to mature (Daiber, 1979a; Opoku-Debrah et al., 2014). They have three different stages of maturity that can be distinguished morphologically (Fig. 1.2), these are: newly laid (1,

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transparent), red eye (2, red colouring inside egg) and black head (3, head capsule of first instar larva visible). Next, the first instar larva (white measuring 1 - 2 mm) bore into the fruit, leaving behind a characteristic pile of frass on the rind of the fruit. The first instar feeds in the fruit, moults multiple times and emerges from the fruit as a fifth instar larva (15 - 20 mm) that has a pinkish red colour (Daiber, 1979b). The larva drops to the soil, and burrows down less than 5 mm (Love et al., 2019) to pupate, the pupa matures for 14 - 21 days (Daiber, 1979c), after which the adult moth (16 - 20 mm wing span) emerges (Fig 1.3).

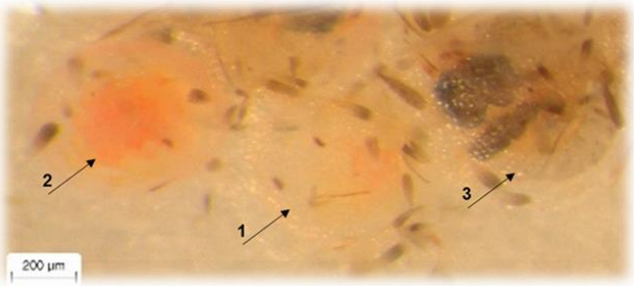

Fig. 1.2 *Thaumatotibia leucotreta* eggs illustrating the different stages of maturity, 1) newly laid egg 2) red eye stage and 3) black head stage just before first instar emerges (Copyright V.M. Steyn).

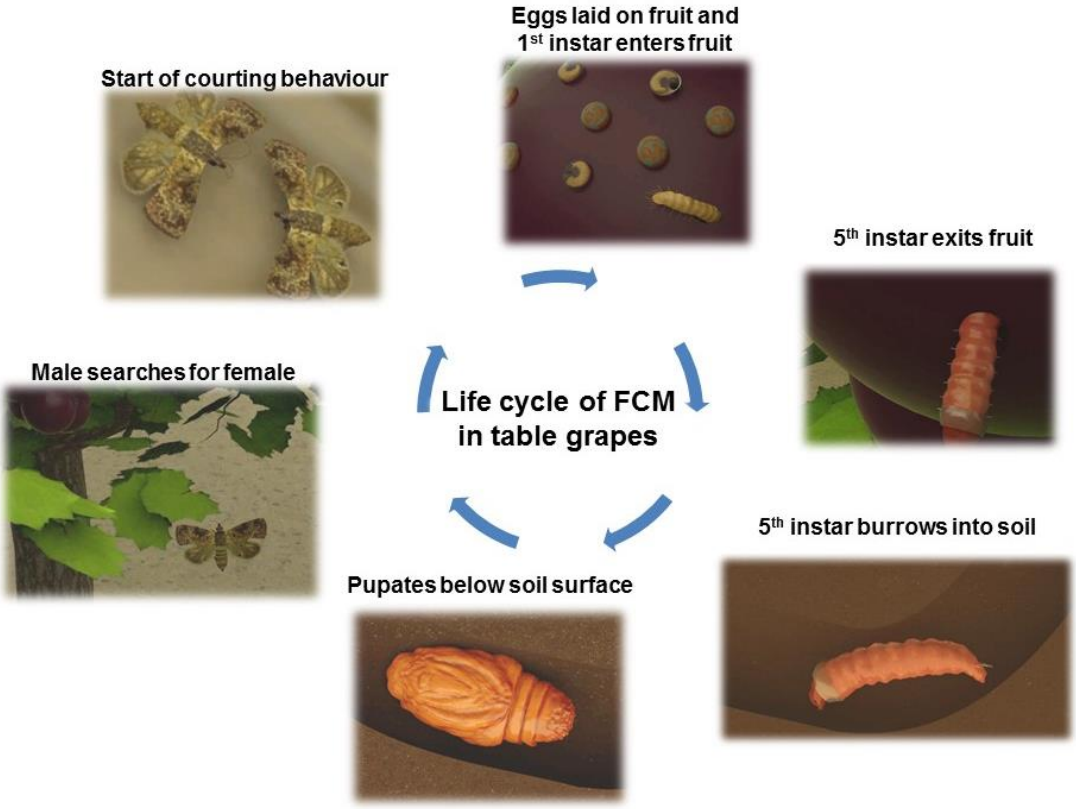

Fig. 1.3 The life cycle of *Thaumatotibia leucotreta* in table grapes, images animated and supplied by R.F. Jacobs according to photos taken by V.M. Steyn.

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1.2.2 Reproduction

After emergence the adults become sexually mature, the females expose their pheromone gland, and release an intraspecific semiochemical (pheromone), defined as a chemical signal that elicits a behavioural response from the same species (Heuskin et al., 2011). Read et al. (1968) first identified FCM's female pheromone. This was artificially synthesised by Henderson and Warren (1970) and later revised and adapted (Persoons, 1977; Newton et al., 1993). Semiochemicals allow males to detect the pheromone plume from afar (Wall and Perry, 1987), normally during their ranging flight (Cardé, 2016), and then navigate to the female's side (Allison and Cardé, 2016). Once the male is in close proximity (2 - 3 cm) to the female he initiates his courtship sequence. Zagatti and Castel (1987) identified six positions FCM males use to expose their three androconial (coremata between abdominal segments, alar notch on wings, and dark hair-like scales on hind tibia) areas to increase mating success with the female. The first position (Fig.1.3) finds the male head to head with the female, whilst fanning his wings, and concludes with successful mating, though not all positions are always utilised by the males (Zagatti and Castel, 1987).

1.2.3 Distribution and population dynamics

FCM is an indigenous African pest, has been reported from neighbouring islands (Cape Verde Islands, Mauritius, Reunion and St Helena), and has recently been introduced to Israel (Wysoki, 1986; Newton, 1998; Grout and Moore, 2015). Along with its extensive host range (excluding their incorrect inclusion of apples, see Stotter, 2009; for a recent and complete list) of agricultural crops, it also has multiple natural hosts including but not limited to acorns, *Quercus robur* (Lam.), Port Jackson galls (Seymour and Veldtman, 2010) buffalo-thorn, *Ziziphus mucronata* (Willdenou) and marula, *Sclerocarya birrea caffra* (Sonder). These natural hosts may offer refuge to FCM and later become a source of reintroductions into the agricultural systems complicating its management (Begemann and Schoeman, 1999; Stotter, 2009). In South Africa, genetic structure analysis has been conducted on FCM to determine the scale to which populations may differ (Timm et al., 2010). Individual differences accounted for most (85%) of the molecular variation in FCM populations. Further variation was explained by geographical distance (7%), among site/farm similarity (8%) and host preference (8%) (Timm et al., 2010). These results were in contrast to early suggestions that FCM may exhibit host preferences (FCM from citrus may prefer citrus) and form host strains (FCM from citrus remain on citrus) (Ford, 1934; Omer-Cooper, 1939). The results from Timm et al. (2010) suggests that FCM may move between hosts according to host availability, increasing the chance of FCM

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immigrating into other crops. However, the matter is further complicated by the among site/farm similarity, the authors suggest that this may indicate limited dispersal of FCM. Though seemingly in contrast, these findings are plausible within the context of the Western Cape that has an agricultural mosaic ensuring a year-round supply of hosts for FCM to utilise without dispersing far. However, the host preference/strain exhibited by FCM may have been diluted (incorrectly estimated) by Timm et al.'s (2010) study, due to two limitations: 1) use of pheromone traps to collect FCM, and 2) host preference was compared between citrus, pears, apples, plums, acorns and litchis. The use of traps to collect the genetic material (FCM) made it impossible to identify the host from which the moth emerged. Therefore, the subsequent host preference analyses were conducted without clear evidence of the host. An example of this is the inclusion of apples and pears in their analyses, these fruits being non-hosts of FCM. Furthermore, Timm has confirmed that individuals collected from apples and pears were retrieved from traps rather than fruit (see De Villiers et al., 2015). Mgocheki and Addison (2016)'s work addressed these concerns and only used FCM collected from fruit. No evidence of genetic divergence between adults from different fruit could be identified, supporting the findings of Timm et al. (2010). However, the extent to which FCM adults move between different hosts remains a subject of debate.

1.2.4 Ecology

Early work on the flight of male FCM using pheromone baited traps in peach, *Prunus persica* (L.) orchards, found that male flight was limited by low temperatures (Daiber, 1978). Stotter (2009) corroborated these findings in citrus and showed that numbers quickly build up after the colder seasons. The first flight of the males is therefore considered to be from late October (crop depending) and continues throughout the summer months, with few males caught from May and during the rest of the winter months (Moore, 2002; Stotter, 2009). The low number of males caught in the colder months is not due to diapause, as no evidence of diapause has been recorded for FCM and laboratory trials failed to induce diapause in FCM (Terblanche et al., 2014). The fewer FCM in the colder months is therefore likely due to slower (68 - 100 days) development of FCM under cold temperatures (Stofberg, 1954; Daiber, 1979 a, b, c). FCM larvae also have low temperature tolerance becoming inactive at 6.7°C and lethal temperatures range between -4.5°C and -0.5°C depending on exposure time (Stotter and Terblanche, 2009; Boardman et al., 2011), further decreasing populations in cold environments. The host may also influence development time of FCM. De Jager (2013) showed that development from egg to adult may take as little as 37 days in grapes at 25°C whereas,

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under the same conditions, development took 40 days in citrus and FCM would not develop in apples.

1.2.5 Current control

Eggs

One of the strongest drivers of population growth is propagule pressure, which is the rate of re-introduction of the pest into an environment (Simberloff, 2009). The egg stage is therefore a very important stage to control. There are two parasitoid species that have been confirmed to attack FCM eggs, these are *Trichogrammatoidea cryptophlebiae* (Nagaraja) (Hymenoptera: Chalcididae) and *Chelonus curvimaculatus* (Cameron) (Hymenoptera: Braconidae), with the former being commercially available (Vital Bugs, Letsitele, South Africa) and the latter also parasitizing the larvae (Searle 1964; Catling and Aschenborn 1974; Malan et al., 2018).

Larvae

After the eggs hatch, the neonate larvae may be controlled by Cryptophlebia leucotreta granulovirus (CrleGV) (Moore, 2002; Moore et al., 2011). In fact, many options have shown promise against the larvae, such as entomopathogenic nematodes (EPNs) (Malan et al., 2011; Malan and Moore, 2016), entomopathogenic fungi (EPF) (Begemann, 1989; Coombes et al., 2013) and parasitoids (Newton, 1998). The EPN enters the host through the insects' natural openings and, once inside, it releases symbiotic bacteria that helps kill the host. The EPN then multiplies and, once the nutrients of the host are used up, the EPN exits the cadaver in search of a new host (Steinhaus, 1949, Lu et al., 2017). In contrast, an EPF spore will land on the insect cuticle, germinate and then penetrate the insect cuticle. The fungus grows throughout the body as blastopores and mycelia, which kill the insect, more conidia are produced on the exterior of the insect and spores are released into the environment (Lacey and Kaya, 2007). Both EPNs and EPF have the potential to persist in the environment after application and are therefore a very good fit for IPM. However, currently the commercially available virus CrleGV (Cryptogran™, River Bioscience, South Africa) and chemical insecticides are the main techniques utilised. Malan et al. (2018) highlights the importance of comparing novel local strains with those that are commercially available so that the potential of EPNs and EPF can be fully realised in IPM systems in South Africa.

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Pupae

The pupal stage should theoretically be the most targeted stage as 1) it is immobile 2) it is the longest stage thereby offering the largest application window (Love et al., 2019). Unfortunately, as FCM pupates beneath the soil it limits the biological agents that can attack it, though EPNs and EPF that occur naturally in the soil environment do show promise in laboratory and semi-field environments (Coombes et al., 2013; Malan et al., 2011). EPNs can even search out these cryptic stages by following a CO_2 gradient (Robinson, 1995).

Adults

The Sterile Insect Technique (SIT) has been deployed by a commercial facility [XSIT (Pty) Ltd, Citrusdal, South Africa] which has been in operation since 2007 targeting FCM in citrus (Barnes et al., 2015; Hofmeyr et al., 2016). SIT works by flooding the landscape with sterile moths (10 sterile for each wild moth); these mate with the wild moths, leading to infertile eggs and therefore a gradual reduction in the population (Hofmeyr et al., 2015). To ensure that the population is suppressed, XSIT monitors wild and sterile FCM on a weekly basis (Boersma et al., 2018). This technique has been successful, but is currently only used in citrus and table grapes.

Two pheromone-mediated control options also exist against FCM. These are "attract-and-kill" and "mating disruption", and both techniques exploit the mate-finding behaviour of FCM. The attract-and-kill technique works by luring the male with a semiochemical to a dispenser impregnated with a pyrethroid gel that kills the male on contact. Mating disruption works by broadcasting the synthetic semiochemical throughout the landscape, so as to interfere with mate-finding behaviour, thereby reducing mating events and decreasing the number of viable eggs in the next generation (Alison and Cardé, 2016). The widespread adoption of mating disruption worldwide is attributed to: 1) reduction of insecticide use for edible crops (Suckling, 2015), 2) its use against pests that cannot be effectively controlled by other means, and 3) low mammalian toxicity (Witzgall et al., 2008, 2010; Ioriatti et al., 2011). Hofmeyr et al. (1991) studied mating disruption in citrus against FCM and showed the successful disorientation of male moths, leading to its registration in South Africa. Mating disruption has not been quantified against FCM in stone fruit and table grapes, but is widely used both in South Africa and in Europe against other Tortricidae [European grapevine moth, *Lobesia botrana* (Denis and Schiffermüller)] in table grapes (14 000 ha) (Iorriatti et al., 2011). Furthermore, it is still unclear how the technique alters the mate-finding behaviour of FCM. Interestingly, despite

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mating disruption's long (50+ years) history worldwide (Miller and Gut, 2015), Miller et al. (2006 a, b) only recently proposed and evaluated the theoretical foundation for the two main classes of lepidopteran disruptive mechanisms, namely competitive and non-competitive disruption. This shifted the research focus from whether mating disruption works to the mechanistic understanding of how Lepidoptera react and thus how the synthetic pheromone alters the insects' behaviour (Miller et al., 2010; Allison and Cardé, 2016). No studies have previously been conducted to determine the underlying disruptive mechanism on FCM. However, studies conducted on closely related species (*Cydia pomonella* and *Grapholita molesta*) have proposed and critically evaluated a framework, along with a dichotomous key, for distinguishing between the two mechanism classes (Miller et al., 2006 a, b; Miller and Gut 2015) and thus provides a protocol to identify the underlying disruption mechanism in other pest species.

1.3 Aim and Objectives

The overall aim of this research was to investigate the use of EPNs, EPF and mating disruption as alternate control methods against FCM in stone fruit and table grapes. To effectively control FCM with alternate methods it would be advantageous to apply a control measure targeting each stage of the life-cycle. To this end, entomopathogens could be used against the immature stages (egg, larva, pupa), whilst mating disruption may be used to reduce mating events so that fewer fertile eggs are available in subsequent generations.

To use entomopathogens against the immature stages of FCM in the South African context, locally isolated EPNs and EPF should be identified and their virulence assessed against the immature stages of FCM in the laboratory. The most promising isolates could then be tested in the field to determine their biological control potential. However, field trials require large quantities of EPNs. Therefore the virulence of different culturing techniques should be compared and the most suited approach used for field assays.

Due to the unknown effect of mating disruption against FCM in stone fruit and table grapes, it would be important to investigate whether mating disruption affects the male FCM's mate-finding behaviour in these crops. If so, it would then be desirable to determine how the addition of the synthetic female pheromone alters the male FCM's behaviour. With this information the optimal dose required to effectively disrupt males can be calculated.

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The current study consists of four data chapters that each addresses one of the questions above. Each objective was written as a separate chapter, and compiled as separate manuscripts for publication in peer-reviewed journals, therefore some repetition was unavoidable. They are:

- Efficacy of South African entomopathogens against *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) immature stages
- Field control of false codling moth, *Thaumatotibia leucotreta*, using *in vitro* cultured *Steinernema jeffreyense* and *S. yirgalemense*
- Quantifying mating disruption of false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in stone fruit and table grapes
- Mechanisms mediating false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) mating disruption, using point-source pheromone dispensers

1.4 References

Allison, J.D., Cardé, R.T., 2016. Pheromone communication in moths: evolution, behavior, and application. Univ of California Press. 401 pp.

Barnes, B.N., Hofmeyr, J.H., Groenewald, S., Conlong, D.E., Wohlfarter, M., 2015. The sterile insect technique in agricultural crops in South Africa: a metamorphosis....but will it fly? African Entomol. 23, 1–18.

Begemann, G.J., 1989. Die isolering en massakultuur van die entomopatogeniese swam *Beauveria bassiana* (Balsamo). Citrus J. 645, 18-19.

Begemann, G.J., Schoeman, A.S., 1999. The phenology of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), *Tortrix capensana* (Walker) and *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) on citrus at Zebediela, South Africa. African Entomol. 7, 131-148.

Boardman, L., Grout, T.G., Terblanche, J.S., 2012. False codling moth *Thaumatotibia leucotreta* (Lepidoptera, Tortricidae) larvae are chill- susceptible. Insect Sci. 19, 315-328.

Boersma, N., Boardman, L., Gilbert, M., Terblanche, J.S., 2018. Sex-dependent thermal history influences cold tolerance, longevity and fecundity in false codling moth *Thaumatotibia leucotreta* (Lepidoptera: T ortricidae). Agr. Forest Entomol. 20, 41-50.

Bloem, S., Carpenter, J.E., Hofmeyer, J.H., 2003. Radiation biology and inherited sterility in false codling moth (Lepidoptera: Tortricidae). J. Econ. Entomol. 96, 1724-1731.

Stellenbosch University https://scholar.sun.ac.za

Blomefield, T.L., 1989. Economic importance of false codling moth, *Cryptophlebia leucotreta*, and codling moth, *Cydia pomonella*, on peaches, nectarines and plums. Phytophylactica 21, 435-436.

Bottrell, D.G., Smith, R.F., 1982. Integrated pest management. Environ. Sci. Technol. 16, 282-288.

Brain, C.K., 1929. Insect pests and their control in South Africa. Nationale Pers, Cape Town, pp. 219-220.

Brown, J.W., 2005. Tortricidae (Lepidoptera). World Catalogue of Insects. Apollo Books, Stenstrup, Denmark, Vol 5, 741 pp.

Cardé, R.T., 2016. Moth navigation along pheromone plumes. In: Pheromone communication in moths: evolution, behavior and application. University of California Press, Berkeley, California, pp. 173-189.

Catling, H.D., Aschenborn, H., 1974. Population studies of the false codling moth, *Cryptophlebia leucotreta* Meyrick, on citrus in the Transvaal. Phytophylactica 6, 31-38.

Coombes, C.A., Hill, M.P., Moore, S.D., Dames, J.F., Fullard, T., 2013. Persistence and virulence of promising entomopathogenic fungal isolates for use in citrus orchards in South Africa. Biocontrol Sci. Techn. 23, 1053-1066.

Daiber, C.C., 1979a. A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: the egg. Phytophylactica 11, 129-132.

Daiber, C.C., 1979b. A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: the larva. Phytophylactica 11, 141-144.

Daiber, C.C., 1979c. A study of the biology of the false codling moth [*Cryptophlebia leucotreta* (Meyr.)]: the cocoon. Phytophylactica 11, 151-157.

De Jager, Z.M., 2013. Biology and ecology of the false codling moth, *Thaumatotibia leucotreta* (Meyrick). M.Sc. thesis, Stellenbosch University.

De Villiers, M., Heunis, J.M., Pringle, K.L., 2015. Phytosanitary host status of apples as a host for false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). African Entomol. 23, 234-238.

Ford, W.K., 1934. Some observations on the bionomics of the false codling moth - *Argyroploce leucotreta*, Meyr.(Family Eucosmidae) - in Southern Rhodesia. Publ. BSAC 3, 9-34.

Stellenbosch University https://scholar.sun.ac.za

Ghimire, N., Woodward, R.T., 2013. Under- and over-use of pesticides: an international analysis. Ecol. Econ. 89, 73-81.

Grout, T.G, Moore, S.D., 2015. Citrus. In: Prinsloo, G.L., Uys, V.M. (eds). Insects of cultivated plants and natural pastures in southern Africa. Entomological Society of Southern Africa, Cape Town, pp. 448-499.

Guedes, R.N.C., Smagghe, G., Stark, J.D., Desneux, N., 2016. Pesticide-induced stress in arthropod pests for optimized integrated pest management programs. Annu. Rev. Entomol. 61, 43-62.

Henderson, H.E., Warren, F.L., 1970. The sex-pheromone of false codling moth *Cryptophlebia leucotreta* Meyr., (formely *Argyroploce leucotrata* Meyr.). Synthesis and bio-assay of tans-dodec-7-en-1-yl acetate and related compounds. J. S.A. Chem. Inst. 23, 9-11.

Heuskin, S., Verheggen, F.J., Haubruge, E., Wathelet, J.P., Lognay, G., 2011. The use of semiochemical slow-release devices in integrated pest management strategies. Biotechnol. Agron. Soc. Environ. 15, 459-470.

Hofmeyr, J.H., Burger, B.V., Calitz, F.J., 1991. Disruption of male orientation to female false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae), using synthetic sex pheromone. Phytophylactica 23, 153-156.

Hofmeyr, J.H., Carpenter, J.E., Bloem, S., Slabbert, J.P., 2015. Development of the sterile insect technique to suppress false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in citrus fruit: research to implementation (Part 1). African Entomol. 23, 180-186.

Hofmeyr, H., Hattingh, V., Hofmeyr, M., Slabbert, K., 2016. Postharvest phytosanitary disinfestation of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in citrus fruit: validation of an ionizing radiation treatment. Florida Entomol. 99, 54-58.

Horak, M. 1998. The Tortricoidea. In: N.P. Kristensen (ed.), Handbook of Zoology, Lepidoptera, Moths and Butterflies, Vol 1: Evolution, Systematics, and Biogeography. Walter de Gruyter, Berlin, New York, pp. 199-215.

Hortgro, 2017. Key deciduous fruit statistics 2017. HORTGRO, Paarl, South Africa.

Ioriatti, C., Anfora, G., Tasin, M., De Cristofaro, A., Witzgall, P., Lucchi, A., 2011. Chemical ecology and management of *Lobesia botrana* (Lepidoptera: Tortricidae). J. Econom. Entomol. 104, 1125-1137.

Stellenbosch University https://scholar.sun.ac.za

Koul, O., Dhaliwal, G.S., Cuperus, G.W. (eds.), 2004. Integrated pest management: potential, constraints and challenges. CABI, 343 pp.

Lacey, L.A., Kaya, H.K. eds., 2007. Field manual of techniques in invertebrate pathology: application and evaluation of pathogens for control of insects and other invertebrate pests. Springer Science & Business Media, pp. 223-248.

Love, C.N., Moore, S.D., Hill, M.P., 2019. The role of abiotic factors in the pupation of *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) in the soil. Agr. Forest Entomol., 21, 38-49.

Lu, D., Macchietto, M., Chang, D., Barros, M.M., Baldwin, J., Mortazavi, A., Dillman, A.R., 2017. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. PLoS Pathog. 13, e1006302. https://doi.org/10.1371/journal.ppat.1006302

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

Malan, A.P., Knoetze, R., Moore, S.D., 2011. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. J. Invertebr. Pathol. 108, 115-125.

Malan, A.P., Moore, S.D., 2016. Evaluation of local entomopathogenic nematodes for the control of false codling moth, *Thaumatotibia leucotreta* (Meyrick, 1913) in a South African citrus orchard. African Entomol. 24, 489-501.

Martin, E.A., Reineking, B., Seo, B., Steffan-Dewenter, I., 2013. Natural enemy interactions constrain pest control in complex agricultural landscapes. PNAS, doi/10.1073/pnas.1215725110.

Meisner, M.H., Harmon, J.P., Ives, A.R., 2014. Temperature effects on long-term population dynamics in a parasitoid-host system. Ecol. Monogr. 84, 457-476.

Mgocheki, N., Addison, P., 2016. Investigating the validity of the species status of the false codling moth in South African deciduous fruit orchards using mating studies and mtDNA. Bull. Entomol. Res. 106, 598-605.

Miller, J.R., Gut, L.J., 2015. Mating disruption for the 21st century: matching technology with

Stellenbosch University https://scholar.sun.ac.za

mechanism. Environ. Entomol. 44, 427-453.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. J. Chem. Ecol. 32, 2089-2114.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): case studies. J. Chem. Ecol. 32, 2115-2143.

Miller, J.R., McGhee, P.S., Siegert, P.Y., Adams, C.G., Huang, J., Grieshop, M.J., Gut, L.J., 2010. General principles of attraction and competitive attraction as revealed by large-cage studies of moths responding to sex pheromone. PNAS 107, 22-27.

Milligan, M.C., Johnson, M.D., Garfinkel, M., Smith, C.J., Njoroge, P., 2016. Quantifying pest control services by birds and ants in Kenyan coffee farms. Biol. Conserv. 194, 58-65.

Moore, S.D., 2002. The development and evaluation of Cryptophlebia leucotreta granulovirus (CrleGV) as a biological control agent for the management of false codling moth, *Cryptophlebia leucotreta*, on citrus. Ph.D. dissertation, Rhodes University.

Moore, S.D., Hendry, D.A., Richards, G.I., 2011. Virulence of a South African isolate of the Cryptophlebia leucotreta granulovirus to *Thaumatotibia leucotreta* neonate larvae. Biocontrol 56, 341-352.

Newton, P. J., 1998. False codling moth, *Cryptophlebia leucotreta* (Meyrick). In: E.C.G. Bedford, van den Berg, M.A. and de Villiers, E.A. (Eds.), Citrus pests in the republic of South Africa, pp. 192-200.

Newton, P.J., Thomas, C.D., Mastro, V.C., Schwalbe, C.P., 1993. Improved two-component blend of the synthetic female sex pheromone of *Cryptophlebia leucotreta*, and identification of an attractant for *C. peltastica*. Entomol. Exp. Appl. 66, 75-82.

Oerke, E.C., 2006. Crop losses to pests. J. Agri. Sci. 144, 31-43.

Omer-Cooper, J., 1939. Remarks on false codling moth. Rhodes University, Grahamstown, South Africa. Mimeograph, pp 1-17.

Opoku-Debrah, J.K., Hill, M.P., Knox, C., Moore, S.D., 2014. Comparison of the biology of geographically distinct populations of the citrus pest, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), in South Africa. Afr. Entomol. 22, 530-537.

Stellenbosch University https://scholar.sun.ac.za

Pimentel, D., 2005. Environmental and economic costs of the application of pesticides primarily in the United States. Environ. Dev. Sustain. 7, 229-252.

Persoons, C.J., Ritter, F.J., Nooyen, W.J., 1977. Sex pheromone of the false codling moth *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae). J Chem. Ecol. 3, 717-722.

Prinsloo, G.L., Uys, V.M., 2015. Insects of cultivated plants and natural pastures in Southern Africa. Entomological Society of Southern Africa, South Africa. 491pp.

Grout, T.G, Moore, S.D., 2015. Citrus. In: Prinsloo, G.L., Uys, V.M. (eds). Insects of cultivated plants and natural pastures in southern Africa. Entomological Society of Southern Africa, Cape Town. pp. 448-499.

Rabb, R.L., 1978. A sharp focus on insect populations and pest management from a wide-area view. Bulletin ESA 24, 55-61.

Read, J.S., Warren, F.L., Hewitt, P.H., 1968. Identification of the sex pheromone of the false codling moth (*Argyroploce leucotreta*). Chem. Comm. 14, 792-793.

Robinson, A.F., 1995. Optimal release rates for attracting *Meloidogyne incognita*, *Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. J. Nematol. 27, 42-50.

Sandler, H.A., 2008. Challenges in integrated pest management for Massachusetts cranberry production: A historical perspective to inform the future. Crop Protection Research Advances. New York, NY: Nova Science Publishers, pp. 21-55.

SATI, 2017. South African Table grape Industry statistics booklet. SATI, Paarl, South Africa.

Schreinemachers, P., Tipraqsa, P., 2012. Agriculture pesticides and land use intensification in high, middle and low income countries. Food Policy 37, 616-26

Schwartz, A., 1979. Ondersoek na die steriele mannetjies tegniek as moontlike beheermaatreël vir valskodlingmot by sitrus: vrylating van steriele motte. Citrus Subtrop. Fruit J. 553, 10-12.

Schwartz, A. 1981. ‘n Bydrae tot die biologie en beheer van die valskodlingmot, *Cryptophlebia leucotreta* (Meyr.) op nawels. Ph.D. dissertation, University of Stellenbosch.

Schwartz, A., Marais, A., van der Kooij, R., 1982. Beheer van valskodlingmot in Citrusdal deur grootskaalse vrylating van ‘n eierparasiet. Subtrop. 3, 17-19.

Stellenbosch University https://scholar.sun.ac.za

Searle, C.M.L., 1964. A list of the insect enemies of citrus pests in southern Africa. Department of agricultural technical services, Republic of South Africa. Tech. Comm. 30, 1-18.

Seymour, C.L., Veldtman, R., 2010. Ecological role of control agent, and not just host-specificity, determine risks of biological control. Austral Ecol. 35, 704-711.

Simberloff, D., 2009. The role of propagule pressure in biological invasions. Annu. Rev. Ecol. Evol. Syst. 40, 81-102.

Steinhaus, E.A., 1949. Principles of Insect Pathology. McGraw-Hill B. Company, INC, pg. 633-665.

Stofberg, F.J., 1954. False codling moth of citrus. Farming in S.A. 29, 273-294.

Stotter, R.L., 2009. Spatial and temporal distribution of false codling moth across landscapes in the Citrusdal area (Western Cape Province, South Africa). M.Sc. thesis, Stellenbosch University.

Stotter, R.L., Terblanche, J.S., 2009. Low-temperature tolerance of false codling moth *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) in South Africa. J. Therm. Biol. 34, 320-325.

Suckling, D.M., 2015. Can we replace toxicants, achieve biosecurity, and generate market position with semiochemicals? Front. Ecol. Evol. 3, 1-17.

Terblanche, J.S., de Jager, Z., Boardman, L., Addison, P., 2014. Physiological traits suggest limited diapause response in false codling moth, Thaumatotibia leucotreta (Lepidoptera: Tortricidae). J. Appl. Entomol. 138, 683-691.

Theron, K.I., 2012. The South African deciduous fruit industry. In: II All Africa Horticulture Congress 1007, pp. 873-879.

Timm, A.E., Geertsema, H., Warnich, L., 2010. Population genetic structure of economically important Tortricidae (Lepidoptera) in South Africa: a comparative analysis. B. Entomol. Res. 100, 421-431.

Timm, A.E., Warnich, L., Geertsema, H., 2007. Morphological and molecular identification of economically important Tortricidae (Lepidoptera) on tropical and subtropical fruit in South Africa. African Entomol. 15, 269-286.

Tscharntke, T., Karp, D.S., Chaplin-Kramer, R., Batáry, P., DeClerck, F., Gratton, C., Hunt, L., Ives, A., Jonsson, M., Larsen, A., Martin, E.A., 2016. When natural habitat fails to

Stellenbosch University https://scholar.sun.ac.za

enhance biological pest control–Five hypotheses. Biol. Conserv. 204, 449-458.

van der Geest, L.P.S., Evenhuis, H.H., 1991. Tortricid Pests: Their Biology, Natural Enemies and Control. Elsevier, Amsterdam, The Netherlands, 808 pp.

Wall, C., Perry, J.N., 1987. Range of action of moth sex-attractant sources. Entomol. Exp. Appl. 44, 5-14.

Witzgall, P., Stelinski, L., Gut, L., Thomson, D., 2008. Codling moth management and chemical ecology. Annu. Rev. Entomol. 53, 503-522.

Witzgall, P., Kirsch, P., Cork, A., 2010. Sex pheromones and their impact on pest management. J. Chem. Ecol. 36, 80-100.

Wysoki, M., 1986. New records of lepidopterous pests of macadamia in Israel. Phytoparasitica 14, 2.

Zagatti, P., Castel, Y., 1987. Courtship behaviour of the false codling moth, *Cryptophlebia leucotreta* (Meyrick): androconial display and mating success (Lepidoptera, Tortricidae). Ann. Soc. Ent. France 23, 113-123.

Zhang, B.C., 1994. Index of Economically Important Lepidoptera. Wallingford: CAB International, 599 pp.

Stellenbosch University https://scholar.sun.ac.za

Chapter 2:

Efficacy of South African entomopathogens against *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) immature stages

Abstract

Thaumatotibia leucotreta (Lepidoptera: Tortricidae) or false codling moth (FCM) is a priority pest on stone fruit and table grapes, posing phytosanitary restrictions on export fruit, as well as direct crop damage. While current control focusses on the adult stage, it is suggested that the integrated pest management (IPM) approach should include a control measure for each stage of the life cycle. This study aims to investigate the potential of biological control agents, sourced locally, against FCM immature stages. Several entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) species were isolated, cultured and identified from soil collected from orchards and vineyards throughout the Western Cape province. These species, along with several other locally collected species, were screened for virulence against the egg, larval and pupal stage. Three *Heterorhabditis* species and *Oscheius microvilli* were isolated from the soil, as well as strains of *Metarhizium* spp. and *Beauveria bassiana.* Eggs proved to be susceptible (30 - 65%) to several EPNs at a concentration of 200 infective juveniles (IJ) per 50 µl. *Xenorhabdus indica*, the symbiotic bacteria of *Steinernema yirgalemense*, caused significantly more mortality than the control, but the secretions of the EPNs were unable to kill the egg without the IJs. EPNs were highly virulent (62 - 100%) against the larvae, with *S. yirgalemense* causing 100% mortality at 50 IJs/insect in all trials. *Metarhizium robertsii*, *M. anisopliae* and *Beauveria bassiana* caused 78%, 50% and 75% mortality respectively against FCM larvae at a spore concentration of 1 x 10^7. The pupae proved to be the most resistant of the immature stages and EPNs caused only low (6 - 33%) mortality at 100 IJs/insect. All the immature FCM stages were susceptible to entomopathogens in the laboratory and should be further tested in the field to illustrate the benefits of their inclusion into the FCM IPM programme.

Keywords: survey, indigenous, entomopathogenic nematodes, fungi, ovicidal, larvae, pupae

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2.1 Introduction

Thaumatotibia leucotreta, the false codling moth (FCM), is an economically important pest for South Africa's fruit, vegetable and nut industries (Blomefield, 1989; Prinsloo and Uys, 2015). When not controlled it can cause severe crop damage in stone fruit leading to crop losses of over 25%, with some incidence of up to 100% (Blomefield, 1989). In 2004, FCM caused an estimated crop loss of R100 million in citrus alone (Moore, 2004). Currently, the integrated pest management (IPM) strategy for FCM comprises of specific chemicals as well as more environmentally friendly options, such as sterile insect technique, mating disruption, bacteria and viruses that are implemented against the pest. However, FCM remains a problem in South Africa with high populations throughout the country. Its control has become particularly problematic as it has developed some resistance against a number of insecticides (chitin inhibitors), and international markets enforcing lower chemical residues (Hofmeyr and Pringle, 1998; Hofmeyr, 2003; Chandler et al., 2011). This encourages the need for more sustainable control which ideally should target every stage in the life cycle of FCM.

The first entomopathogenic fungi (EPF) were described from silkworm colonies nearly 200 years ago (Bassi, 1835). The accelerated growth of this field came after the publication of the extensive synthesis on insect pathology by Steinhaus (1949), which was later used for course work and forms the cornerstone of this field. The author highlights that entomopathogens have the unique advantage over insect predators, in that they complete their life cycle by finding a single host, whereas insect predators need to find multiple subsequent hosts to complete their life cycle. This ensures the relative ease of proliferation and persistence of the entomopathogens in the environment, making it a valuable addition to an IPM strategy (Steinhaus, 1949). Entomopathogens, in particular, entomopathogenic nematodes (EPNs) and EPF have more recently shown to be host specific, with no discernible non-target effects (Babendreier et al., 2015), leading to their use as an environmentally-friendly control option against many insect pests (Campos-Herrera, 2015; Hatting et al., 2018).

Previous studies advocate for the need to target the soil borne stages of FCM (Malan et al., 2011; 2018). Increased interest in the use of entomopathogens, for the control of FCM, has led to many new species of EPN being described from South Africa (Hatting et al., 2009; Çimen et al., 2015; Malan and Hatting 2015; Çimen et al., 2016; Malan and Ferreira, 2017; Steyn et al., 2017). A recent review specifically highlights the potential of EPNs and EPF for the control of FCM life stages (Malan et al., 2018). EPNs are particularly suited as they are able to seek

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out and kill insects in cryptic habitats (Gumus et al., 2015). In fact, the susceptibility of FCM larvae to EPNs has been well documented (Malan et al., 2011; Manrakhan et al., 2013; Malan and Moore, 2016; Steyn et al., 2017) and cause high mortality in the laboratory (70 - 100%). Unfortunately, the window of application to control FCM larvae is relatively short. The 5th instar larva emerges from the fruit and drops to the soil, where it will pupate, remaining as a prepupa for 3 days and finally becoming a hardened pupa (Daiber, 1979a, b, c). The pupal stage of FCM is the longest stage of the life cycle (Daiber, 1979c), between 14-21 days at 25 °C, and therefore has the longest application window of all the stages and is the ideal stage to target using EPNs (Love et al., 2019). Theoretically, the pupa should be the most resilient or resistant to attack of the soil borne stages, as this is its primary habitat and therefore should have developed some inherent resistance against naturally occurring soil borne pathogens. Few studies have considered the virulence of EPNs and EPF against the pupal stage and have found encouraging results (Malan et al., 2011; Coombes et al. 2013).

Experience from other disciplines, such as invasion biology, highlight the importance of limiting the propagule pressure to ensure effective pest control (Simberloff, 2009). Therefore, the ovicidal activity or egg stage is an important stage to control, as it reduces new entries into the population. In FCM there is an additional benefit for controlling this stage, as the egg is laid on the outside of the fruit (De Jager, 2013), preventing damage to the fruit for sale and export. Even though the value of controlling eggs is clear, there are few reliable options (e.g. egg parasitoids) to target this stage. A number of EPN strains have been shown to be virulent against other immature stages of FCM (Malan and Moore, 2011), though EPNs have never been tested against the egg stage. The virulence of EPNs against insect eggs has had mixed results, some showing no virulence, such as for Phthiraptera (De Doucet et al., 1998) and Diptera (Kim et al., 2004) and others reporting success against Coleoptera and Lepidoptera (Shahina et al., 2009; Kalia et al., 2014).

The main aim of this study was to test the virulence of locally isolated entomopathogens against the immature life stages of FCM. Here several locally-sourced EPNs and EPF collected from orchards and vineyards throughout the Western Cape province were evaluated, for their efficacy against the soil borne stages (larvae and pupae) of FCM. Secondly, this is the first attempt to test virulence of EPNs, their symbiotic bacteria and their secretions against the eggs of FCM. It is hypothesised that the symbiotic bacteria will be able to cause egg mortality

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without their vector, however, that it will be more virulent when it has the aid of its vector (IJ) to buffer environmental conditions and to aid transportation.

2.2 Material and methods

2.2.1 Source of insects

The immature stages of *T. leucotreta* were collected from a mass reared colony maintained at XSIT, Citrusdal (-32° 36' 0.00" S, 19° 01' 0.00" E), South Africa. These stages included newly laid eggs on wax paper, 5th instars with their diet and 48 h old pupae, which were transported in a cooler box to Stellenbosch (33° 56' 1.327" S, 18° 51' 47.536" E). Upon arrival in Stellenbosch the insects were transferred to 27°C ± 1°C. Thereafter, virulence/pathogenicity of EPNs, symbiotic bacteria and EPF against the various stages were tested.

2.2.2 Source of entomopathogens

The different species of entomopathogens used in the study were obtained from a survey of the Western Cape soils and the Stellenbosch University collection, which include the EPN and EPF species as indicated in Table 2.1. These species were collected from soils in South Africa from a variety of different habitats and are currently maintained at Stellenbosch University for research purposes.

Survey

A survey of the Western Cape in stone fruit, grapevine and citrus orchards was conducted in search of local EPNs and EPF. Soil samples were collected from 20 different farms. A hectare on each farm was sampled by dividing it into four quadrants. Within each quadrant five subsamples of 50 g of soil was collected in a 1 L plastic container. Each block (one ha of an orchard) consisted of four plastic containers, of which each constituted a sample, with a total of 80 samples. Each of these soil samples were baited with the greater wax moth larvae [(*Galleria mellonella* L.) (Lepidoptera: Pyralidae)] to allow isolation of EPNs (Moore and Malan, 2016) and EPF (Goble et al., 2010).

Molecular characterisation of nematodes

Molecular characterization of EPNs followed protocols described by Nguyen (2007). *Galleria mellonella* larvae were inoculated with IJs from each of the isolated EPNs, the IJs were allowed to develop for 2 days, after which the *G. mellonella* larvae were dissected to retrieve young

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females that were used for DNA extraction. Each of four females was placed individually in 30 µl drop of lysis buffer (16 mM [NH4]2SO4, 67 mM Tris–HCl, pH 8.8, 0.1% TWEEN®-20 and Proteinase K) on the side of an Eppendorf tube, then cut into pieces using a sterile hypodermic needle tapped to the tip of the tube. Eppendorf tubes were immediately placed in a -85°C freezer for a minimum of 20 min, after which the DNA was extracted by using a thermocycler at 65°C for 1 h, followed by 95°C for 10 min. Using the KAPA2G ReadyMix PCR Kit a PCR cycle of 3 min at 95°C, followed by 35 cycles of 20 s at 95°C, 20 s at 48°C, and 30 s at 72°C, and a final cycle of 5 min at 72°C. The reaction was completed with a final extension for 7 min at 72°C and then cooled and held at 4°C (Malan et al., 2011). PCR samples were separated on a 1% agarose gel, stained with ethidium bromide and visualised using ultraviolet light. The un-purified PCR product was sent to the Central Analytical Facilities (CAF), DNA Sequencing Unit, Department of Genetics at Stellenbosch University, for a post PCR clean-up and sequencing.

The generated forward and reverse sequences of each isolate were aligned and edited using CLC Main Workbench (version 7.9.1). The consensus sequence were blasted at NCBI (National Centre for Biotechnology Information), Nucleotide BLAST®, to be able to compare them with closely related sequences deposited in GenBank for species identification.

Molecular characterisation of fungi

Spores (50 - 100 µg) from pure fungal cultures were scraped from Sabouraud dextrose agar (SDA; LAB, Neogen®) plates under sterile conditions into an Eppendorf tube. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp., CA, USA). Gene regions used for the comparisons included the internal transcribed spacer region (ITS) with the primers ITS 1 (forward) (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (reverse) (5'-CTCCTCCGCTTATTGATATGC-3') (White et al., 1990). To distinguish between species of the PARBE clade (Justin et al., 2018) of *Metarhizium* the elongation factor 1–α (EF 1–α) genes with the primers EF2F (forward) (5'-GTCGGTGGTATCGACAAGCGT-3') and EF2R (reverse) (5'-AGCATGTTGTCGCCGTTGAAG-3') (Jacobs et al., 2004) were used. The PCR conditions were 94ºC for 10 min, followed by the performing of 36 cycles of 94ºC for 30 sec, at 56ºC for 30 sec (for the ITS primers), or at 53ºC for 30 sec (for the EF primers), and then at 72ºC for one min (Abaajeh and Nchu, 2015). The generated PCR products were handled as described above.

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Table 2.1. Species, isolates, nearest town, habitat and GenBank number of entomopathogenic nematodes (*Heterorhabditis* and *Steinernema*) and entomopathogenic fungi (*Metarhizium*) used in this study from the Stellenbosch University collection.

Species	Isolate	Nearest town	Habitat	GenBank no.	Reference
H. bacteriophora	SF351	Wellington	Grapevine (*Vitis vinifera*)	FJ455843	Malan et al. 2006
H. indica	SGS	Bonnievale	Grapevine (*Vitis vinifera*)	KU945293	Unpublished
H. noenieputensis	SF669	Noenieput	Fig (*Ficus carica*)	JN620538	Malan et al. 2014
H. baujardi	MT19	KwaZulu-Natal	Natural vegetation	MF535520	Abate et al. 2018
S. jeffreyense	J194	Jeffrey's Bay	Guava (*Psidium guajava*)	KP164886	Malan et al. 2016
S. yirgalemense	157C	Nelspruit	Citrus (*Citrus x aurantium*)	EU625295	Malan et al. 2011
M. robertsii	MR3	Grabouw	Apple (*Malus pumila*)	-	-

2.2.3 Source of bacteria

Xenorhabdus indica Stackebrand, from the Stellenbosch University EPN symbiotic bacterial collection, was cultured by adding 200 µl of a 15% glycerol stock culture, stored at -80°C, to 30 ml tryptic soy broth (TSB) in a 250 Erlenmeyer flask (Ferreira et al., 2014; 2016). The shake flasks were kept on an orbital shaker (OrbiShake, Labotec ™, Midrand, South Africa) in a dark growth chamber for 48 h at 28°C, to allow bacterial cells to multiply. The flasks where then transferred to an orbital shaker at 14°C to stop growth, prior to use. Bacteria were streaked on NBTA plates to ensure purity, blue colonies and uptake of dye, after which its virulence was screened against FCM eggs, larvae and pupae. In pilot trials the bacterial broth showed no mortality against the larvae and pupae, therefore subsequent tests focused on the eggs only.

2.2.4 Bioassay protocol

Filter paper discs were placed in alternate wells, to limit contamination between samples, of a 24-well bioassay plate. All treatments were a pre-determined nematode concentration in distilled water, of which 50 µl was pipetted into each well, which ensured the filter paper was moist, without any free water. A glass sheet was placed over the wells to prevent larvae from escaping. The bioassay plates were placed on moist paper towels, inside two litre plastic containers and kept in a dark room for 48 h at 27°C. Mortality was recorded after 48 h.

2.2.5 Entomopathogenic nematode bioassays

Eggs

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A better understanding of the mode of action that the IJs employ to kill the egg is required, so it was necessary to observe the egg development and then inoculate the IJs, symbiotic bacteria and IJs secretions during the different stages of the eggs' development. EPN treatments were administered using eggs of two different ages, which include either newly laid eggs or eggs that have matured at 27°C for 48 h. The 48 h of maturation was indicated by the visible head capsule of the first instar (referred to as black head) within the egg. The egg sheets were then cut into 10 mm square blocks and the number of eggs that had reached the black head stage were recorded (n = 50) on each of the squares. The eggs that had not reached this stage were removed from the wax paper squares with a sharp needle. Using the bioassay protocol (section 2.2.4), a total of 12 squares of either the newly laid or the 48 h old eggs were placed on moist filter paper in alternate wells of a 24-well bioassays plate for each EPN treatment. The treatments consisted of five EPN species, as indicated in (Table 2.1) (except for *H. bacteriophora*) at a concentration of 200 IJ/50 µl, compared to distilled water only as control. Emergence of the neonate larvae were recorded every 24 h for the first 72 h. If the neonate larvae had not emerged 72 h after inoculation it was regarded as a mortality. The experiment was repeated on a different test date using a fresh batch of pathogens.

A second experiment to determine the role of the symbiotic bacteria, *X. indica* was conducted. This bacterium was chosen as it was shown to control other lepidopteran species (Kalia et al., 2014). The treatments were compared against two controls, the first was distilled water only and the second consisted of the water part of the 200 IJ/50 µl inoculum that had been siphoned off after the EPN suspension stood for 15 min. The second control was included to ensure that it was the EPN causing the mortality and not its secretions. Egg sheets of approximately 50 eggs per sheet were prepared, as described above, and placed on moist filter paper in alternate wells of a 24-well bioassay plate. A volume of 50 µl of the bacterial broth, distilled water, secretions and 200 IJ/well was inoculated onto each of the sheets and emergence of the 1st instar were recorded every 24 h for the first 72 h. If the neonate larvae had not emerged 72 h after inoculation it was recorded as a mortality. The experiment was repeated on a different test date using a fresh batch of pathogens.

Larvae

Different EPN species as indicated in Table 2.1 (excluding *H. baujardi* and *H. noenieputensis*) as well as *Heterorhabditis zealandica* Poinar VS3 (Table 2.2) were tested against the late instar of FCM. Using the described bioassay protocol (section 2.2.4), 5 bioassay wells with a total of

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60 FCM larvae were inoculated with 50 IJs/insect of each of the EPN species. The experiment was repeated on a different test date using a fresh batch of the pathogen.

Pupae

FCM are in the pre-pupal stage for 2-3 days before pupation. Therefore, pupae were allowed to age to five days before inoculation to ensure full maturity. EPN species indicated in Table 2.1, including isolates VS5 and VS18 (recovered from survey, Table 2.2) were tested against 5-day-old FCM pupae. Following the bioassay protocol (section 2.2.4), 5 bioassay wells with a total of 60 pupae were inoculated with 100 IJs/insect of each of the EPN species. The experiment was repeated on a different test date using a fresh batch of pathogens.

2.2.6 Entomopathogenic fungi bioassays

Fungal preparation

Spores from pure fungal cultures were scraped from SDA plates under sterile conditions into a 10 ml McCartney bottle, containing distilled autoclaved water, which constituted the stock. A drop of TWEEN®-20 was added, after which the inoculum was vortexed and poured through a chiffon cloth to ensure that there were no clumps or hyphae in the spore suspension. A serial dilution was made from the stock bottles, by adding 1 ml of stock to 9 ml of distilled water. The spore concentration was then counted with a haemocytometer, allowing specific concentrations to be calculated for subsequent trials.

Viability of the spores were checked for each fungal strain, by spreading 50 µl of 1×10^7 conidia/ml suspension on a SDA plate. Each SDA plate was sealed with Parafilm and placed in the dark. After 12 h spore germination was determined with the aid of a 20 x magnification of a compound microscope (Leica, 2000). The percentage germination was determined by 100-spore counts taken from each plate (Ekesi et al., 2002). The spores were considered viable if the length of the germ-tube was found to be at least twice the diameter of the propagule (Inglis et al., 2012). The counts were repeated three times for each of the strains collected. All strains used in the trials showed > 85% viability.

Fungal screening

A screening trial was conducted with *Metarhizium robertsii* Bisch., Rehner and Humber (Hypocreales: Clavicipitaceae) from the Stellenbosch University collection (Table 2.1), as well

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as all fungal isolates recovered from the survey (Table 2.2). A total of 12 FCM larvae were dipped into a 1 x 10^7 spore suspension (prepared as previously described, section 2.2.4 and then transferred to moist filter paper in alternate wells of a 24-well bioassay plate. The control received distilled water with TWEEN®-20 only. Mortality was assessed by inspecting larvae for mortality with visible mycosis five days after dipping. The trial was repeated on separate dates and with a freshly cultured inoculum. The experiment was repeated on a different test date using a fresh batch of pathogens.

Fungal concentration

A concentration trial (concentrations calculated as described, section 2.2.4) was conducted using *M. robertsii*. 60 FCM larvae were dipped into one of five spore suspensions each with its own concentration (0, 1 x 10^4, 1 x 10^5, 1 x 10^6 and 1 x 10^7 conidia/ml) and then transferred to moist filter paper in alternate wells of a 24-well bioassay plate. The control was distilled water with a drop (50 µl) of TWEEN®-20 only. Mortality was assessed by inspecting larvae for mortality, with visible mycosis, five days after dipping.

2.2.7 Statistical analyses

The statistical analysis was performed using Statistica 12 (Stat-Soft Inc., 2012). If the residuals were not normally distributed, the data were log(X + 1)-transformed for further analysis. If the assumptions (normality and equality) of a one-way analysis of variance (ANOVA) were violated irrespective of transformation, the Kruskal-Wallis H-test was used to separate the means at $p < 0.05$. If there were no significant test date versus treatment interactions, data from different test dates were pooled and analysed using ANOVA. When applicable, means were separated using Fisher's LSD post-hoc test. Abbott's correction factor was used to compensate for natural mortality (Abbott, 1925). All further statistical analyses were undertaken in RStudio version 1.0.143 and R version 3.5.1 (R, 2018). A generalized linear model, with a gaussian distribution and an identity link function, was used to determine the most susceptible FCM stage to the EPNs tested. It was also used to test the prediction that IJs would be more effective against the egg than its symbiotic bacteria or secretions. Several packages were used to illustrate our findings graphically, these were ggplot2, plotly and forcats (Wickham, 2016; Sievert, 2018).

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2.3 Results

2.3.1 Survey

A total of 12 EPN and nine EPF strains were isolated from the soil using *G. mellonella* as bait insects. Eight (40%) of the 20 locations tested positive for EPNs, whereas seven (35%) locations tested positive for EPF, with some of these locations offering up multiple isolates. Success of recovery and culture to inoculum differed between the entomopathogens, with 18.75% of the EPN samples leading to an isolate, whereas only 11.25% of the EPF samples yielded a useable strain. No new EPF species were found, however *M. anisopliae* and *B. bassiana* were successfully recovered. No new EPN species or any *Steinernema* species were found during the survey, however three species of heterorhabditids were found and one Rhabditidae species identified as *Oscheius microvilli* Zhou, Yang, Wang, Bao, Wang, Hou, Lin, Yedid and Zhang. The heterorhabditids included *H. bacteriophora* (most common, 55.65%), *H. zealandica* (22.22%) and *H. indica* (11.11%). Entomopathogenic fungal species from *Beauveria* were recovered more frequently (77.78%) than from *Metarhizium*. *Beauveria bassiana* was isolated (VMS1, VSM4-6) more frequently from vineyards (75%) than from stone fruit orchards. *Metarhizium anisopliae* was recovered from a stone fruit orchard (Table 2.2).

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Table 2.2. Species and isolates of entomopathogenic nematodes and entomopathogenic fungi identified from a survey of sites from stone fruit, vineyards and citrus blocks in the Western Cape, South Africa during 2016-2017 fruit growing seasons.

Entomopathogens	Species	Isolate no.	Colour	Genbank Number	Nearest town	Habitat
Nematode	*H. zealandica*	VS1	Green	Pending	De Doorns	Table grapes
	H. zealandica	VS2	Green	Pending	De Doorns	Table grapes
	H. zealandica	VS3	Dark red	Pending	De Doorns	Citrus
	H. zealandica	VS4	Dark red	Pending	De Doorns	Citrus
	H. indica	VS5	Pale yellow	Pending	Halfmanshof	Table grapes
	H. bacteriophora	VS6	Red	Pending	Franschhoek	Peaches
	H. bacteriophora	VS7	Red	Pending	Robertson	Wine grapes
	H. indica	VS8	Red	Pending	Halfmanshof	Table grapes
	H. bacteriophora	VS9	Red	Pending	Riebeek kasteel	Table grapes
	H. bacteriophora	VS10	Red	Pending	Riebeek kasteel	Table grapes
	H. bacteriophora	VS11	Red	Pending	Riebeek kasteel	Table grapes
	H. bacteriophora	VS12	Red	Pending	Halfmanshof	Table grapes
	H. bacteriophora	VS13	Red	Pending	Halfmanshof	Table grapes
	H. bacteriophora	VS15	Red	Pending	Riebeek kasteel	Table grapes
	H. bacteriophora	VS16	Dark red	Pending	Riebeek kasteel	Table grapes
	Oscheius microvilli	VS17	Red	Pending	Paarl	Table grapes
	H. bacteriophora	VS18	Red	Pending	Halfmanshof	Table grapes
Fungi	*Beauveria bassiana*	VMS1	White	Pending	Stellenbosch	Peaches
	Metarhizium anisopliae	VMS2	Green	Pending	Paarl	Plum
	Metarhizium sp.	VMS3	White	-	Franschhoek	Peaches
	B. bassiana	VMS4	White	Pending	Halfmanshof	Table grapes
	B. bassiana	VMS5	White	Pending	Robertson	Wine grapes
	B. bassiana	VMS6	White	Pending	Riebeek kasteel	Table grapes
	Metarhizium sp.	VMS7	Green	-	Paarl	Peaches
	B. bassiana	VMS8	White	-	Halfmanshof	Table grapes
	B. bassiana	VMS9	White	-	Paarl	Plum

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2.3.2 Entomopathogenic nematodes

The EPNs caused significantly higher mortality of FCM larvae, compared to the eggs (T = 5.338, df = 128, $p < 0.001$) and pupae (T = 13.71, df = 117, $p < 0.001$) at low IJs concentration of 50 IJs/insect. Additionally, the EPNs were significantly less effective against pupae than the eggs (T = -7.321, df = 126, $p < 0.001$).

Eggs

The screened EPN species caused no mortality to newly laid FCM eggs, however, the eggs that were allowed to age to the black head stage before inoculation, were found to be susceptible. Subsequent results therefore only pertain to eggs that were inoculated once they had reached the black head stage. The older eggs proved to be susceptible to all five EPN species (Fig. 2.1), which were all significantly ($F_{5, 100}$ = 19.605, $p < 0.001$) different from the control and caused 30 - 65% mortality. *Steinernema jeffreyense* performed the worst (30.22% ± 4.65%), while *H. baujardi* caused the highest mortality (64.57% ± 6.88%) and differed significantly from *H. indica* (p = 0.032) (47.04% ± 9.27%) and *S. jeffreyense* ($p < 0.001$) against the egg stage.

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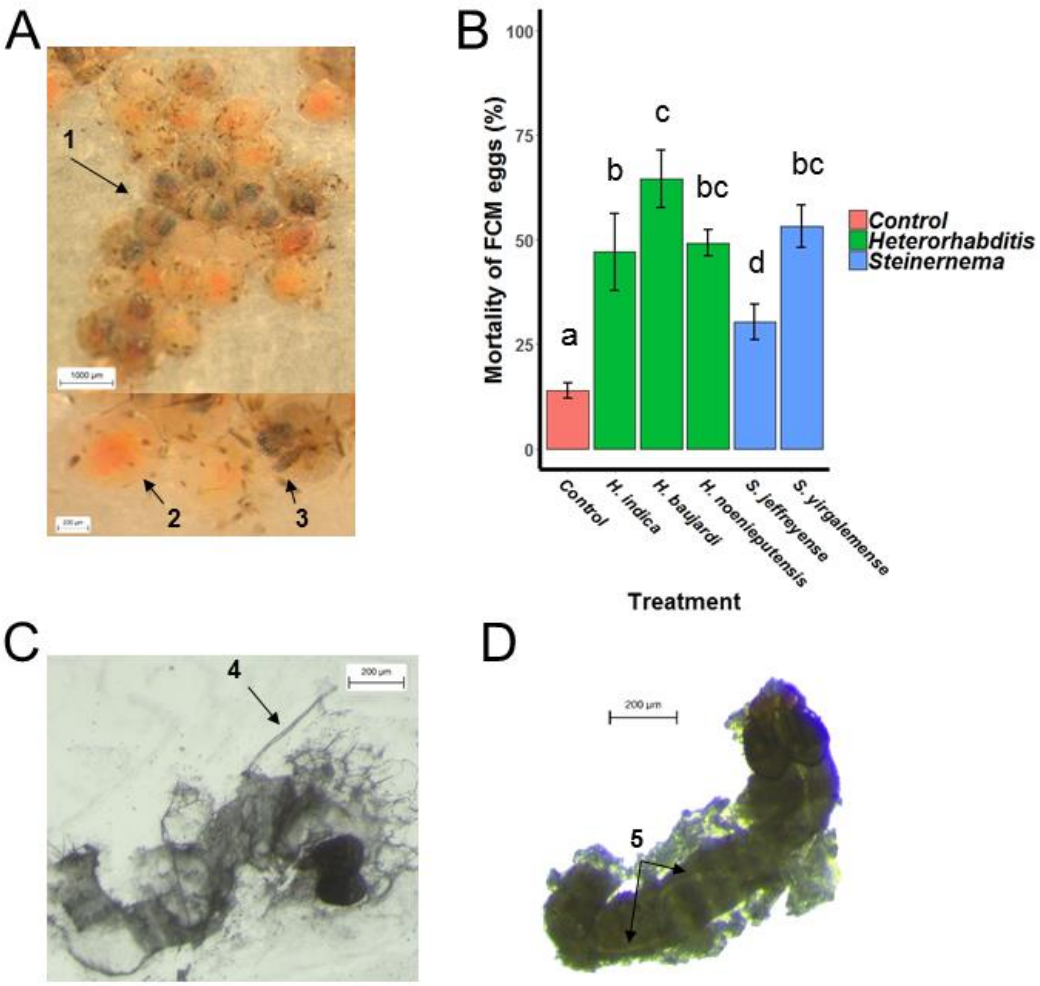

Fig. 2.1. Panel (A) the egg sheets that were inoculated with IJs (1), the young red egg that was not susceptible to IJs (2) and the older egg (black head) that was susceptible to IJs. Panel (B) mean percentage mortality (± SE) of *Thaumatotibia leucotreta* eggs at black head stage, inoculated with *Heterorhabditis* and *Steinernema* (200 IJs/50 µl). Different letters above the vertical bars indicate significant differences ($p < 0.05$). Panel (C) dissected egg and neonate instar *Thaumatotibia leucotreta* larva, with *H. noenieputensis* IJ (4). Panel (D) two *H. noenieputensis* IJs inside the 1st instar that was killed inside the egg (5).

Potential secretion of *S. yirgalemense* caused marginal mortality (24.93% ± 6.61%) of FCM eggs, however, the *S. yirgalemense* inoculum was significantly ($Z = 2.441$, $N = 72$ $p = 0.044$) more effective (44.06% ± 5.78%) than its secretions (Fig. 2.2). There was no significant ($Z = 1.014$, $N = 72$, $p = 0.932$) difference between the virulence of *S. yirgalemense* and its symbiotic bacteria, *X. indica* (36.92% ± 6.74%).

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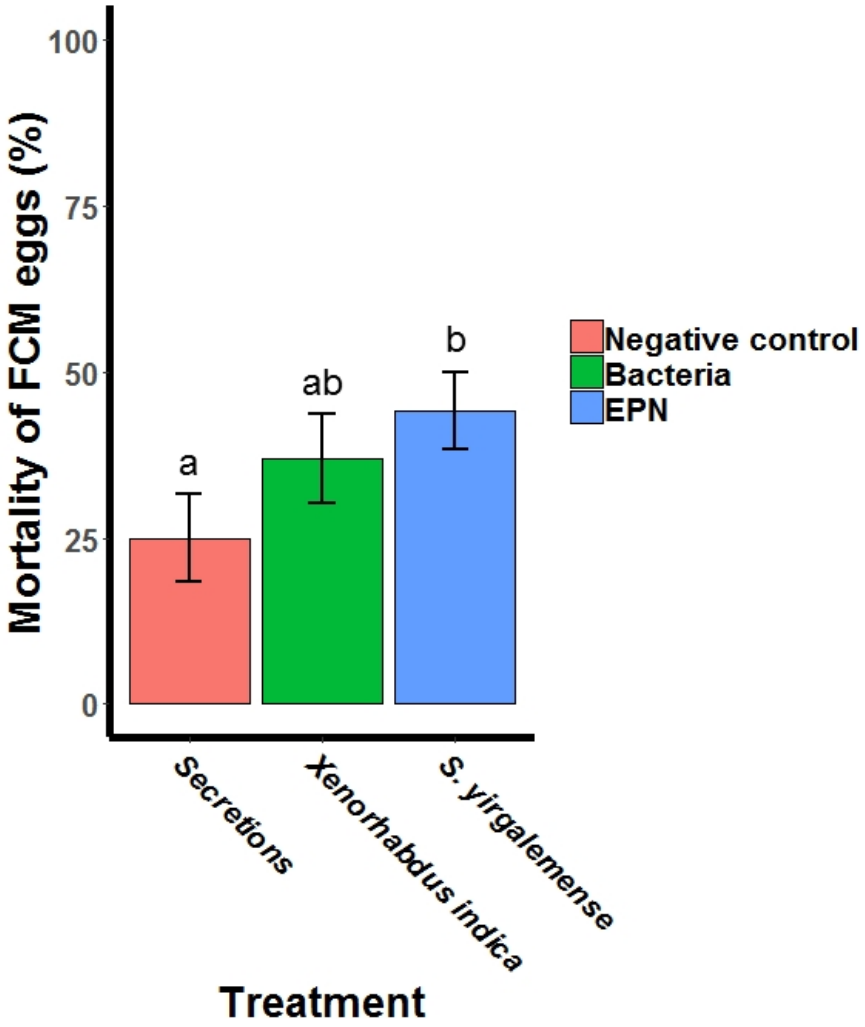

Fig. 2.2. Mean percentage (Abbott's corrected) mortality (± SE) of *Thaumatotibia leucotreta* eggs, distilled water with EPN secretions (negative control), a bacterial broth of *Xenorhabdus indica* (50 µl/well) or *Steinernema yirgalemense* (200 IJ/well). Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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Larvae

The different EPN species caused between 62 - 100% mortality of FCM larvae (Fig. 2.3). *Heterorhabditis indica* (98.33% ± 0.89%) and *S. yirgalemense* (100%) caused significantly higher mortality than any of the other species, with *S. yirgalemense* repeatedly causing 100% mortality in every trial. The remaining EPN species showed virulence (> 65%) against the FCM larval stage and did not differ significantly (T = 1.221, df = 58, p = 0.227) from each other, except for *H. zealandica* (VS3) and *S. jeffreyense*.

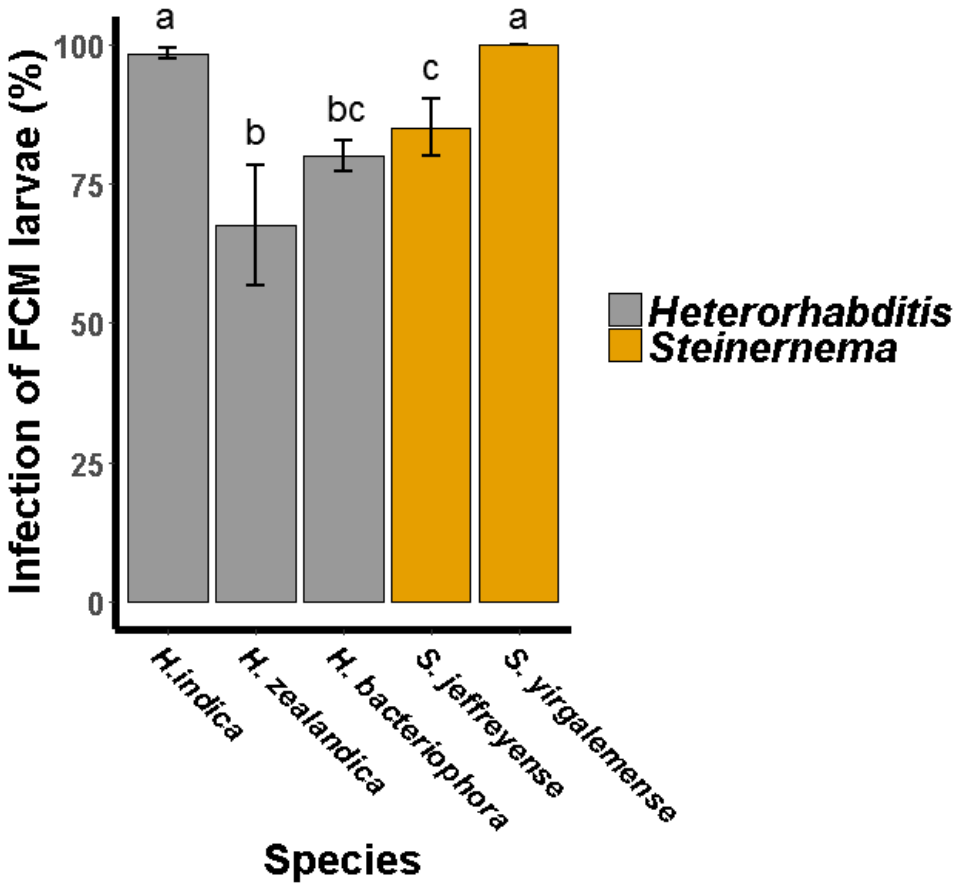

Fig. 2.3. Mean percentage infection (± SE) of *Thaumatotibia leucotreta* larvae inoculated with *Steinernema* and *Heterorhabditis*. The late instars were inoculated with 50 IJs/insect for each of the species. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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Pupae

The EPN species tested caused 6 - 33% mortality against FCM pupae (Fig. 2.4). *Heterorhabditis baujardi* caused the highest mortality (33.33% ± 3.51%) of all the tested species, but did not significantly differ from *H. indica* SGS ($p = 0.116$) (26.19% ± 3.48%) and *H. indica* VS5 ($p = 0.266$) (26.67% ± 5.53%).

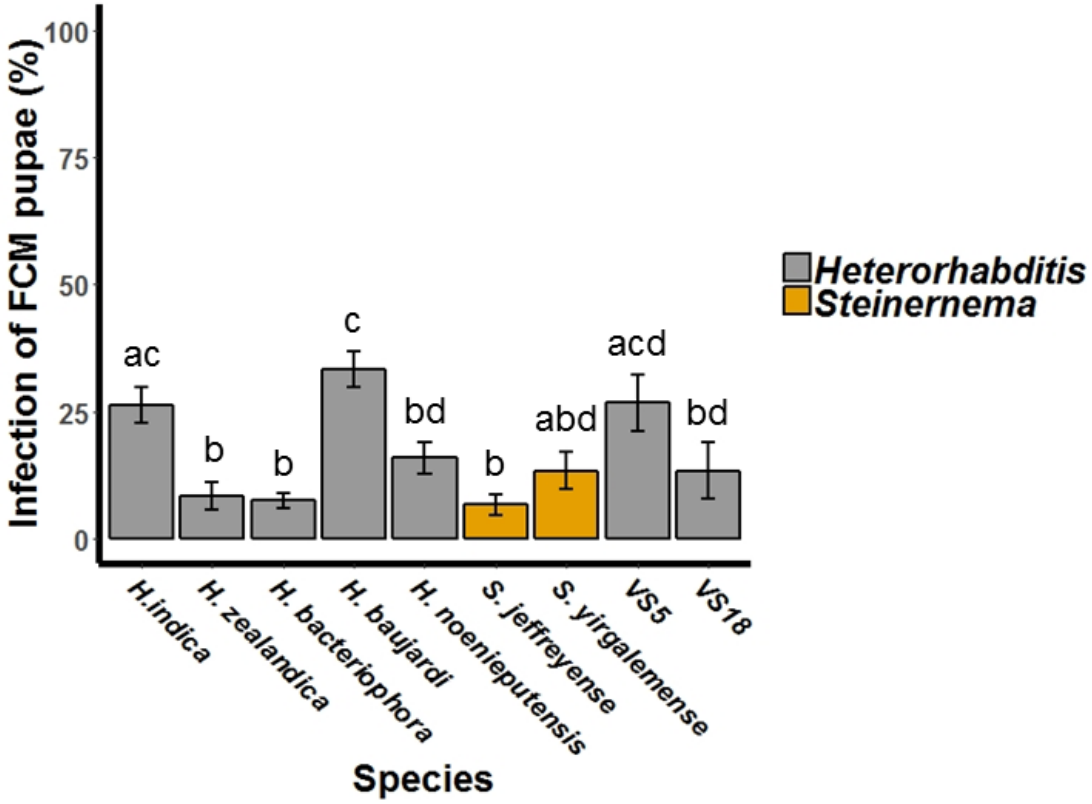

Fig. 2.4. Mean percentage infection (± SE) of *Thaumatotibia leucotreta* pupae inoculated with entomopathogenic nematodes. The pupae were inoculated with 100 IJs/insect for each of the species. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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2.3.3 Entomopathogenic fungi against larvae

Fungal screening

Eight of the EPF isolates proved virulent against FCM and caused 10 - 78% infection five days after application (Fig. 2.5). There was no significant ($F_{1, 39} = 2.4469$, $p = 0.126$) difference in infection between *Metarhizium* and *Beauveria* species, with species from both genera performing well (> 65% infection) and poorly (< 25% infection). *Metarhizium robertsii*, VS1, VS2, VS4 and VS5 strains were significantly ($F_{9, 31} = 22.094$, $p < 0.001$) more virulent against FCM larvae than the other strains tested. *Metarhizium robertsii* was significantly ($p = 0.001$) more virulent than the *M. anisopliae* (VS2) strain collected from our survey.

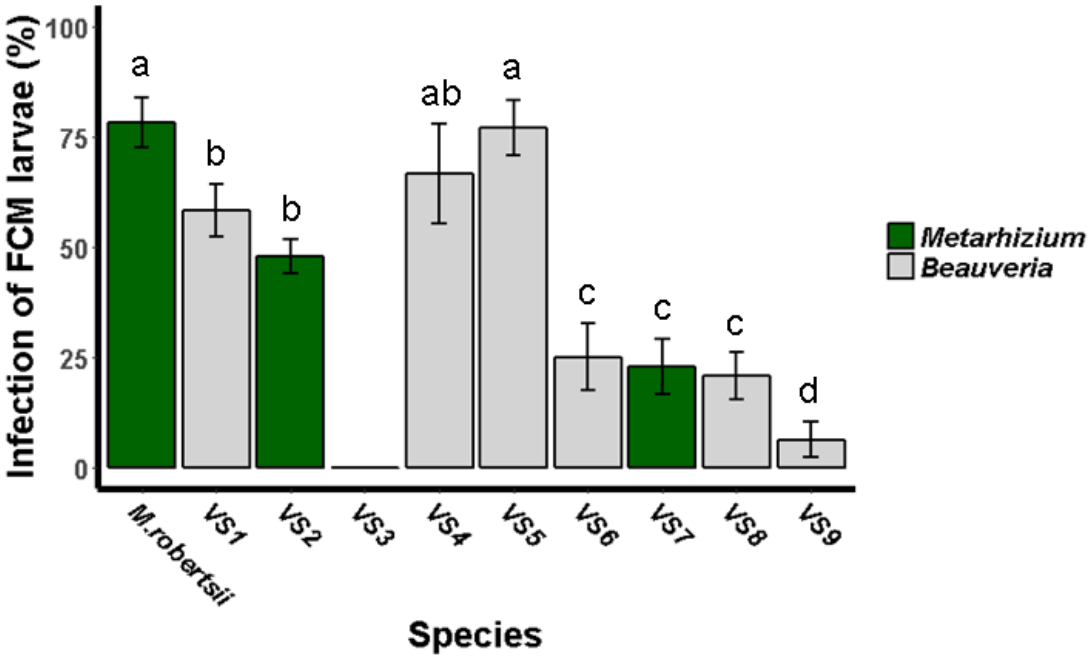

Fig. 2.5. Mean percentage infection (± SE) of late instar *Thaumatotibia leucotreta* larvae after dunk test application of several entomopathogenic fungi strains with a spore concentration of 1 x 10^7. Different letters above vertical bars indicate significant differences ($p < 0.05$).

Fungal concentration

Metarhizium robertsii did not cause FCM larval infection at low (1 x 10^4) spore concentrations. The higher fungal spore concentrations (1 x 10^5, 1 x 10^6 and 1 x 10^7 conidia/ml) yielded 11 - 78% mortality (Fig. 2.6). The mortality increased significantly ($F_{4, 20} = 86.146$, $p < 0.001$) with each order of magnitude increase in spore concentration.

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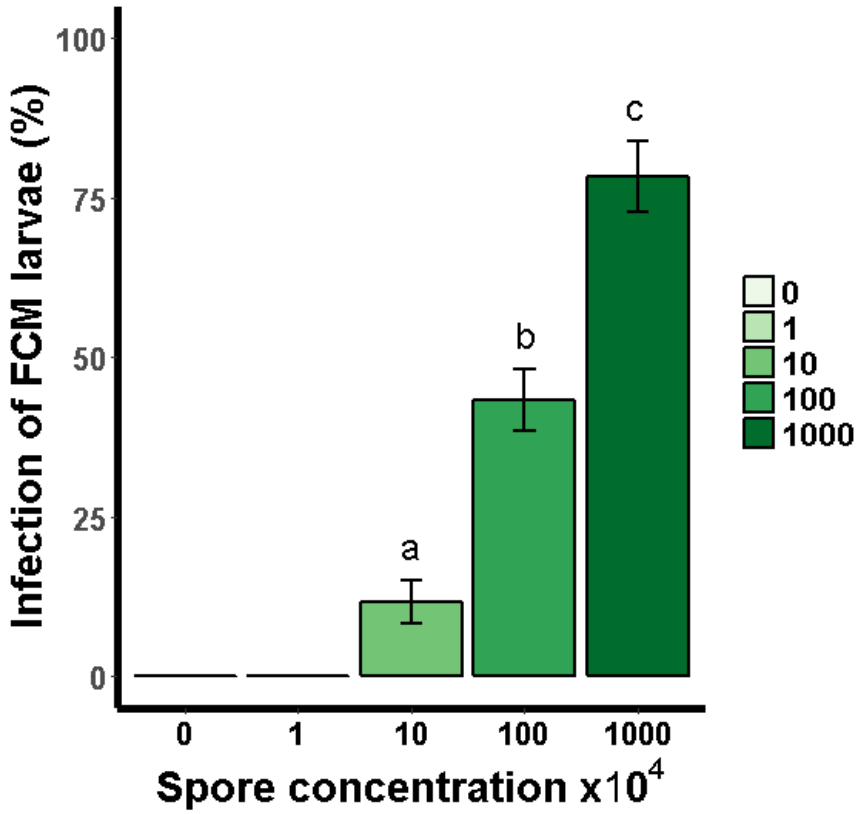

Fig. 2.6. Mean percentage infection (± SE) of late-instar *Thaumatotibia leucotreta* larvae after dunk test application of several concentrations of *Metarhizium robertsii* spores. Different letters above vertical bars indicate significant differences ($p < 0.05$).

2.4 Discussion

The survey of entomopathogens resulted in the recovery of multiple EPN and EPF isolates from soil samples collected from vineyards and orchards in the Western Cape. Most EPNs were recovered from table grape vineyards, as compared to stone fruit orchards, which yielded only two isolates. *Heterorhabditis indica* was isolated from a table grape vineyard in Halfmanshof, while the only previous report of this species in South Africa was from Bonnievale (Table 2.1), also from the Western Cape. Though *H. indica* is widespread in other parts of the world (Burnell and Stock, 2000), it seems to be rare in the surveys from South Africa conducted so far as it has only been recovered in one study other than the current (Malan et al., 2011). Interestingly, bacteria from *H. indica* VS5 turned wax moth larvae yellow upon infection, while the other isolate VS8 recovered from this survey and the isolate SGS turned wax moth larvae brick red. *Heterorhabditis indica* is associated with *Photorhabdus luminescens* (Poinar, 1983; Burnell and Stock, 2000). Future studies should confirm the identity of the associated bacteria with locally isolated *H. indica* and the discrepancy with regard to colour change of the infected wax

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moth larvae. Ferreira et al. (2014) showed that locally isolated *H. zealandica* was associated with a new symbiotic bacteria, *Photorhabdus zealandica*, which turned *G. mellonella* larvae steel grey. During this study *H. zealandica*, which turned *G. mellonella* larvae red and greenish, were found. This result indicates that there may be three different bacteria associated with the three different isolates of *H. zealandica* in South Africa. During a recent South African survey, James et al. (2018) isolated *H. zealandica* turning *G. mellonella* larvae both red and green.

Oscheius microvilli from the family Rhabditidae was recovered from a table grape vineyard and is the first record of its presence in South Africa. The species was first described from China in 2017 (Zhou et al., 2017). It is an interesting species as the *Oscheius* genus may associate with several different bacteria (often *Serratia* spp.; Zhou et al., 2017) and thus does not have such a close relationship with its bacteria as do species from *Heterorhabditis* and *Steinernema*. Their bacterial association is referred to as facultative and therefore its inclusion as an EPN is still debated in the literature (Dillman et al., 2012).

Unlike other studies in this region no *Steinernema* spp. were isolated (see Malan et al., 2011) and *S. carpocapsae*, which is the most widespread worldwide (Gaugler, 2002), has never been recovered in South Africa. A possible explanation for the reported absence of *S. carpocapsae* from South African soils could be the sampling method employed by others (Malan et al., 2006, 2011; Steyn et al., 2017) and in the current survey. All these studies share a soil sampling method where the soil is sampled by scraping away leaf litter and top soil. Then soil is sampled ± 10 - 20 cm below the soil surface. This method may bias EPN recovery, especially the recovery of *S. carpocapsae*, as it has been shown to mostly occur very close to the soil surface (Chandler et al., 1998; Gaugler, 2002). It is therefore suggested that future studies should include top soil and leaf litter in their sampling effort for EPNs.

Galleria mellonella is generally used as a trap insect for the isolation of EPNs and EPF (Goble et al., 2011; Coombes et al., 2013; Abaajeh and Nchu, 2015). Goble et al. (2010), found that *G. mellonella* had the highest (19%) success for isolating EPF during their survey, compared to *Ceratitis capitata* (Wiedemann; Diptera: Tephritidae) and FCM. During the present study, low (11%) recovery of EPF was obtained by using *G. mellonella* larvae as a bait insect. This may be ascribed to the methodology as, unlike the aforementioned study, the soil container was not tipped after placing the bait insect onto the soil. As the larvae do not burrow in soil, they had less chance to contract the EPF spores and this most likely caused the lower recovery of EPF strains. *Beauveria bassiana* was isolated most frequently of the EPF strains, which supports

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previous surveys (Goble et al., 2011). Various *Metarhizium anisopliae* strains have also been recovered previously by other studies (Goble et al., 2011; Coombes et al., 2013).

The first evidence of Lepidoptera [*Helicoverpa armigera* (Hübner) and *Spodoptera litura* Fabricius] eggs being susceptible to EPNs was provided by Kalia et al. (2014), who showed *Steinernema abbasi* Elawad, Ahamad and Reid (syn. *S. thermophilum*) with the symbiotic bacteria *X. indica* to be virulent. This finding is surprising as there are no natural openings in the cell wall of Lepidoptera eggs. The author suggests that the infective juveniles (IJs) penetrate the eggs. This is unlikely as the IJs do not have the necessary morphological structures with which to break through the cell wall (Kaya and Stock, 1997). However, it is plausible that EPNs are effective against lepidopteran eggs, especially as the authors provide photographic evidence for their finding. It is possible that the symbiotic bacteria or secretions (that may contain antibiotics or secondary metabolites) of the EPNs is causing the observed mortality. However, how the IJs manage to penetrate the egg is not well justified and still remains open for debate.

This study contributes novel evidence to the understanding of EPN virulence against the egg stage of FCM. The newly laid eggs proved to be impenetrable by the IJs of all the screened EPN species however, if inoculation occurred atthe black head stage, the eggs proved to be susceptible. This finding can be explained by the observation of the FCM egg every 12 h throughout its development, which showed that the neonate larva chews the wall of the egg and then remains in the egg for another 6 - 12 h before emerging. It is during this time that the IJs enter the egg through the cell wall and infect the FCM neonate larva before it emerges. This greatly reduces the time that the eggs are susceptible to attack and may explain the lower mortality seen in FCM when compared to other Lepidoptera, which reported 30% or higher mortality with the same IJs concentration (Kaila et al., 2014). The use of EPNs as a control will only be feasible practically if their limitations can be overcome, which include; 1) FCM eggs having a short window of susceptibility to EPNs, 2) EPNs being soil dwelling and will struggle to survive arboreally.

The EPN secretions, symbiotic bacteria and the nematodes themselves (Lu et al., 2017), played a role in the observed mortality of the egg stage. Interestingly, the present study showed that, under controlled conditions, *X. indica* is capable of killing the insect host irrespective of whether its vector is present or not. Further research specifically looking towards the potential of using the symbiotic bacterial cells or its secreted metabolites in isolation as a control could be promising, as it may be easier and cheaper to mass produce. However, before the symbiotic bacteria could be used in the field, the cells would have to be removed leaving only the

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supernatants which have been used and are effective as a control technique (Kepenekci et al., 2016). It is argued that in a field environment its performance is likely to be worse as the symbiotic bacteria or supernatants may be more sensitive to UV, heat and desiccation when it does not have its vector to buffer these environmental conditions. However, its potential may still be realised in orchards under netting or green house environments that have less harsh conditions or even post-harvest treatment of fruit.

As expected, the larvae were the most susceptible (90 - 100%) of the three life stages to EPNs and remains the best immature stage to target with control strategies. The EPN species tested had little success in controlling the pupae. The seemingly paradoxical finding of high virulence against larvae and low virulence against pupae can indicate some persistence (either mechanical or through the immune system) and may be evolutionary resistance of the pupae to soil dwelling pathogens, as they would have stronger selection on survival in this medium than larvae. Malan et al. (2011) reported higher susceptibility of the pupae than was shown here, one reason for this may be pupal age. The FCM remain prepupae for 3 days, at which time they are still soft and may have less resistance than the older pupae. It is suggested that age is an important factor to consider when determining the susceptibility of pupae to EPNs. Furthermore, EPNs detect the insect by following a CO_2 gradient (Robinson, 1995; Hallem et al., 2011; Gang and Hallem, 2016) and, as the pupae have a lower metabolic activity than larvae, they should produce less CO_2 and be less attractive to the EPNs.

The lower susceptibility of the pupal stage to EPN species, was not unexpected and has been shown before (Malan et al., 2011). However, as mentioned above, this may be overcome by targeting the pre-pupal stage. This would decrease the length of the application window, but should drastically increase the efficacy of the IJs to penetrate the pupae.

The collected EPF strains (*B. bassiana* and *M. anisopliae*) proved to be successful in causing mortality in FCM larvae. This supports previous findings that found various EPF strains (*B. bassiana* and *M. anisopliae*) to be highly (80%) effective against the 5th instar FCM (Goble et al., 2010, 2011; Coombes et al., 2017). Additionally, this study showed that *M. robertsii* is effective (78%) against FCM larvae. However, the high mortality, even with the short exposure time used in this study, suggests that EPF may be a viable control option for the larvae, even with its short application window, as they are easy to culture and remain viable for an extensive period of time in the soil (Coombes et al., 2017).

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The high larval infection from EPF strains screened in this study along with the evidence of previous studies (Goble et al., 2010, 2011) suggests that EPF has potential as a highly effective control agent against the soil borne stages of FCM. However, it should be noted that the EPF is a less favourable choice when targeting non-mobile stages of the life cycle as it is stationary and relies on its application coverage to control the pest. However, as FCM larva only burrow for a few millimetres into the soil to spin a cocoon (Love et al., 2019), spores washing into the soil would have an opportunity to target the prepupal stage of FCM, as well as during eclosion of the moth.

From this and previous studies it is clear that FCM larvae are highly susceptible to EPNs in the laboratory. Future research should test these EPN species in the field or semi-field environments. Recent work by Steyn et al. (2018), tested several EPN species that were cultured in *G. mellonella* in a semi-field environment, with promising results. However, larger field trials and commercial application of EPNs will require EPNs to be massed cultured *in vitro*, but how these EPNs perform against FCM in laboratory and semi field environments is still unknown.

2.5 References

Abaajeh, A.R., Nchu, F., 2015. Isolation and pathogenicity of some South African entomopathogenic fungi (Ascomycota) against eggs and larvae of *Cydia pomonella* (Lepidoptera: Tortricidae). Biocontrol Sci. Technol. 25, 828-842. doi:10.1080/09583157.2015.1019831

Abate, B.A., Slippers, B., Wingfield, M.J., Malan, A.P., Hurley, B.P., 2018. Diversity of entomopathogenic nematodes and their symbiotic bacteria in south African plantations and indigenous forests. Nematol. 20, 355-371.

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265-267.

Babendreier, D., Jeanneret, P., Pilz, C., Toepfer, S., 2015. Non-target effects of insecticides, entomopathogenic fungi and nematodes applied against western corn rootworm larvae in maize. J. Appl. Entomol. 139, 457–467. https://doi.org/10.1111/jen.12229

Bassi, A., 1835. Del Mal del segno calcinaccio o moscardino, malattia che afflige i bachi da seta, e sul modo di liberarne le bigattaje anche le più infestate, opera del dottore Agostino Bassi. Teoria tip. Orcesi.

Stellenbosch University https://scholar.sun.ac.za

Burnell, A.M., Stock, S.P, 2000. *Heterorhabditis*, *Steinernema* and their bacterial symbionts - lethal pathogens of insects. Nematol. 2, 31-42.

Campbell, J.F., Orza, G., Yoder, F., Lewis, E., Gaugler, R., 1998. Spatial and temporal distribution of endemic and released entomopathogenic nematode populations in turfgrass. Entomol. Exp. Appl. 86, 1-11.

Campos-Herrera, R., 2015. Nematode pathogenesis of insects and other pests. Series: Sustainability in Plant and Crop Protection (Ciancio, A., Series Ed.). Springer International Publishing, Switzerland, 530 pp.

Chandler, D., Bailey, A.S., Tatchell, G.M., Davidson, G., Greaves, J., Grant, W.P., 2011. The development, regulation and use of biopesticides for integrated pest management. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 366, 1987-1998.

Çimen, H., Lee, M.M., Hatting, J., Hazir, S., Stock, S.P., 2015. *Steinernema innovationi* n. sp. (Panagrolaimomorpha: Steinernematidae), a new entomopathogenic nematode species from South Africa. J. Helminthol. 89, 415-427.

Çimen, H., Půža, V., Nermuť, J., Hatting, J., Ramakuwela, T., Faktorova, L., Hazir, S., 2016. *Steinernema beitlechemi* n. sp., a new entomopathogenic nematode (Nematoda: Steinernematidae) from South Africa. Nematol. 18, 439-453.

Coombes, C.A., Hill, M.P., Moore, S.D., Dames, J.F., 2017. Potential of entomopathogenic fungal isolates for control of the soil-dwelling life stages of *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) in citrus. African Entomol. 25, 235-238.

Coombes, C.A., Hill, M.P., Moore, S.D., Dames, J.F., Fullard, T., 2013. Persistence and virulence of promising entomopathogenic fungal isolates for use in citrus orchards in South Africa. Biocontrol Sci. Technol. 23, 1053-1066.

De Doucet, M.M.A., Miranda, M.B., Bertolotti, M.A., 1998. Infectivity of entomogenous nematodes (Steinernematidae and Heterorhabditidae) to *Pediculus humanuscapitis* De Geer (Anoplura: Pediculidae). Fundam. Appl. Nematol. 21, 13-16.

De Jager, Z.M., 2013. Biology and ecology of the false codling moth, *Thaumatotibia leucotreta* (Meyrick). M.Sc. thesis, Stellenbosch University.

Stellenbosch University https://scholar.sun.ac.za

Dillman, A.R., Chaston, J.M., Adams, B.J., Ciche, T.A., Goodrich-Blair, H., Stock, S.P., Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. PLoS Pathog. 8, p.e1002527.

Ekesi, S., Maniania, N.K., Lux, S.A., 2002. Mortality in three African tephritid fruit fly puparia and adults caused by the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. Biocontrol Sci. Technol. 12, 7-17. doi:10.1080/09583150120093077

Ferreira, T., Malan, A.P., 2014. Potential of entomopathogenic nematodes for the control of the banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae). J. Helminthol. 88, 293-301. https://doi.org/10.1017/S0022149X13000175

Ferreira, T., Van Reenen, C.A., Endo, A., Spröer, C., Malan, A.P., Dicks, L.M.T., 2014. Description of *Photorhabdus zealandica* sp. nov., a symbiont of the entomopathogenic nematode *Heterorhabditis zealandic*a. Int. J. Syst. Evol. Microbiol. 64, 1540-1545.

Ferreira, T., Van Reenen, C.A., Tailliez, P., Pagès, S., Malan, A.P., Dicks, L.M.T., 2016. First report of the symbiotic bacterium, *Xenorhabdus indica*, associated with the entomopathogenic nematode *Steinernema yirgalemense*. J. Helminth. 90, 108-112. https://doi:10.1017/S0022149X14000583.

Gang, S.S., Hallem, E.A., 2016. Mechanisms of host seeking by parasitic nematodes. Mol. Biochem. Parasitol. 208, 23-32.

Gaugler, R. (ed.), 2002. Entomopathogenic nematology. CABI.

Goble, T.A., Dames, J.F., Hill, M.P., Moore, S.D., 2010. The effects of farming system, habitat type and bait type on the isolation of entomopathogenic fungi from citrus soils in the Eastern Cape Province, South Africa. BioControl 55, 399-412.

Goble, T.A., Dames, J.F., Hill, M.P., Moore, S.D., 2011. Investigation of native isolates of entomopathogenic fungi for the biological control of three citrus pests. Biocontrol Sci. Technol. 21, 1193-1211. doi:10.1080/09583157.2011.608907

Gumus, A., Karagoz, M., Shapiro-Ilan, D., Hazir, S., 2015. A novel approach to biocontrol: Release of live insect hosts pre-infected with entomopathogenic nematodes. J. Invertebr. Pathol., 130, 56–60. https://doi.org/10.1016/j.jip.2015.07.002

Hallem, E.A., Dillman, A.R., Hong, A.V., Zhang, Y., Yano, J.M., De Marco, S.F., Sternberg,

Stellenbosch University https://scholar.sun.ac.za

P.W., 2011. A sensory code for host seeking in parasitic nematodes. Curr. Biol. 21, 377-383.

Hatting, J., Stock, S.P., Hazir, S., 2009. Diversity and distribution of entomopathogenic nematodes (Steinernematidae, Heterorhabditidae) in South Africa. J. Invertebr. Pathol. 102, 120-128.

Hatting, J.L., Moore, S.D., Malan, A.P., 2018. Microbial control of phytophagous invertebrate pests in South Africa: Current status and future. J. Invertebr. Pathol. (In Press) https://doi.org/10.1016/j.jip.2018.02.004

Hofmeyr, J.H. 2003. Integrated pest and disease management. In: Integrated Production Guidelines for Export Citrus. Citrus Research International, Nelspruit. Volume 3, 95-101.

Hofmeyr, J.H., Pringle, K.L., 1998. Resistance of false codling moth, *Cryptophlebia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), to the chitin synthesis inhibitor, triflumuron. African Entomol. 6, 373-375.

Inglis, D.G., Enkerli, J., Goettel, M.S., 2012. Laboratory techniques used for entomopathogenic fungi: Hypocreales. In: Lacey, L.A. (ed) Manual of techniques in invertebrate pathology, Elsevier, San Diego.

Jacobs, K., Bergdahl, D.R., Wingfield, M.J., Halik, S., Seifert, K.A., Bright, D.E., Wingfield, B.D., 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycol. Res. 108, 411-418. doi:10.1017/S0953756204009748

James, M., Malan, A.P., Addison, P., 2018. Surveying and screening South African entomopathogenic nematodes for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Crop Prot. 105, 41-48.

Kalia, V., Sharma, G., Shapiro-Ilan, D.I., Ganguly, S., 2014. Biocontrol potential of *Steinernema thermophilum* and its symbiont *Xenorhabdus indica* against lepidopteran pests: virulence to egg and larval stages. J. Nematol. 46, 18-26.

Kaya, H.K., Stock, P.S., 1997. Techniques in insect nematology. Man. Tech. Insect Pathol. 281-324. https://doi.org/10.1016/B978-012432555-5/50016-6

Kepenekci, I., Hazir, S., Lewis, E.E., 2016. Evaluation of entomopathogenic nematodes and the

Stellenbosch University https://scholar.sun.ac.za

supernatants of the *in vitro* culture medium of their mutualistic bacteria for the control of the root-knot nematodes *Meloidogyne incognita* and *M. arenaria*. Pest Manag. Sci. 72, 327-334. https://doi.org/10.1002/ps.3998

Kim, H.H., Choo, H.Y., Kaya, H.K., Lee, D.W., Lee, S.M., Jeon, H.Y., 2004. *Steinernema carpocapsae* (Rhabditida: Steinernematidae) as a biological control agent against the fungus gnat *Bradysia agrestis* (Diptera: Sciaridae) in propagation houses. Biocontr. Sci. Technol. 14, 171-183.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: Back to the future. J. Invertebr. Pathol. 132. https://doi.org/10.1016/j.jip.2015.07.009

Love, C.N., Moore, S.D., Hill, M.P., 2019. The role of abiotic factors in the pupation of *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Tortricidae) in the soil. Agr. Forest Entomol., 21, 38-49.

Lu, D., Macchietto, M., Chang, D., Barros, M.M., Baldwin, J., Mortazavi, A., Dillman, A.R., 2017. Activated entomopathogenic nematode infective juveniles release lethal venom proteins. PLoS Pathog. 13, e1006302. https://doi.org/10.1371/journal.ppat.1006302

Malan, A.P., Nguyen, K.B., Addison, M.F., 2006. Entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from the southwestern parts of South Africa. African Plant Protect. 12, 65-69.

Malan, A.P., Knoetze, R., Moore, S.D., 2011. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. J. Invertebr. Pathol. 108, 115-125. https://doi.org/10.1016/j.jip.2011.07.006

Malan, A.P., Knoetze, R., Tiedt, L., 2014. *Heterorhabditis noenieputensis* n. sp. (Rhabditida: Heterorhabditidae), a new entomopathogenic nematode from South Africa. J. Helminthol. 88, 139-151.

Malan, A.P., Hatting, J.L., 2015. Entomopathogenic nematode exploitation: case studies in laboratory and field applications from South Africa. In: Campos-Herrera, R. (eds). Nematode pathogenesis of insects and other pests. Springer, Cham, pp. 477-508.

Malan, A.P., Moore, S.D., 2016. Evaluation of local entomopathogenic nematodes for the

Stellenbosch University https://scholar.sun.ac.za

control of false codling moth, *Thaumatotibia leucotreta* (Meyrick, 1913), in a Citrus Orchard in South Africa. African Entomol. 24, 489-501.

Malan, A.P., Ferreira, T., 2017. Entomopathogenic nematodes. In: Fourie, H., Spaull, V.W., Jones, R.K., Daneel, M.S., De Waele, D. (eds). Nematology in South Africa: A View from the 21st Century. Springer, Cham. pp. 459-480.

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

Manrakhan, A., Daneel, J.-H., Moore, S.D., 2013. The impact of naturally occurring entomopathogenic nematodes on false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in citrus orchards. Biocontr. Sci. Technol. 24, 241-245. https://doi.org/10.1080/09583157.2013.854316

Moore, S.D., Fullard, T., Dames, J.F., Hill, M.P., Coombes, C.A., 2015. *Beauveria* and *Metarhizium* against false codling moth (Lepidoptera: Tortricidae): a step towards selecting isolates for potential development of a mycoinsecticide. African Entomol. 23, 239-242.

Nguyen, K.B., 2007. Methodology, morphology and identification. In: Nguyen, K.B.,Hunt, D.J. (eds.), Entomopathogenic Nematodes: Systematics, Phylogeny and Bacterial Symbionts. Brill, Leiden-Boston, pp. 59-119.

Poinar, G.O., 1983. The natural history of nematodes. Prentice-Hall, NJ: Englewood Cliffs.

Prinsloo, G.L., Uys, V.M., 2015. Insects of cultivated plants and natural pastures in Southern Africa. Entomological Society of Southern Africa, South Africa. 491pp.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Robinson, A.F., 1995. Optimal release rates for attracting *Meloidogyne incognita*, *Rotylenchulus reniformis*, and other nematodes to carbon dioxide in sand. J. Nematol. 27, 42-50.

Shahina, F., Gulsher, M., Javed, S., Khanum, T.A., Bhatti, M.A., 2009. Susceptibility of

Stellenbosch University https://scholar.sun.ac.za

different life stages of red palm weevil, *Rhynchophorus ferrugin*eus, to entomopathogenic nematodes. Int. J. Nematol. 19, 232-240.

Shapiro-Ilan, D.I., Gouge, D.H., Piggott, S.J., Fife, J.P., 2006. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. Biol. Control 38, 124–133. https://doi.org/10.1016/j.biocontrol.2005.09.005

Shapiro-Ilan, D.I., Jackson, M., Reilly, C.C., Hotchkiss, M.W., 2004. Effects of combining an entomopathogenic fungi or bacterium with entomopathogenic nematodes on mortality of *Curculio caryae* (Coleoptera: Curculionidae). Biol. Control 30, 119-126. https://doi.org/10.1016/j.biocontrol.2003.09.014

Sievert, C., 2018. plotly for R. https://plotly-book.cpsievert.me

Simberloff, D., 2009. The role of propagule pressure in biological invasions. Annu. Rev. Ecol. Evol. Syst. 40, 81-102.

Steinhaus, E.A., 1949. Principles of Insect Pathology. McGraw-Hill B. Company, INC 764.

Steyn, W.P., Malan, A.P., Daneel, M.S., Slabbert, R.M., 2017. Entomopathogenic nematodes from north-eastern South Africa and their virulence against false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). Biocontrol Sci. Technol. 27, 1265-1278. https://doi.org/10.1080/09583157.2017.1391174

Steyn, W.P., Malan, A.P., Daneel, M.S., Slabbert, R.M., 2018. Entomopathogenic nematodes (Rhabditidae and Heterorhabditidae) for the control of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) under South African conditions (Ph.D. dissertation, Tshwane University of Technology).

Warton, D.I., Hui, F.K., 2011. The arcsine is asinine: the analysis of proportions in ecology. Ecology, 92, 3-10.

Wickham, H. 2018. forcats: Tools for Working with Categorical Variables (Factors). R package version 0.3.0. https://CRAN.R-project.org/package=forcats

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Zhou, G., Yang, H., Wang, F., Bao, H., Wang, G., Hou, X., Lin, J., Yedid, G., Zhang, K., 2017. *Oscheius microvilli* n. sp. (Nematoda: Rhabditidae): A facultatively pathogenic nematode

Stellenbosch University https://scholar.sun.ac.za

from Chongming Island, China. J. Nematol. 49, 33-41.

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Chapter 3:

Control of false codling moth, *Thaumatotibia leucotreta*, using in vitro cultured *Steinernema jeffreyense* and *S. yirgalemense*

Abstract

False codling moth (FCM), *Thaumatotibia leucotreta*, is a priority pest of citrus, stone fruit and table grapes, as it causes direct crop damage. Biological control of entomopathogenic nematodes (EPNs) has not been explored for use against FCM in stone fruit and table grapes. However, EPNs have been shown to provide exceptional efficacy against the larvae of FCM in laboratory bioassays, compared to other biocontrol agents. EPNs are particularly attractive for the control of FCM as they attack the soil-dwelling stages. In this study, the mortality, quality and age of *in vitro* liquid cultured *Steinernema jeffreyense* was assessed in the laboratory and tested in the field. Additionally, pre- and post-application pathogenicity tests with FCM larvae were conducted to assess EPN virulence of *in vitro* cultured nematodes. Field trials were conducted by means of applying infective juveniles (IJs) to the vineyard floor. Four different *S. jeffreyense* concentrations (0, 10, 20 and 30 IJs/cm^2) were applied to forty 1 m^2 experimental plots that were artificially infested with FCM larvae. The insects were retrieved from the soil 48 h after application, to allow for the assessment of the immediate effect. FCM loaded cages were replaced over a period of four weeks, to determine the persistence of the original application. A second trial, following the same procedure, *S. jeffreyense* and *Steinernema yirgalemense* were compared with regard to virulence and persistence over a 4-week period. In the laboratory, the *in vitro* cultured EPNs proved to be of similar quality as the *in vivo* cultured *S. jeffreyense* with a high percentage mortality of > 80%. The semi-field study showed promising results, with the immediate effect yielding up to 77% mortality of FCM larvae and remained > 35% over the 4-week period after application. These results compare favourably with previous field studies, using *in vivo* EPN, proving that EPNs would be a valuable addition to the current integrated pest management programme to control FCM.

Keywords: entomopathogenic nematodes, *in vitro*, field, IPM

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3.1 Introduction

False codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), is an economically important pest and has additional phytosanitary restrictions (Prinsloo and Uys, 2015). However, there are limited chemical control options that effectively control this pest, especially as harvest draws near, encouraging the need for environmentally friendly control options (Malan et al., 2018). Entomopathogenic nematodes (EPNs) are roundworms that have a mutualistic symbiotic relationship with gram negative bacteria that can work together to kill insects within 48 h (Gaugler, 2002). There is potential for the use of these organisms for the biological control of the soil stage of FCM (Chapter 2; Malan et al., 2011; Steyn et al., 2017). Biological control through the augmentative release of EPNs has been practised worldwide for several decades (Campos-Hererra, 2015), but has not been implemented against FCM in stone fruit and table grapes in South Africa. In citrus, Cryptonem (L9251) [*Heterorhabditis bacteriophora*, River Bioscience (Pty.) Ltd, Port Elizabeth, South Africa] is used against FCM at 10 IJ/cm^2 and is currently the only EPN product available (imported) in South Africa (Hatting et al., 2018).

To make augmentative releases a reality, the EPNs need to be massed produced, which can be achieved by one of three methods. *In vivo* production, is thought to yield the highest quality EPNs (Gaugler and Georgis, 1991; Yang et al., 1997) and requires lower technology input and capital outlay (Shapiro-Ilan et al., 2012). *In vitro* production comes in two forms, solid culture and liquid culture. Solid culture is seen as the intermediate between *in vivo* and liquid in terms of capital outlay and EPN quality (Shapiro-Ilan et al., 2012). However, *in vitro* liquid culture has the advantage of economy of scale, (successful production in 80 L bioreactors, Georgis et al., 1995), making the production less expensive and as such is the most (95%) adopted method worldwide (Shapiro-Ilan et al., 2012).

The mass production of organisms may lead to the selection of attributes that increase their production potential, which may include increased fecundity and survivability. Unfortunately, the increased production is one trait that may cause a marked decrease (cost) in other aspects of the organism's survival or behaviour that is not continuously selected for during the rearing process. This leads to trait deterioration and most frequently manifests as life-history trade-offs between reproduction and performance as was found for invertebrates, Lepidoptera and EPNs (Phillips et al., 2006; Bilgrami et al., 2006; Hanski et al., 2006). Consequently, it is likely that the virulence of EPNs against insects may decline over time in mass reared systems and may be more pronounced with *in vitro* culture (as this trait is not selected for) causing the quality of

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the EPNs to be reduced (Shapiro-Ilan and Gaugler, 2002). In fact, previous studies have provided evidence that there are differences in the virulence between *in vitro* and *in vivo* cultured EPNs (Gaugler and Georgis, 1991; Shapiro-Ilan and Gaugler, 2002; Ferreira et al., 2014, 2016).

The suppression of pest insects, with the application of mass reared EPNs, has been reviewed by Shapiro-Ilan et al. (2002) and Lacey et al. (2015). The authors highlight several factors (biological and economic) that allow some interventions to lead to success rather than failure. The biological factors include the phenology (life stages susceptible to the EPN), environmental factors (especially soil moisture and temperature), timing (cooler seasons more favourable), application (methods that deploy the EPN into the soil are favoured) and formulation. Formulation is of considerable importance as it offers several benefits, it lengthens shelf life, aids in transportation and may benefit in field survival (Kagimu et al., 2017). Often, however, even when all the biological factors are favourably met, the use of EPNs may still not be adopted due to economic factors, such as competition with other biological control techniques (ease of use, price/ha, ability to control other pests) (Shapiro-Ilan et al., 2002). In lieu of this, these biological and economic factors should first be validated, before an EPN can be considered as a potential biological control option.

Numerous biological and economic factors that lead to the success of biological control have been validated for the use of EPNs to control lepidopteran pests in the South African context. For instance, phenology (Chapter 2, Malan et al., 2011), environmental factors (De Waal et al., 2011) and formulation (Kagimu et al., 2017) have been studied and show suitability for this control method. The method is also economically competitive as EPNs have been shown to target multiple South African pest insects and can be sprayed with conventional farm equipment creating high demand from growers (Malan et al., 2018). Additionally, semi-field experiments using *in vivo* reared EPNs against FCM larvae show promising results (Malan and Moore, 2016; Steyn et al., 2018). However, these validations have all been done with *in vivo* cultures that do not translate well to economies of scale (relatively high price/ha). It is therefore vital to determine how liquid *in vitro* reared EPNs perform in comparison to *in vivo* reared EPNs.

The objective of this study was to determine the virulence of *in vitro* liquid mass cultured EPNs, against late instar FCM larvae in the laboratory and semi-field environment. In the laboratory, the effect of age of *in vitro* cultured and stored *S. jeffreyense* on virulence against FCM larvae was tested. It was hypothesised that initially there would be no difference in virulence against FCM, but with storage time, virulence will decrease. Additionally, it was aimed to determine

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the quality of *in vitro* liquid cultured *S. jeffreyense*, in comparison with those cultured *in vivo*. It was hypothesised that the *in vivo* cultured infective juveniles (IJs) are more natural and will outperform the *in vitro* cultured IJs in terms of FCM pathogenicity. Additionally, it was predicted that *S. yirgalemense* will outperform (measured as infection of FCM larvae) *S. jeffreyense* in the field environment, as was shown previously (Chapter 2, *in vivo* culture) in the laboratory. Lastly, the effect of handling was tested on the field inoculum and whether these effects can be offset by formulation was investigated. It was hypothesised that by formulating the IJs it will decrease the deterioration of the nematodes and ensure high virulence of the IJs against FCM larvae irrespective of handling in field.

3.2 Material and methods

3.2.1 Source of insects

The late instar larval stage of *T. leucotreta*, collected from a mass reared colony maintained at XSIT in Citrusdal, South Africa, were transported in a cooler box to Stellenbosch. Upon arrival in Stellenbosch, the insects were placed at 27°C ± 1°C. *Galleria mellonella* L (Lepidoptera: Pyralidae) or wax moth larvae were cultured according to the technique of Van Zyl and Malan (2015) and used to culture *in vivo* EPNs.

3.2.2 Source of entomopathogenic nematodes

The Department of Conservation Ecology and Entomology, Stellenbosch University, supplied the *in vitro* liquid cultured IJs of *S. jeffreyense* (J192) (KC897093) and *S. yirgalemense* (157-C) (AY748450) used in this study. The IJs were cultured using the technique of Dunn et al. (2018) and stored, 30 ml of the liquid diet, in 250 ml sterile Erlenmeyer flasks, plugged with cotton wool, on a platform orbital shaker at 120 rpm, at a temperature of 14°C, for different times. *In vivo S. jeffreyense* were cultured using *G. mellonella* larvae as host and a modified White trap was used to collect the IJs (Kaya and Stock 1997). The formulation used, contained the *in vitro* cultured *S. jeffreyense* and was formulated according to Kagimu (2018).

3.2.3 Bioassay protocol

Filter paper discs were placed in alternate wells of a 24-well bioassay plate. All treatments were inoculated with a suspension containing IJs in distilled water and 50 µl of the inoculum was pipetted into each well, which ensured the filter paper was moist, without free water. Control treatments received 50 µl of water only. Sixty late instar FCM larvae per treatment were placed on moist filter paper, in 12 alternative wells of five 24-well bioassay plates. A glass sheet in the lid ensured no larvae escaped. The bioassay plates were placed in 2 L plastic containers on

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moist paper towels to maintain moisture, closed with the lid and kept in a dark room for 48 h at 27°C. Mortality by infection was assessed with the aid of a dissecting microscope after 48 h, by opening the larvae and visually observing the nematodes inside of the cadaver.

3.2.4 Baseline sampling and trapping

Each vineyard was divided into four quadrants, within each quadrant five subsamples of 50 g of soil were collected and placed into a 1 L plastic container, so that each block had four plastic containers, each of which constituted a sample so that the baseline trapping had a total of 8 samples. Each of these soil samples were baited with10 *G. mellonella* larvae, to trap for resident EPN occurrence (Malan and Moore, 2016).

3.2.5 Pathogenicity of age and formulation of in vitro cultured nematodes

The effect of age and quality of the *in vitro* liquid cultured IJs was determined by comparing the virulence of *S. jeffreyense* against larvae of FCM, following the bioassay protocol outlined above, at a concentration of 50 IJs/50 µl. Three *in vitro* cultured IJs ages were tested, namely fresh formulation (1 - 2 days old), 86 days old (the intermediate) and 136 days old. The age of the inoculum was calculated 14 days after inoculation of the liquid culture, with a new cohort of IJs available in the flasks, which were then moved from 25°C to storage at 14°C. To determine the quality of the *in vitro* culture, its virulence against FCM larvae was compared to a fresh *in vivo* IJs culture. Above trials were repeated on a different test date and with another batch of inoculum.

3.2.6 Laboratory pathogenicity of field inoculum

The inoculum of the *in vitro S. jeffreyense*, used for the semi-field trial was tested for pathogenicity against FCM larvae, 12 h before application, using the bioassay protocol. *Steinernema jeffreyense* cultured *in vivo* and *in vitro* were applied at 50 IJs/insect. After 48 h mortality by infection was determined as this will indicate a baseline for the quality and maximum performance (calculated as infection) to be expected in the field from the applied nematodes.

3.2.7 Field trial to assess concentration

Two semi-field trials were conducted during summer (January - March) 2018 in table grape vineyards in Paarl (33°43'07.1"S 18°57'54.7"E). Each field trial consisted of forty 1 m^2 experimental plots, each plot was assigned to a treatment, following a completely randomised design (Fig. 3.1.). The field trials followed the protocol outlined in Malan and Moore (2016). At each experimental plot a metal mesh cage was buried just below the soil surface 20 cm away

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from the stem. Each cage was filled with sifted soil from the orchard and 20 FCM larvae. The vineyard was irrigated once per week to ensure adequate moisture.

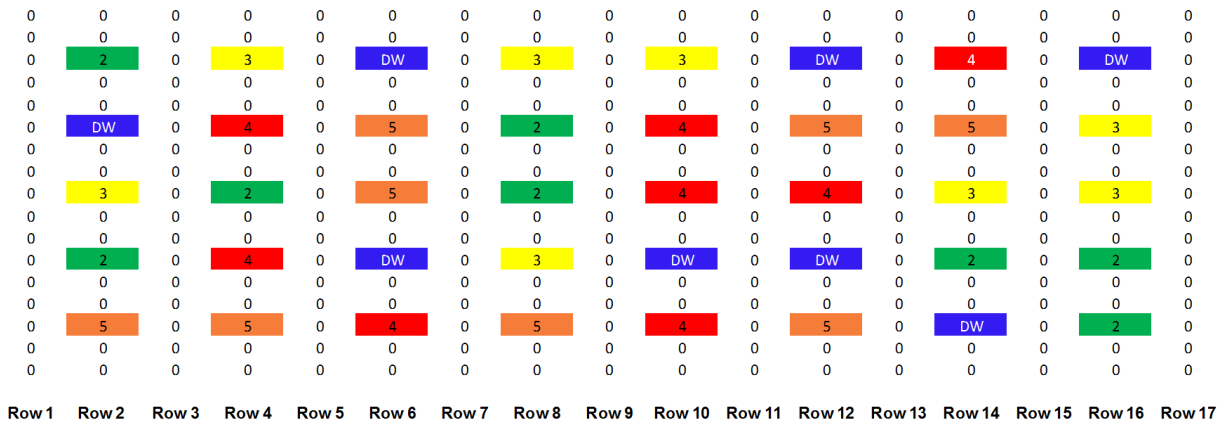

Fig. 3.1. The completely randomised design of the semi-field trials. Each zero represents a grapevine plant which was not treated and served as a buffer between experimental plots. The experimental plots are represented by the different coloured blocks with each colour is a different treatment.

An inoculum with a low IJs concentration (2000 IJs/ml or 100 IJs/50 µl) of each treatment (except the formulation, as it was in solid form) was transported to the field in a 2 L glass bottle and then diluted further for field use. To avoid oxygen deprivation, only 1.2 L of inoculum was placed in the bottle. Additionally, the bottles were agitated every 5 min to ensure adequate oxygen distribution. The dry formulation was added directly to the tank of the pressure sprayer in the field. The transported inoculum was diluted to a 1 L inoculum and the contents applied to each experimental block. Before each EPN application, 30 ml was sprayed into a glass jar and inspected to ensure effective nematode dispensing. The IJs were then applied to the vineyard floor with a 5 L pressure sprayer, after the vineyard floor had been artificially infested with FCM larvae in buried cages. The insects were retrieved from the soil 48 h later to allow for assessment of the immediate effect. Newly filled cages (containing soil and larvae) were then re-buried 7, 14, 21 and 28 days after application and retrieved 48 h later. After retrieval of the cages, they were taken to the laboratory where the soil was sifted and the larvae or prepupae were inspected for mortality and dissected to confirm infection by EPNs and determine persistence.

Three temperature Thermochron iButton® data loggers (Dallas Semiconductors, Model DS1920; 0.5°C accuracy) were equally spaced in the experimental plot. These consisted of two

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that were placed in an identical cage as the larvae, just below the soil surface in row five and row 13. These iButtons therefore recorded the temperature the FCM larvae experienced. The third ibutton was placed in row nine, one metre above the soil surface allowing it to measure the ambient temperature. All ibuttons recorded the temperature (± 0.5°C) every 15 min over the 28-day trial period.

The treatment included four different *in vitro* liquid cultured *S. jeffreyense* concentrations (0, 10, 20, 30 IJs/cm^2) and the formulation at 30 IJs/cm^2. Each treatment was randomly assigned and applied to eight of the 1 m^2 experimental plots. To achieve the desired concentrations the transported inoculum was diluted into a 1 L IJs suspension.

3.2.8 Species comparison field trial

The trial (followed a similar design as section 3.2.7) compared both *in vitro* liquid cultured *S. jeffreyense* and *S. yirgalemense*, previously shown to be virulent against FCM (Chapter 2). The two species were compared to a water only control and each of the treatments were applied at 20 IJs/cm^2 to 16 of the 1 m^2 experimental blocks. Cages were retrieved and immediately replaced with fresh FCM loaded cages, on day 7, 14 and 28, to determine the persistence between the two nematode species. Soil temperature was recorded as described above.

3.2.9 Post application viability of inoculum

The inoculum that was kept in the field and then used for the application, was transported back to the laboratory after use and tested against FCM. The treatments included *in vitro* cultured IJs (lowest field concentration = 22.7 IJs/50 µl and 50 IJs/50 µl), the formulation (still in dry form and only diluted prior to test, 50 IJs/50 µl) and a water only control (50 µl). This allowed the assessment of whether handling of the inoculum decreased the IJs pathogenicity against FCM.

3.2.10 Statistical analyses

The statistical analysis was performed using Statistica 12 (Stat-Soft Inc., 2012). The data were tested for normality using Shapiro Wilk's test. If the data were not normally distributed they were log(X+1)-transformed for further analysis. If the assumptions (normality and equality) of a one-way analysis of variance (ANOVA) were violated irrespective of transformation, the Kruskal-Wallis H-test was used to separate the means at $p < 0.05$. If there were no significant test date versus treatment interactions, data from different test dates were pooled and analysed using ANOVA. When applicable, means were separated using the Fisher's LSD method. All further statistical analyses were undertaken in RStudio version 1.0.143 and R version 3.5.1 (R, 2018). A generalized linear model, with a gaussian distribution and an identity link function,

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was used to determine the effect of handling the inoculum in the field during field application. It was also used to compare the soil and ambient temperature over the 4-week field trial. Several packages were used to illustrate our findings graphically, including ggplot2, plotly and forcats (Wickham, 2016; Sievert, 2018).

3.3 Results

3.3.1 Baseline sampling and trapping

No EPNs were isolated from any of the soil samples collected from the table grape vineyards. This indicates that there were no or very low levels of naturally occurring EPNs present.

3.3.2 Pathogenicity of age and formulation of in vitro cultured nematodes

There was a significant ($F_{4, 50}$ = 61.036, p < 0.001, df = 50) effect of age amongst the *in vitro* cultures (Fig. 3.2), the older cultures of 136 days (*in vitro* B) (86.67% ± 2.83%) performed better than the fresh formulation (72.5% ± 5.13%, p = 0.036) and 86-day-old (*in vitro* A) (47.5% ± 4.82%, p < 0.001) cultures. The 136-day-old *in vitro* reared IJs did not significantly (p = 0.782) differ from the *in vivo* (85% ± 5.09%) cultured *S. jeffreyense.*

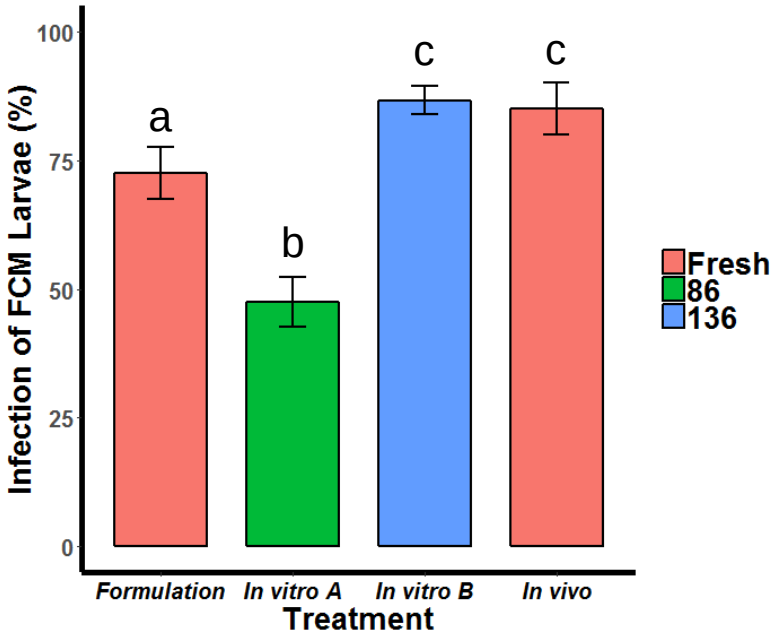

Fig. 3.2. Mean percentage infection (± SE) of *Thaumatotibia leucotreta* larvae inoculated with *Steinernema jeffreyense* produced either by *in vitro* liquid (A = 86 days old and B = 136 days old) culture, formulated and *in vivo* (fresh), mortality was assessed 48 h after treatment. The last instar FCM larvae were inoculated with 50 IJs/insect for each of the treatments. Different letters above the vertical bars indicate significant differences (p < 0.05).

3.3.3 Laboratory pathogenicity of field inoculum

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There was no significant difference ($H_{1, 25} = 0.479$, $p = 0.489$) found between the *in vivo* and the *in vitro* cultured *S. jeffreyense*, used in the field trials (Fig. 3.3). The *in vitro* caused high infection (86.67% ± 1.36%) of FCM larvae and was the maximum performance or mortality that was expected in the field.

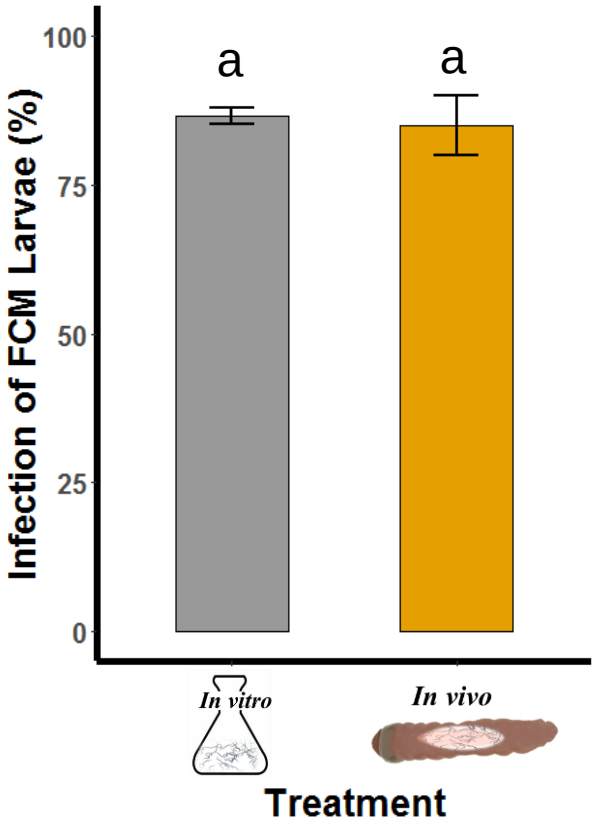

Fig. 3.3. Mean percentage infection (± SE) of last instar *Thaumatotibia leucotreta* larvae inoculated with *Steinernema jeffreyense* (50 IJs/insect) cultured for the semi-field trials, either by *in vitro* or *in vivo* cultured IJs. Mortality was assessed 48 h after treatment. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.3.4 Field trial to assess concentration

The average soil temperature, (23.18°C) during the exposure of the FCM larvae in the soil, was significantly ($T = -8.97$, $df = 5811$, $p < 0.001$) lower than the average ambient temperature (24.92°C), over the 4-week field trial (Fig. 3.5).

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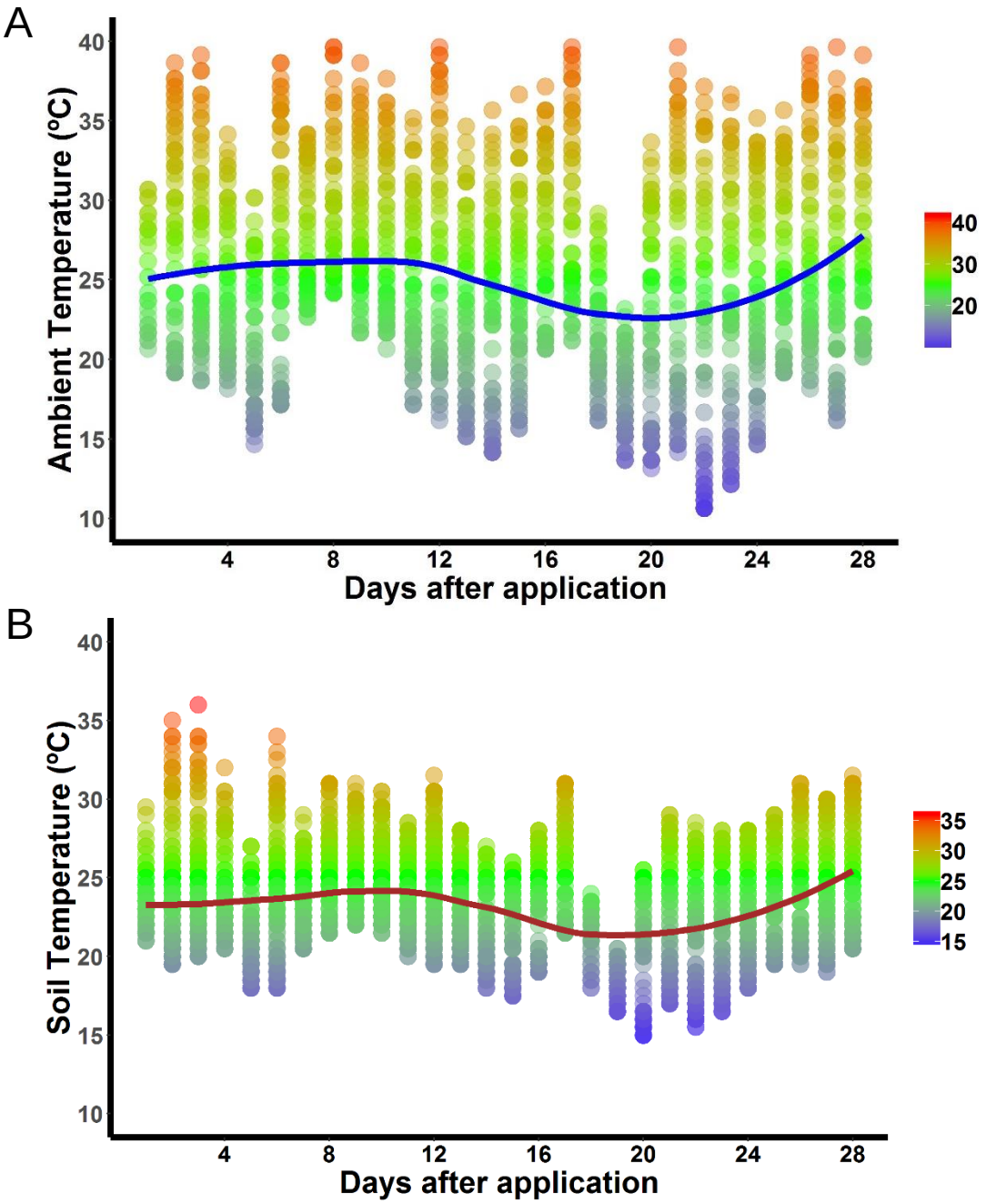

Fig. 3.4. The temperature recorded every 15 min (dots) over the 28-day exposure period of the concentration field trial in Paarl. The vertical dots are coloured on scale from low (blue, 15°C) to high (red, 40°C) temperatures. Panel (A) the ambient temperature measured in the middle of the experimental plot (row nine). Panel (B) the soil temperature measured in row five and 13 of the experimental plot. The solid lines represent the average ambient (blue) and soil (brown) temperature.

There was a positive dose effect, with the highest concentration causing significantly ($F_{3, 24}$ = 3.683, p = 0.004) higher infection of FCM larvae, for the immediate effect (48 h), than the

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lowest dose (Fig. 3.6A). *Steinernema jeffreyense* at the highest concentration caused mortality of 76.99% ± 6.59% and performed the best for the immediate effect (48 h), but did not significantly differ from medium concentration (p = 0.062, 58% ± 8.25%) or formulation (p = 0.267, 67.16% ± 5.14%). Overall there was a significant ($F_{2, 46}$ = 4.639, p = 0.015) decrease in persistence as time passed (Fig. 3.6B). However, there was no significant ($F_{8, 46}$ = 1.083, p = 0.392) difference between the *S. jeffreyense* treatments, but the formulation did significantly (p = 0.009) differ from *in vitro* high concentration at each time point post application. The infection of the *in vitro* low, did not significantly ($p > 0.05$) decrease with time [immediate (48 h), 14, 21, 28 days] and its infection remained the most stable (overall mean 36.5%) throughout the 28 day trial. The *in vitro* high concentration caused infection of a third (33.07% ± 7.92%) of the FCM larvae 28 days after the application, whereas the formulation was unable to infect a fifth (17.59% ± 2.18%) of the FCM larvae after the same time.

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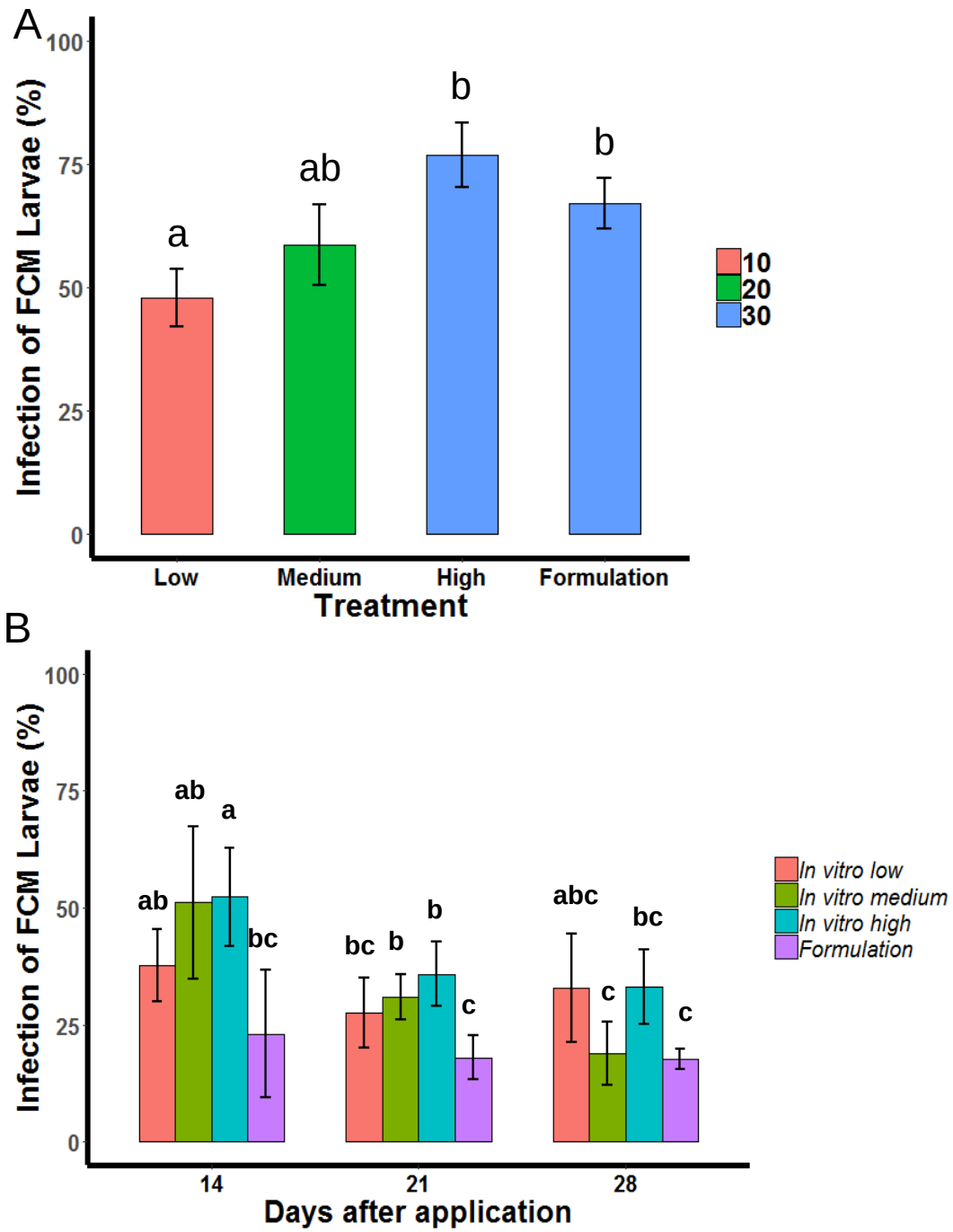

Fig. 3.5. Mean percentage infection (± SE) of *Thaumatotibia leucotreta* larvae applied with *in vitro Steinernema jeffreyense* in semi-field conditions. Treatments were liquid *in vitro* low (red, 10 IJs/cm^2), medium (green, 20 IJs/cm^2), high concentrations (blue, 30 IJs/cm^2) and an *in vitro* formulation (Panel A blue, Panel B purple at 30 IJs/cm^2). Panel (A) shows the immediate effect (48 h after application) of the treatments on the FCM larvae. Panel (B) shows the persistence of the treatments to infect FCM larvae 14, 21 and 28 days after the initial application. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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3.3.5 Species comparison field trial

The average soil temperature (21.71°C), during the exposure of the FCM larvae in the soil, was significantly (Z = -10.93, N = 4894, $p < 0.001$) colder (2°C less) than in the concentration trial (Fig. 3.7).

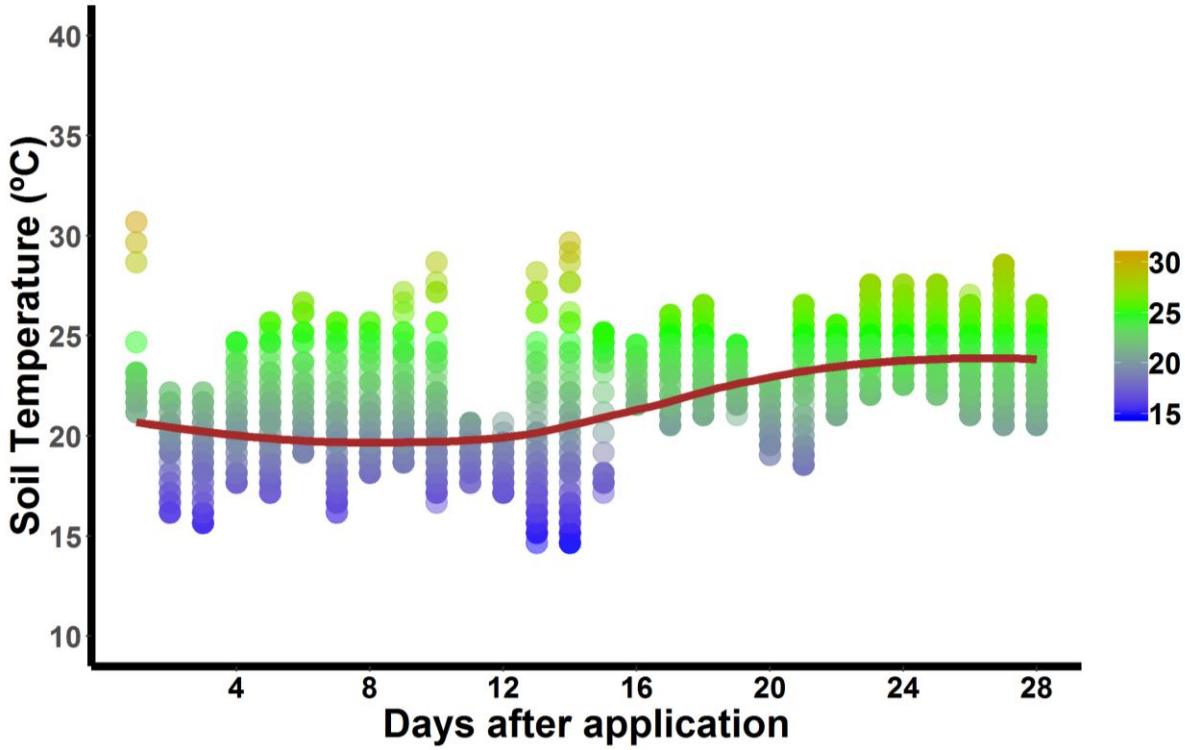

Fig. 3.6. The temperature recorded every 15 min (dots) over the 28-day period exposure of the *Steinernema yirgalemense* and *S. jeffreyense* comparison field trial in Paarl. The soil temperature was measured in row five and 13 of the experimental plot. The solid line (brown) represents the average soil temperature, while the vertical dots are coloured on a scale from low (blue, 15°C) to high (red, 30°C) temperatures.

There was no overall significant ($F_{1, 18}$ = 2.038, p = 0.171) difference of infection of FCM larvae between the two species over the 28-day field trial (Fig. 3.7). The overall infection of FCM larvae significantly ($F_{2, 36}$ = 19.436, $p < 0.001$) decreased as time increased although the persistence of the species differed. In the case of *S. jeffreyense*, infection of FCM larvae significantly ($F_{2, 31}$ = 16.365, $p < 0.001$) decreased with every succeeding week after the application. However, *S. yirgalemense* infection only significantly (p = 0.023) decreased from day 7 (73.34% ± 7.89%) to 14 (44.94% ± 3.98%) after application, but did not significantly (p = 0.544) decrease from 14 to 28 days (37.84% ± 10.18%) after the initial application.

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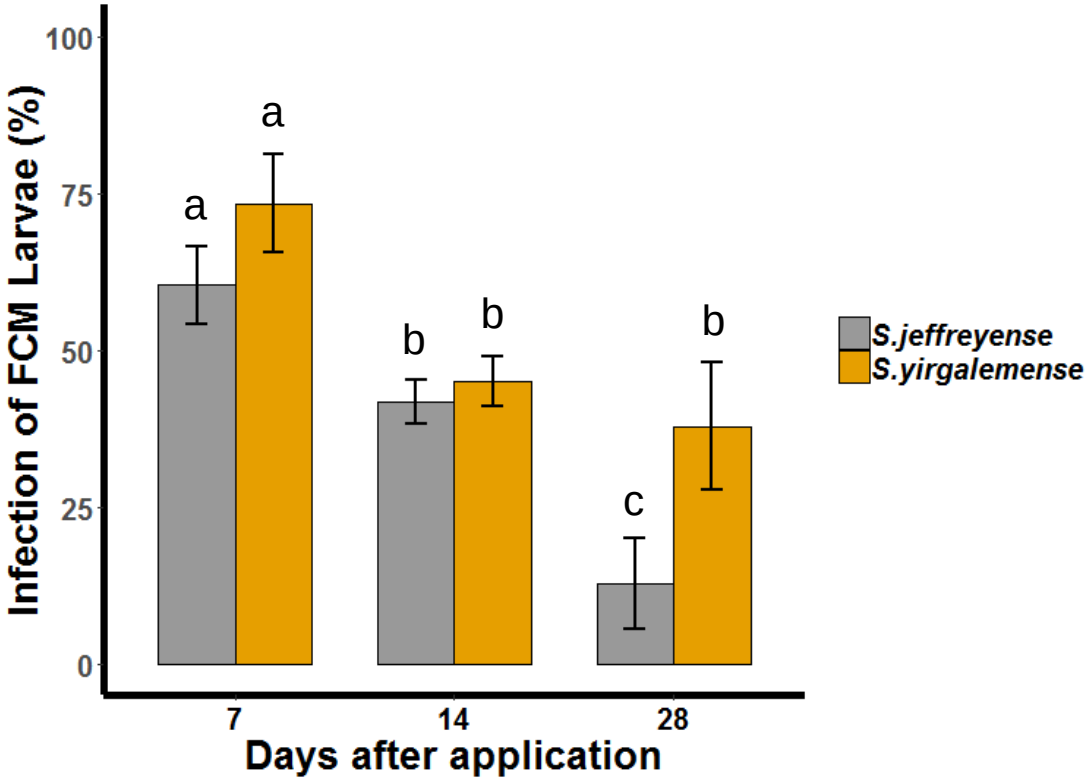

Fig. 3.7. Mean percentage infection (± SE) of *Thaumatotibia leucotreta* larvae 7, 14 and 28 days after entomopathogenic nematode application in a semi-field trial. Treatments were *in vitro* liquid cultured *Steinernema jeffreyense* (grey) and *S. yirgalemense* (gold) at a concentration of 20 IJs/cm^2. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.3.6 Post application viability of inoculum

The infection of FCM larvae caused by *in vitro* post application inoculum (63.33% ± 9.35%) had significantly ($T = 3.172$, $df = 14$, $p = 0.007$) lower performance than the pre-application inoculum (86.67% ± 1.36%) (Fig. 3.4). There was a significant ($F_{2, 12} = 13.052$, $p = 0.037$) effect of dose, with the higher IJs concentrations causing higher infection rates. However, there was no significant ($p = 0.654$) difference between the performance (rate of infection) of the *in vitro* inoculum and the formulation of *S. jeffreyense*.

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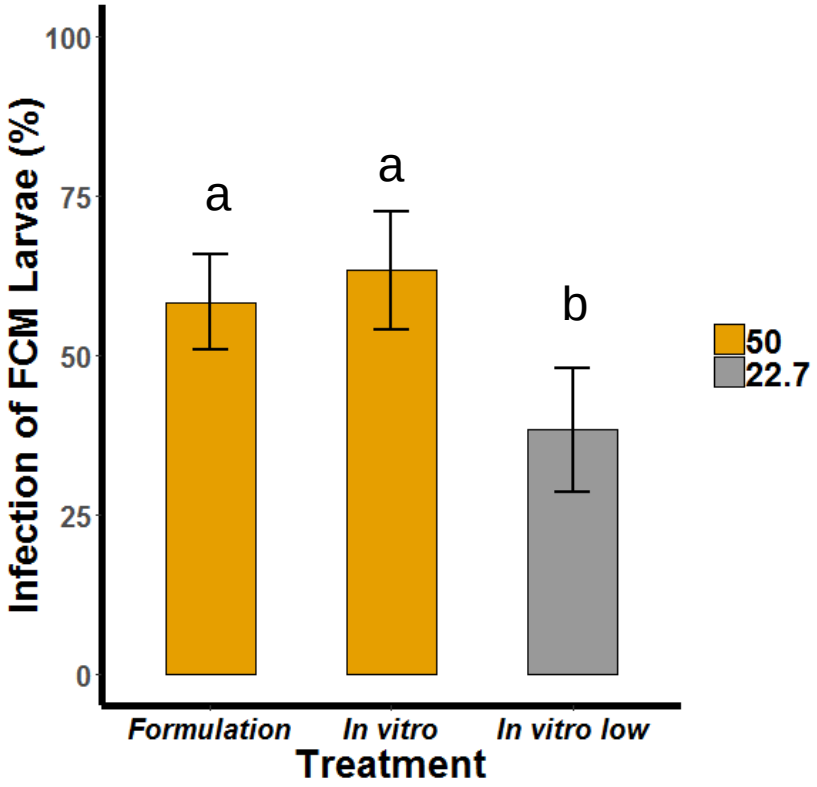

Fig. 3.8. Mean (± SE) percentage infection of *Thaumatotibia leucotreta* larvae inoculated with the same inoculum that had been used for field application. Treatments were the formulation (50 IJs/50 µl) and *in vitro Steinernema jeffreyense*, at concentrations of 22.7 IJs/50 µl and 50 IJs/50 µl, mortality was assessed 48 h after treatment. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

3.4 Discussion

Both *in vitro* liquid cultured *S. jeffreyense* and *S. yirgalemense* have not previously been tested for pathogenicity against FCM larvae. The IJs inoculum used in this study was either freshly cultured *in vivo* or *in vitro*. In the case of *S. jeffreyense*, the IJs were stored at 14°C for up to 136 days. No significant differences were found during the screening of the older *in vitro* and freshly *in vivo* cultured *S. jeffreyense*, with a mortality of 87% and 85% respectively, using a low concentration (50 IJs/insect). The quality of the *in vitro* cultured EPNs is often assumed and has been previously proven, to be inferior to *in vivo* produced EPNs (Yang et al., 1997; Shapiro-Ilan et al., 2012; Ferreira et al., 2014, 2016). Contrary to the expectation, the virulence of *S. jeffreyense* did not differ between the different production methods under laboratory conditions. This may be explained by Bilgrami et al. (2006), who showed that it is the deterioration of the symbiotic bacteria that cause the most notable reduction in EPN quality (e.g. virulence). *In vitro* liquid culture grows the bacteria in an optimized complex medium

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(Dunn and Malan, 2018) and, if the technique is successful, it should lead to sufficient lipids to be stored, to aid in longevity and infectivity in field trails, which are comparable with those of *in vivo* cultured nematodes.

Age affected the virulence of the EPNs as expected, however, the direction of the effect found during this study was not anticipated. The older *in vitro* cultured *S. jeffreyense* performed better (87%) than the freshly cultured nematodes (47%), which may be ascribed to the physiological maturity of the IJs being required to infect/colonise the insect. This process would happen naturally in a White trap when the nematodes are ready to move from the cadaver to the water trap, which is not the case in artificial culturing of the nematodes. This finding is important for *in vitro* production, as it indicated that caution should be taken in using or formulating IJs too soon after production. Further research on the age of *in vitro* cultured IJs is required, to ensure that the IJs are ready for formulation.

In the field trial, the medium concentration (20 IJs/cm^2) of EPNs led to infection of 50 - 68% of the FCM larvae and the high concentration (30 IJs/cm^2) to mortality of 70 - 85%. Depending on the cost involved, the lower concentration could be used for future trials, to ensure the efficiency of the method. The persistence of the medium and high concentrations did not significantly differ over the four-week period. However, though there was an initial decline (loss of 24% virulence) for the high concentration from the initial to two-week post application period, the persistence remained reasonably stable for the rest of the period and still provided 33% infection after four weeks. The persistence of the medium nematode concentration steadily declined each week after the initial application and only provided 18% infection after the four week period. Unfortunately, it is not possible to make comparisons between the species (*S. jeffreyense*) tested here and the commercially available product Cryptonem (L9251) (Hatting et al., 2018), as data for the product are not available. However, Malan and Moore (2016) tested the virulence of a local (*in vivo* cultured) *Heterorhabditis bacteriophora* Poinar 1976 (the active ingredient of Cryptonem) in the field with a similar design as our field experiments. The authors reported very high (91%) initial mortality of FCM larvae with 20 IJs/cm^2, but the persistence was considerably lower than found in the present study (Malan and Moore, 2016). The formulation also had similar infection rates to that of the medium nematode concentration and did not increase the performance of the *in vitro* cultured *S. jeffreyense*. The *in vitro* inoculum's virulence decreased by 20% from pre- to post application, indicating the detrimental effects of handling and heat in the field. None of these handling effects that were experienced during the day in the field were mediated by the formulation. A reason for the lower than expected performance of the formulation is thought to be as a result of small number (< 5 million) of IJs

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available for formulation, as the formulation tends to desiccate the IJs if the ratio of IJs to diatomaceous earth is incorrect (Kagimu, 2018), as was the case here. This study was unable to culture large enough numbers of IJs to ensure the formulation was perfect, these results should therefore be seen only as the first step and more work is required to confirm our results. Nevertheless, it is suggested that the virulence of the formulation should always be tested against target insects (not only *G. mellonella*) to ensure it is effective for field use.

Both *in vitro* reared *S. jeffreyense* and *S. yirgalemense* performed well (> 60% infection) in the field environment after a 7-day exposure to field conditions. Our prediction that *S. yirgalemense* would be significantly better than *S. jeffreyense* proved to be incorrect. However, it was shown that differences may arise between the species as time in the field increases. *Steinernema yirgalemense* still infected more than a third of the FCM larvae, four weeks after the application, whereas *S. jeffreyense* infected less than 13%. Environmental factors such as soil temperature, moisture and availability of the host are known to affect the survival or persistence of EPNs in the field (Glazer, 2002). However, the IJs stage and our design (both species experienced the same conditions) should ensure that these stressors do not influence the differences seen between the species. The effect of host availability is therefore the most important factor to consider with our design (the removal of hosts in our field trials) as the essential energy available that allows EPNs to persist is limited (Glazer, 2002). Lipid reserves of IJs have been shown to diminish with time (Andaló et al., 2011) and limited energy sources should affect the species differently. *Steinernema jeffreyense*, which is relatively large (> 1000 µm, Malan et al., 2016), may have greater energy (lipid) reserves, allowing it to function better for longer periods (Hazir et al., 2001). Another consideration in relation to body size is dehydration, with the larger EPNs having the advantage to persist longer (Nguyen et al., 2006) due to better surface to volume ratio, which is important for ectotherms to curb evaporative water loss and thermal inertia (Peters, 1986). It is therefore contradictory that *S. yirgalemense* (the smaller EPN) persisted longer in our field trials. This apparent anomaly may be because of behavioural differences between the species, such as being inactive and rolling into coils to reduce water loss (Womersley, 1987) and should be explored for these species. It was observed that during storage, *S. jeffreyense* is always active, while in the case of *S. yirgalemense* the IJs tends to be curled in a question-mark position when not disturbed. Furthermore, though *S. jeffreyense* has the larger body, it may also be more active (or have higher energy requirements), which may require it to use its energy resources quicker. Therefore, the more active or larger body length could become a drain (rather than a benefit) on the nematode and may cause it to become less virulent or die more readily. In the FCM field trial of Malan and Moore (2016), the same trend

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was experienced with regard to persistence of another large IJ, *Steinernema khoisanae* (Nguyen et al., 2006).

Our semi-field trial results compared favourably with that of Malan and Moore (2016) and Steyn et al. (2018) that tested the potential of EPNs in the semi-field environment against the larvae of FCM. These authors used *in vivo* cultured nematodes, while the current study showed that the *in vitro* cultured EPNs are as virulent in the field environment. The persistence for *S. yirgalemense* was similar (35% after 4 weeks) to that reported by Malan and Moore (2016), but shorter than reported by Steyn et al. (2018). This is likely to be as a result of more favourable prevailing subtropical conditions (higher temperature and more frequent irrigation) in the latter trial. As the *in vitro* cultured EPNs used here have only gone through a few generations (less than 100) of sub-culture, it is suggested that their virulence should be periodically evaluated in the future. This will ensure that virulence, host finding and infection abilities, are not lost, through genetic drift, during continuous *in vitro* culturing of the EPNs. This finding provides novel support for the potential of liquid *in vitro* mass production of these species, for the control of FCM.

It is important to determine whether the high mortality or suppression (70%) reported in the semi-field trials for FCM will translate to equivalent suppression in large-scale field applications. Compared to EPN field trials, our semi-field trials resulted in high suppression of the pest insect. The suppression is likely to be lower in the field environment, targeting natural populations of FCM, than measured under these controlled semi-field conditions. However, it is important to remember that the *in vitro* reared EPNs used in this study will benefit from optimization (Yang et al., 1997, Dunn and Malan, 2018) and formulation (Gaugler, 2002; Kagimu et al., 2017). Another consideration is the persistence of EPNs measured in this study, which is regarded to be a low estimate for two reasons. Firstly, EPNs are mobile and are diluted due to diffusion into surrounding areas that were not treated. Secondly, the removal of the host does not allow for subsequent generations of the EPNs to be produced in the field. It is concluded from this study that *S. jeffreyense* and *S. yirgalemense* have potential as biological control agents of FCM, and the development of EPNs as a commercial product in South Africa should be encouraged.

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3.5 References

Andaló, V., Moino, A., Maximiniano, C., Campos, V.P., Mendonca, L.A., 2011. Influence of temperature and duration of storage on the lipid reserves of entomopathogenic nematodes. Rev. Colomb. Entomol. 37, 203-209.

Bilgrami, A.L., Gaugler, R., Shapiro-Ilan, D.I., Adams, B.J., 2006. Source of trait deterioration in entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* during in vivo culture. Nematol. 8, 397-409.

Campos-Herrera, R., 2015. Nematode pathogenesis of insects and other pests. Series: Sustainability in Plant and Crop Protection (Ciancio, A., Series Ed.). Springer International Publishing, Switzerland, 530 pp.

De Waal, J.Y., Malan, A.P., Addison, M.F., 2011. Efficacy of entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) against codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae) in temperate regions. Biocontrol Sci. Technol. 21, 1161-1176.

Dunn, M.D., Malan, A.P., 2018. Production optimization of a novel entomopathogenic nematode, *Steinernema jeffreyense*, in shake flasks. Appl. Microbiol. Tech. (Submitted).

Ferreira, T., Addison. M.F., Malan, A.P., 2014. *In vitro* liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. African Entomol. 22, 80-92.

Ferreira, T., Addison, M.F., Malan, A.P., 2016. Development and population dynamics of *Steinernema yirgalemense* and growth characteristics of its associated *Xenorhabdus* symbiont in liquid culture. J. Helminth. 90, 108-112. doi:10.1017/S0022149X15000450.

Gaugler R, Han R., 2002. Production technology. In: R. Gaugler, (ed.). Entomopathogenic Nematology. Wallingford, UK: CABI, pp. 289-310.

Gaugler, R, Georgis, R., 1991. Culture method and efficacy of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae). Biol. Control 1, 269-274.

Gaugler, R. (ed.), 2002. Entomopathogenic nematology. CABI.

Georgis, R., Dunlop, D.B., Grewal, P.S., 1995. Formulation of entomopathogenic nematodes. In: Hall, F.R., Barry, J.W., (eds.). Biorational Pest Control Agents: Formulation and Delivery. Washington, DC: American Chemical Society, pp. 197-205.

Glazer, I., 2002. Survival biology. In: R. Gaugler, (ed.). Entomopathogenic Nematology.

Stellenbosch University https://scholar.sun.ac.za

Wallingford, UK: CABI, pp., 169-182.

Hanski, I., Saastamoinen, M., Ovaskainen, O., 2006. Dispersal-related life-history trade-offs in a butterfly metapopulation. J. Animal Ecol. 75, 91-100.

Hatting, J.L., Moore, S.D., Malan, A.P., 2018. Microbial control of phytophagous invertebrate pests in South Africa: Current status and future. J. Invertebr. Pathol. https://doi.org/10.1016/j.jip.2018.02.004

Hazir, S., Stock, S.P., Kaya, H.K., Koppenhöfer, A.M., Keskin, N., 2001. Developmental temperature effects on five geographic isolates of the entomopathogenic nematode *Steinernema feltiae* (Steinernematidae). J. Invert. Pathol. 77, 243-250.

Kagimu, N., Ferreira, T., Malan, A.P., 2017. The attributes of survival in the formulation of entomopathogenic nematodes utilised as insect biocontrol agents. African Entomol. 25, 275-291.

Kagimu, N., 2018. Formulation of *Steinernema yirgalemense*, *S. jeffreyense* and *Heterorhabditis bacteriophora* in alginate beads and diatomaceous earth. BioControl (In prep.).

Kaya, H.K., Stock, P.S., 1997. Techniques in insect nematology. Man. Tech. Insect Pathol. 281-324. https://doi.org/10.1016/B978-012432555-5/50016-6

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1-41.

Malan, A.P., Knoetze, R., Moore, S.D., 2011. Isolation and identification of entomopathogenic nematodes from citrus orchards in South Africa and their biocontrol potential against false codling moth. J. Invertebr. Pathol. 108, 115-125. https://doi.org/10.1016/j.jip.2011.07.006

Malan, A.P., Moore, S.D., 2016. Evaluation of local entomopathogenic nematodes for the control of false codling moth, *Thaumatotibia leucotreta* (Meyrick, 1913), in a citrus orchard in South Africa. Afr. Entomol. 24, 489-501. https://doi.org/10.4001/003.024.0489

Malan, A.P., Knoetze, R., Tiedt, T.R., 2016. *Steinernema jeffreyense* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. J. Helminth. 90, 262-278. doi:10.1017/S0022149X15000097

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with

Stellenbosch University https://scholar.sun.ac.za

emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

Moore, S.D., Kirkman, W., Stephen, P. 2004. CRYPTOGRAN, a virus for the biological control of false codling moth. S.A. fruit J. 3, 35-39.

Nguyen, K.B., Malan, A.P., Gozel, U., 2006. *Steinernema khoisanae* n. sp. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from South Africa. Nematol. 8, 157-175.

Phillips, B.L., Brown, G.P., Webb, J.K., Shine, R., 2006. Invasion and the evolution of speed in toads. Nature 439, 803.

Peters, R.H., 1986. The ecological implications of body size (Vol. 2). Cambridge University Press.

Prinsloo, G.L., Uys, V.M., 2015. Insects of cultivated plants and natural pastures in Southern Africa. Entomological Society of Southern Africa, South Africa.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Shapiro-Ilan, D.I., Gaugler, R., 2002. Production technology for entomopathogenic nematodes and their bacterial symbionts. J Ind. Microbiol. Biotechnol. 28, 137-146.

Shapiro-Ilan, D.I., Gouge, D.H., Koppenhöfer, A.M., 2002. Factors affecting commercial success: Case studies in cotton, turf and citrus. In: Entomopathogenic nematology 16, 333-349.

Shapiro-Ilan, D.I., Han, R, Dolinksi, C., 2012. Entomopathogenic nematode production and application technology. J Nematol. 44, 206-217.

Sievert, C., 2018. plotly for R. https://plotly-book.cpsievert.me

Steyn, W.P., Malan, A.P., Daneel, M.S., Slabbert, R.M., 2017. Entomopathogenic nematodes from north-eastern South Africa and their virulence against false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). Biocontrol Sci. Technol. 27, 1265-1278. https://doi.org/10.1080/09583157.2017.1391174

Steyn, W.P., Malan, A.P., Daneel, M.S., Slabbert, R.M., 2018. Entomopathogenic nematodes (Rhabditidae and Heterorhabditidae) for the control of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) under South African conditions (Ph.D. dissertation, Tshwane University of Technology).

Stellenbosch University https://scholar.sun.ac.za

Van Zyl, C., Malan, A.P., 2015. Cost-effective culturing of *Galleria mellonella* and *Tenebrio molitor* and entomopathogenic nematode production in various hosts. African Entomol. 23, 361-375.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Womersley, C., 1987. A re-evaluation of strategies employed by nematode anhydrobiotes in relation to their natural environment. In: Veech, J.A., Dickson, D.W. (eds.) Vista on Nematology. Society of Nematology, Hyattsville, Maryland, pp. 165-173.

Yang, H., Jian, H., Zhang, S., Zhang, G., 1997. Quality of the entomopathogenic nematode *Steinernema carpocapsae* produced on different media. Biol. Control 10, 193-198.

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Chapter 4:

Quantifying mating disruption of false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in stone fruit and table grapes

Abstract

Mating disruption is the broadcasting of female pheromones within an agricultural system so as to interfere with, or decrease, the reproductive potential of the pest insect. Seen as a sustainable, non-chemical management method, it is used in citrus orchards against the false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick). Research to support its efficacy against FCM is limited, especially in stone fruit and table grapes. Previous attempts to quantify mating disruption against FCM has led to inconsistent results, encouraging the need to investigate mating disruption experimentally. Here an experimental design that provides reliable results was evaluated. Sterile males were released in stone fruit and table grapes and recaptured with a trapping grid, every 24 hours for five days. Trapping efficiency, trap interference and recaptures of wild and sterile moths were compared. Factors that improved the reliability of results, included the use of mark-release-recapture experiments, nine trap layout and a paired control that ensured that only one variable was changed. Experimental blocks were then either treated with 400 pheromone dispensers/ha or 0 pheromone dispensers. This study reports the first experimental evidence that mating disruption is a viable control option against FCM in stone fruit and table grapes. Mating disruption was calculated as 86% in stone fruit and 93% in table grapes, with less than the recommended field dose. Evidence from this study indicated that mating disruption shows promise as a control technique against FCM in stone fruit and table grapes. This study therefore sets a solid foundation for further research into the mechanistic understanding of how the pest's behaviour is affected, as well as to calculate the dosage to cause the highest level of mating disruption with the most economic amount of pheromone.

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4.1 Introduction

Mating disruption is the broadcasting of the synthetic female pheromone throughout the landscape so as to interfere with mate-finding ability and decrease reproductive events (Miller and Gut, 2015). Fewer reproductive events results in fewer viable offspring and thus smaller populations. Calling lepidopteran females expose their pheromone glands, which release the sex pheromone into the surroundings; the male moths are attracted to the pheromone and after they find the female, mating occurs (Allison and Cardé, 2016). This makes Lepidoptera a perfect target for mating disruption, which has successfully been used for control and in eradication programmes for several lepidopteran pests (Lance et al., 2016; Suckling et al., 2017). Mating disruption is also seen as a sustainable, non-chemical management method (Suckling, 2015). False codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) is a key pest in stone fruit, table grapes and citrus, though the majority of work focussing on its control has been conducted on citrus (Malan et al., 2018). To the author's knowledge, only one peer-reviewed study has been published to date on fruit crops that tested mating disruption against FCM (Hofmeyr et al., 1991), however, a recent study has been conducted in cotton (Ochou et al., 2017). Though the study by Hofmeyr et al. (1991) was conducted in citrus, many studies cite mating disruption as a viable control method of FCM in a variety of crops (Moore and Hattingh, 2012; Hofmeyr et al., 2016; Malan et al., 2018). Four products are registered [RB SPLAT, L10259; X-MATE FCM, L10320; Check Mate FCM-F, L8384; Isomate FCM, L76692 (see Agri-Intel, 2018)].

The attraction and disruption potential of FCM's semiochemicals were tested in citrus orchards nearly two decades ago (Hofmeyr and Calitz, 1991, Hofmeyr et al., 1991; Hofmeyr and Burger, 1995). The success, (measured as males unable to find traps/females), that the authors reported has led to its widespread use throughout the citrus-growing regions in South Africa. Only one peer reviewed study (Ochou et al., 2017) on cotton and several semi-popular articles have been published on the mating disruption of FCM since (Schoeman and De Beer, 2008; Moore and Kirkman, 2011). However, most peer-reviewed work has focussed on refining the synthetic female pheromone and shedding light on the exact blend of the pheromone (Persoons et al., 1977; Angelini et al., 1981; Newton and Mastro, 1989; Newton et al., 1993). As yet no peer reviewed work is available to confirm its effectiveness against FCM in either stone fruit or table grapes. This is problematic as different crops have various growth forms and may differ according to density of foliage canopy or height of the plant and number of trees planted/ha. Several abiotic factors such as temperature, light intensity and wind speed are known to affect

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trap effectiveness (McNeil, 1991; Williams et al., 2013). These abiotic factors are likely to differ between dense and sparse growth types, making it important to quantify mating disruption on a variety of crops. Despite the lack of real evidence for this control method's success in stone fruit and table grapes, mating disruption is now widely used and registered on various fruit crops (Agri-Intel, 2018; and prescribed by fruit unions, Hortgro, 2017) throughout South Africa.

Attempts to quantify the potential of mating disruption against FCM have had mixed results, with some authors reporting very high (> 80%) suppression of trap catches, but the fruit damage remained unchanged (Hofmeyr et al., 1991) and others reporting less fruit damage in treated block (Ochou et al., 2017). The variability of results makes inferences regarding its success difficult and in some cases has led to a loss of confidence in the method. The unpublished (Hofmeyr and Hofmeyr, 2002; Moore and Kirkman, 2010, 2011) studies make it difficult to review or reproduce experiments, as "in house" results (without methods) are often reported and subsequently cited (Malan et al., 2018). This encourages the need to test mating disruption experimentally.

Previous work conducted on FCM mating disruption have shared a similar design, several blocks were treated with or without the pheromone dispensers and then naturally occurring (wild) FCM were caught with a central trap. Long term trap catches between treated and untreated blocks were compared to determine the treatments' success (Ochou et al., 2017), while some (Schoeman and De Beer, 2008) used only damage assessments. This design has several drawbacks, firstly the central trap only catches a small percentage of the population. Secondly, the wild populations of FCM are often not quantified before the trial and it is difficult to quantify their numbers during these trials. Another limitation is due to FCM's polyphagous feeding habits and the mobility of adults, damage has not been correlated with FCM presence. This makes comparisons difficult between the treated and untreated blocks. The problem with this design is that more than one factor is changed, making it extremely difficult to draw reliable conclusions. A more logical and experimentally suited approach would be to only change one variable at a time, in this case pheromone present or not, allowing for strong inference (Platt, 1964; Miller and Gut, 2015). I propose that mating disruption of FCM could be better quantified by releasing a known number of moths (into the treated and untreated blocks) and recapture them to quantify the effect of the pheromone on mating disruption. This method has successfully been used to determine the potential of mating disruption in stone fruit against Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera:Tortricidae) (Reinke et al., 2014)

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and other fruit pests [Codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera:Tortricidae); McGhee et al., 2014].

The aim of this study was to quantify mating disruption against FCM in stone fruit and table grapes. Here, an adapted experimental design is validated that provides reliable results, evaluation of trap position, efficiency of trap layout and comparisons between trapping of wild and released moths. As well as being the first attempt to calculate mating disruption against FCM in stone fruit and table grapes.

4.2 Material and methods

4.2.1 Source of insects

Thaumatotibia leucotreta were collected as 1-day-old sterile adults from a colony maintained at XSIT (Pty) Ltd in Citrusdal, South Africa. Sterile adults were used for the experiments as FCM is a priority pest and it would not be acceptable to release unsterilized individuals into the fruit orchards. The sterile adults were from the mass reared population maintained at XSIT that are used for the sterile insect technique (SIT). The moths were transported in a cooler box to a stone fruit orchard either plums, (33°54'26.9"S 18°47'42.7"E, Stellenbosch), or table grapes (33°10'09.0"S 18°59'20.6"E, Halfmanshof) to ensure the cold chain was not interrupted. Upon arrival, at the experimental plots, the moths were allowed to warm up for 10 minutes after which they were released.

4.2.2 Mark-release-recapture protocol

Two stone fruit (plum) or four table grape blocks of 0.5 ha were used to conduct all experiments. The treatment blocks may be considered relatively small for measuring mating disruption, as product labels (Isomate, Nulandis, South Africa) suggest areas larger than 5 Ha should be treated for best results. However, when testing mating disruption experimentally it may be impractical to use large experimental blocks. Previous studies testing mating disruption experimentally use smaller or similar experimental plot sizes than used during this study (Miller et al., 2006, Table 1). Smaller (<1 Ha) experimental plots, also have several advantages; 1) multiple (at least 4) replications were possible with available time and resources, and 2) there is less variability amongst variables that are not being tested, ensuring more consistent results. A total of 16 mark recapture releases were conducted in each of the crops. The stone fruit releases took place from November 2016 - February 2017 and the table grape releases took place from November 2017 - December 2017. Each block consisted of a grid of nine delta traps

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loaded with FCM female pheromone (Chempac, Simondium, South Africa) and four release points (Fig. 4.1). The grid of traps was placed around the release points, all traps were placed at least 20 m from the nearest release point and the nine-trap layout was chosen to ensure there was no pseudo-replication of inner traps (Suckling et al., 2015). Four release points were chosen to ensure that the male moths were evenly distributed throughout the experimental block. Two hundred sterile FCM (100 males and 100 females) were released at each of the release points and recaptured every 24 h for five days. Sterile moths were marked with different colours (pink, yellow, and blue) of fluorescent powder (Day Glo®) that were randomised for each release or treatment. No moths were caught in the traps of a different treatment for any of the pre-treatment stone fruit trials. Therefore, moths were not marked for subsequent releases. Subsequently, the sterile moths were identified by inspection of gut contents, as the rearing facility colours their artificial diet with Calco Oil Red® (Royce International, Sarasota, Florida) that colours the gut red. Half of the blocks for each crop (i.e. one for stone fruit and two for table grapes) were treatment blocks, the other half of the blocks were paired controls. Two releases were done simultaneously, one in the treatment block and one in the paired control block. The treatment block and paired control block had the exact same design, slope, wind direction (control plot upwind from treatment plot) and were chosen 100 m apart. The treatment blocks consisted either of a no pheromone treatment (pre-treatment) or 400 point-source polyethylene tube dispensers (Isomate FCM 240, lot no. FCM-50393, Shin-Etsu Chemical Co., Ltd., Japan) that are formulated commercially and loaded with 240 mg of active ingredients (166.8 mg of E-8-dodecenyl acetate + 70.8 mg of Z-8-dodecenyl acetate + 2.4 mg of E / Z-8-dodecenol) (post-treatment) per hectare and the paired control block had no dispensers. The dispensers were hung every 7.5 m in the upper (at least 1.8 m high) branches or trellis and corresponded to every ~5th tree or vine as they were planted 1.5 m apart and rows were 3 m apart.

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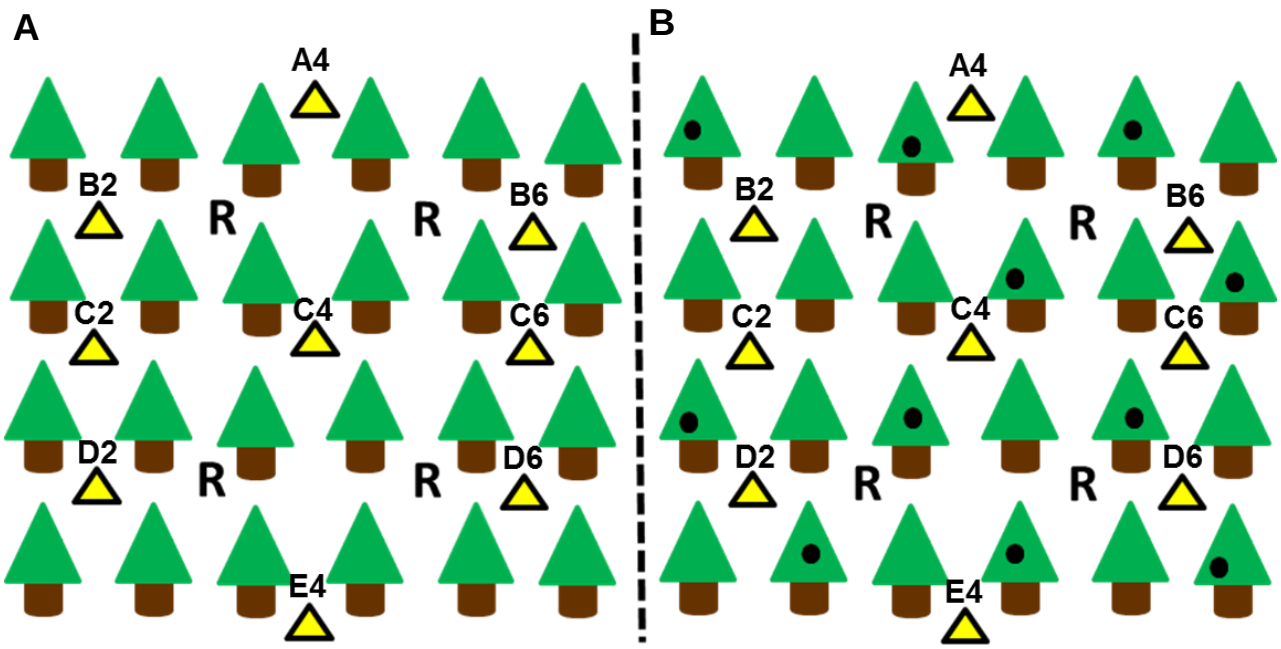

Fig. 4.1. The layout of the field releases in stone fruit and table grapes to test mating disruption against *Thaumatotibia leucotreta*. Delta traps (yellow triangles) were hung at least 20 m from the release points (R). The trap positions of the traps are shown by the code above each trap, for instance C4 is the central trap. Panel A) is the layout of the paired control. Panel B) is the layout of the treated block, the black dots represent pheromone dispensers.

4.2.3 Temperature data

Weather station data

The maximum and minimum temperatures were recorded daily for the stone fruit experiments with weather stations that were administrated by the Agricultural Research Council (Agro-Climatology division) weather stations within a five km radius of the field trial. The average daily temperature was calculated by adding the max and min temperatures and dividing by two.

Microclimate data

A temperature Thermochron iButton® (Dallas Semiconductors, Model DS1920; 0.5°C accuracy) data logger was centrally placed within each of the six table grape experimental plots. Temperature was recorded every 15 min and the average temperature was calculated for day (06:00 - 19:45) and night (20:00 - 05:45) in table grapes for the duration of the trial.

4.2.4 Evaluation of experimental design

Only blocks that received no pheromone were used to evaluate the experimental design.

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Trap placement and efficiency

All traps were named according to their position within the trapping grid. The mean number of moths caught by each position (e.g. central trap, C4, Fig. 4.1) was calculated for each release and compared to that of other trap positions. The total distance (m) the trap positions were away from the release points was calculated to determine if distance had an effect on recapture rates. The number of moths recaptured in the central trap (C4) was compared to border traps (A4 and E4). Trap interference was calculated following Suckling et al. (2015), by dividing the number of moths caught in the border traps by the number of moths caught in the central trap.

Along or across rows

Moths may fly more readily along a row of trees or vines rather than through the tree row. This hypothesis was tested by comparing the number of male moths caught in the traps that ran along the row from the release points (Fig. 4.1) to the number of moths caught in traps that required the moths to cut through the rows. The moths caught in the central trap (C4) were excluded, so that there was a balanced (four traps along and four across) design.

Wild vs released moths

To compare the trapping profile of wild and sterile moths, the mean number of recaptured moths was calculated for each of the trap positions in the stone fruit blocks that were not treated with pheromone. No wild moths were present in the table grape blocks, therefore only data from the stone fruit releases were used for this analysis.

4.2.5 Mating disruption

The data from the stone fruit and table grape releases were analysed separately. Traps loaded with FCM female pheromone were used as a proxy for calling females, as it was assumed that, if males were not caught in the traps, they would also not be able to find a calling female. This is the standard technique for quantifying mating disruption and has been used for many insects including other lepidopterans (Hofmeyr et al., 1991, Stelinski et al., 2004; Miller et al., 2006 a, b; Allison and Cardé, 2016). Mating disruption was calculated following Miller et al. (2006b) and McGhee et al. (2014) and consisted of two steps.

Formula 1: Standardized catch

$$\text{Catch} = \left(\frac{males\ caught\ in\ treatment\ block}{males\ caught\ in\ paired\ release}\right)$$

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The standardization accounted for the effect of moth quality, temperature, crop type and other possible influences on the recapture rates. The level of mating disruption was then calculated:

Formula 2: Mating disruption

$$\text{Mating disruption} = \left(1 - \frac{males\ caught\ in\ treatment\ block}{males\ caught\ in\ paired\ release}\right) \times 100$$

When more individuals were caught in the treatment block than in the control block, formula 2 provided a negative answer, however, as a negative value is nonsensical, these values were therefore reported as 0.

4.2.6 Statistical analyses

The statistical analyses were performed using Statistica 12 (Stat-Soft Inc., 2012). If the residuals were not normally distributed the data were log(X + 1)-transformed for further analysis. If the assumptions (normality and equality) of a one-way analysis of variance (ANOVA) were violated irrespective of transformation, the Kruskal-Wallis H-test and Mann-Whitney U test were used to separate the means at $p < 0.05$. When applicable, means were separated using the Fisher's LSD method. RStudio version 1.0.143 and R version 3.5.1 (R, 2018), was used to illustrate the findings graphically, the packages used were ggplot2, plotly and forcats (Wickham, 2016; Sievert, 2018).

4.3 Results

4.3.1 Temperature

Weather station data

The average temperature in the stone fruit orchard (Fig. 4.2) during November - December 2016 for the pre-treatment releases (18.77°C ± 0.37°C, Fig. 4.2A) was significantly ($Z = -6.09$, $N = 120$, $p < 0.001$) lower than during the post-treatment releases (22.33°C ± 0.31°C, Fig. 4.2B) which were conducted during January - February 2017.

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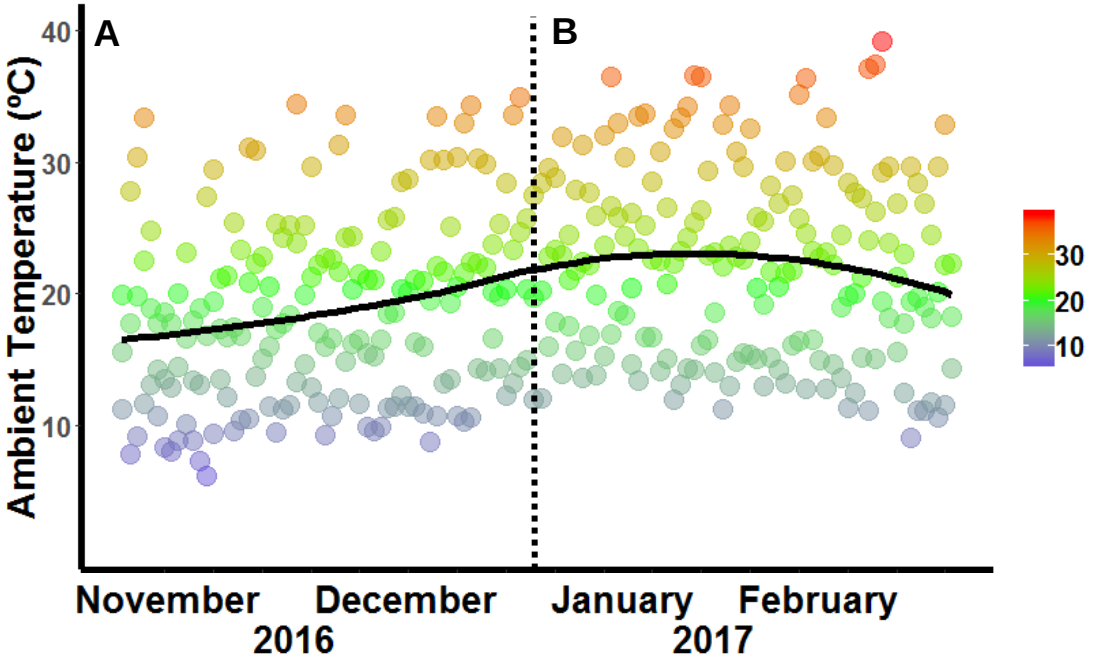

Fig. 4.2. The daily minimum, maximum and average temperature recorded by several weather stations within a five km radius of the stone fruit field trial in Stellenbosch. Panel (A) the ambient temperature measured during the pre-treatment releases. Panel (B) the ambient temperature measured during the post-treatment releases. The solid (black) line represents the average ambient temperature.

Microclimate data

The average temperature (21.54°C ± 0.12°C) was marginally higher during the table grape trials (November-December 2017), than during the stone fruit trials (20.52°C ± 0.29°C). The average night temperature (16.77°C ± 0.1°C), during the table grape field trial (Fig. 4.3) was expectedly significantly (Z = -31.37, N_{night} = 1295, N_{day} = 2257, $p < 0.001$) lower than the average day temperature (24.28°C ± 0.15°C), but the day and night temperatures did not differ between the pre-treatment (Fig. 4.3A) and post-treatment (Fig. 4.3B) trials.

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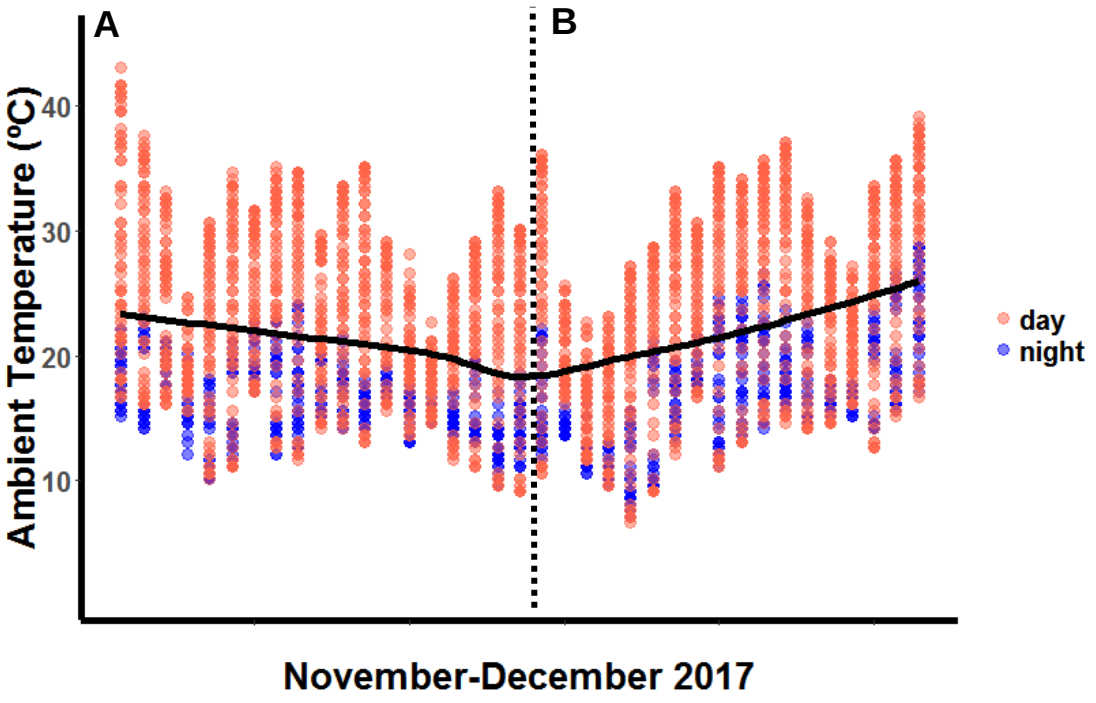

Fig. 4.3. The temperature recorded every 15 min over the 38-day exposure period of the table grape field trial in Halfmanshof. Points were coloured for day (red, 06:00-19:45) and night (blue, 20:00-05:45). Panel (A) the ambient temperature measured during the pre-treatment releases. Panel (B) the ambient temperature measured during the post-treatment releases. The solid (black) line represents the average ambient temperature.

4.3.2 Evaluation of experimental design

Trap placement and efficiency

Significantly ($F_{1, 133}$ = 25.362, p < 0.001) more moths were caught per trap per release in table grapes (Fig. 4.4A, 8.69 ± 1.02) than in stone fruit orchards (Fig. 4.4B, 2.92 ± 0.37). However, there was no significant ($F_{8, 117}$ = 1.113, p = 0.360) effect of trap position on the number of moths caught between the crops. Position C2 (2.2 ± 0.46) caught the fewest moths in the trapping grid, and captured significantly fewer moths than positions A4 (8.07 ± 1.69, p = 0.026) and E4 (9.0 ± 2.81, p = 0.039). The position (C4) closest to the release sites captured a similar number of moths to the trap positions further away, causing no significant ($F_{3, 131}$ = 2.172, p = 0.094) effect of distance on the number of moths recaptured. There was a very weak negative association in stone fruit (r = -0.343, p = 0.006) and table grapes (r = -0.216, p = 0.068) of trap catches with distance from release site, but it explained almost none of the variation in the system (stone fruit, R^2 = 0.118; table grapes, R^2 = 0.047). The moths caught in the central trap did not significantly ($F_{2, 42}$ = 0.158, p = 0.854) differ from the number caught in border traps.

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The trapping interference proved to be low (1.1 - 1.23) in our trapping design as the central trap caught only 14% less than the border traps.

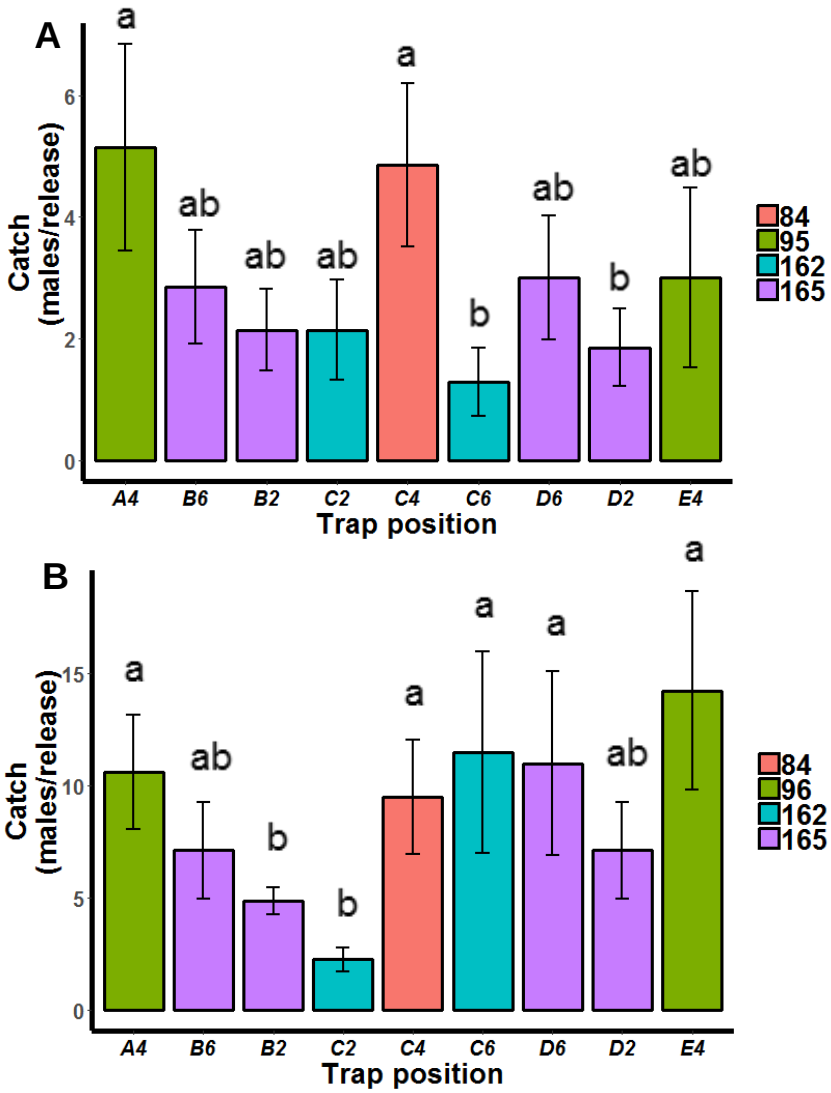

Fig. 4.4. Mean (± SE) number of *Thaumatotibia leucotreta* males caught per release (Catch) for every position in the trapping grid for the blocks not treated with pheromone. Panel A) moths caught in stone fruit, panel B) moths caught in table grapes. Bars are coloured by total distance (m) the trap is away from release sites. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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Along and across rows

There was no overall significant ($F_{1, 26}$ = 0.239, p = 0.469) difference between the number of moths caught in the traps along (37 ± 10.06) or across (45.57 ± 11.72) from the release points (Fig. 4.5). There was also no significant (p = 0.629) placement (along/across) x crop effect.

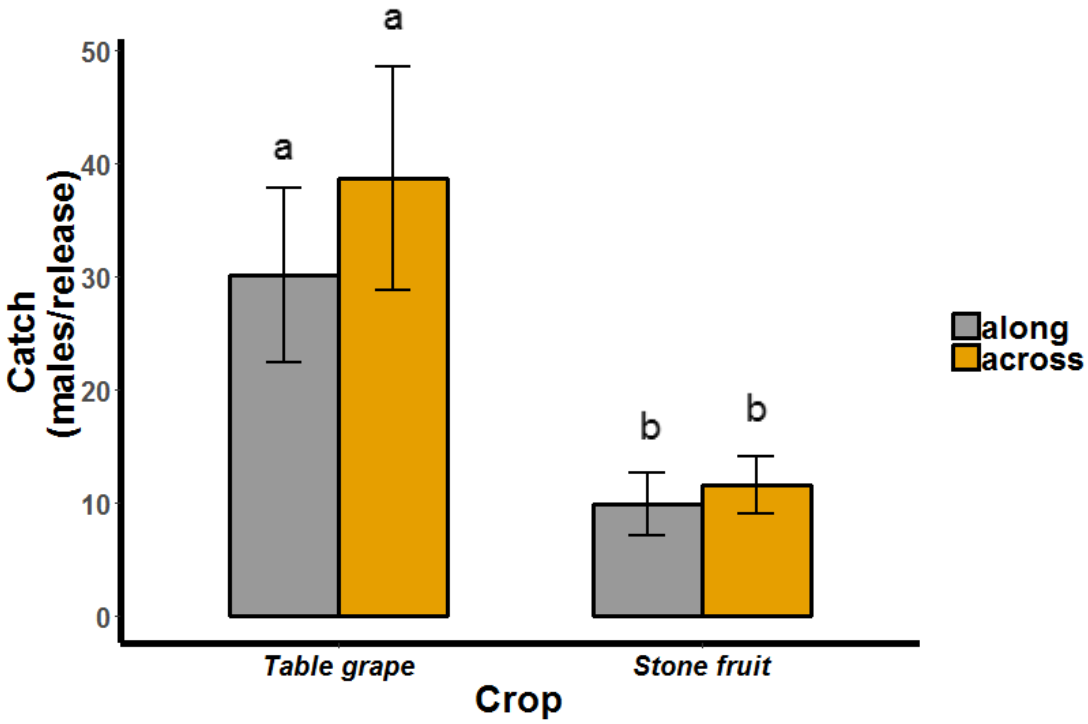

Fig. 4.5. Mean (± SE) number of *Thaumatotibia leucotreta* caught per release (Catch) in traps that were in the same (along, grey) or different (across, gold) rows as the release point for each crop. Different letters above the vertical bars indicate significant differences (p < 0.05).

Wild vs released moths

The trapping pattern of wild moths and sterile released moths were similar ($F_{8, 108}$ = 1.246, p = 0.230) in the pre-treatment releases (Fig. 4.6). Only position C4 caught significantly (p = 0.046) more released moths than wild moths during the two month period.

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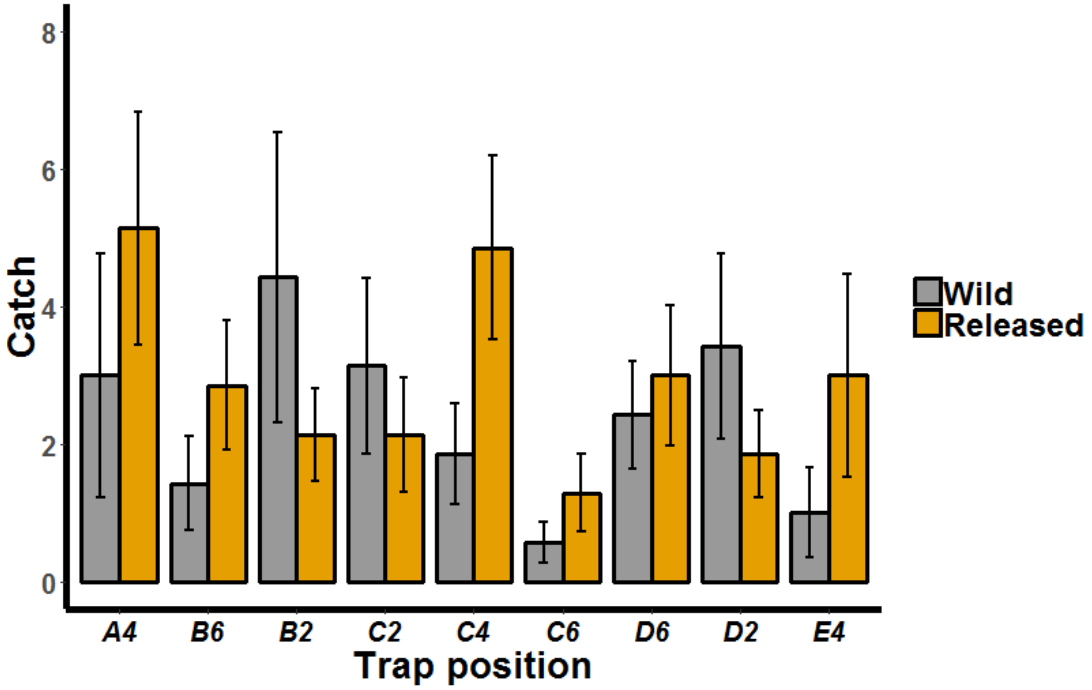

Fig. 4.6. Mean (± SE) number of *Thaumatotibia leucotreta* males caught per release (Catch) for every position in the trapping grid. Trapping was conducted in stone fruit blocks not treated with pheromone and illustrates data from eight releases over a period of two months. Grey bars are wild moths and the gold represents the released sterile moths.

4.3.3 Mating disruption

A relatively large proportion of the released sterile moths were recaptured in the control blocks (Table 4.1), on average the trapping grid recaptured 13.5% (± 3.14%). The recapture percentage was significantly ($F_{1, 13}$ = 5.712, p = 0.033) higher in table grapes (19.56% ± 4.88%) than in stone fruit (6.57% ± 1.53%). The number of moths caught in the pre-treatment control blocks was significantly lower than number of moths caught in the post-treatment control blocks in stone fruit (Z = -2.924, N = 72, p = 0.003) and table grapes (Z = -3.367, N = 72, p < 0.001). The pre-treatment releases all had higher catch in the treatment blocks than in the control blocks (Table 4.1), causing mating disruption to be calculated as a negative value and therefore taken as 0 (Fig. 4.7). The addition of pheromone dispensers caused a significant reduction in trap catches in stone fruit ($F_{1, 70}$ = 8.975, p = 0.004) and in table grapes ($F_{1, 61}$ = 16.632, p < 0.001). The mating disruption was the highest in table grapes (92.7% ± 1.34%), but did not significantly ($F_{1, 11}$ = 2.488, p = 0.143) differ from the disruption in stone fruit (Fig. 4.7). The post-treatment mating disruption of wild moths (79.31% ± 2.07%) was similar to the mating disruption of the released moths (86.38% ± 3.43%) in stone fruit orchards (Fig. 4.7).

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Table 4.1. The number of released sterile male *Thaumatotibia leucotreta* recaptured per treatment and the standardized catch as a percentage in stone fruit and table grapes. The number and standardized catch of wild moths recaptured in stone fruit is provided in brackets.

	Pre-treatment (0 dispensers)			Post-treatment (400 dispensers)		
Crop	**Control**	**Treatment**	**Catch[a]**	**Control**	**Treatment**	**Catch[a]**
Stone fruit	14 (39)	18 (41)	128.57 (105.13)	28 (64)	5 (14)	17.86 (21.88)
Stone fruit	3 (9)	7 (9)	233.33 (100)	42 (6)	4 (1)	9.52 (16.67)
Stone fruit	33 (5)	59 (6)	178.79 (120)	16 (17)	1 (4)	6.25 (23.53)
Stone fruit	26	28	107.69	48	10	20.83
Table grapes	32	35	109.38	62	5	8.06
Table grapes	53	59	111.32	60	2	3.33
Table grapes	32	38	118.75	189	17	8.99
Table grapes	62	76	122.58	136	12	8.82

[a]Calculated following McGhee et al. (2014), standardized catch = $(\frac{males\ caught\ in\ treatment\ block}{males\ caught\ in\ control\ block})$ x 100

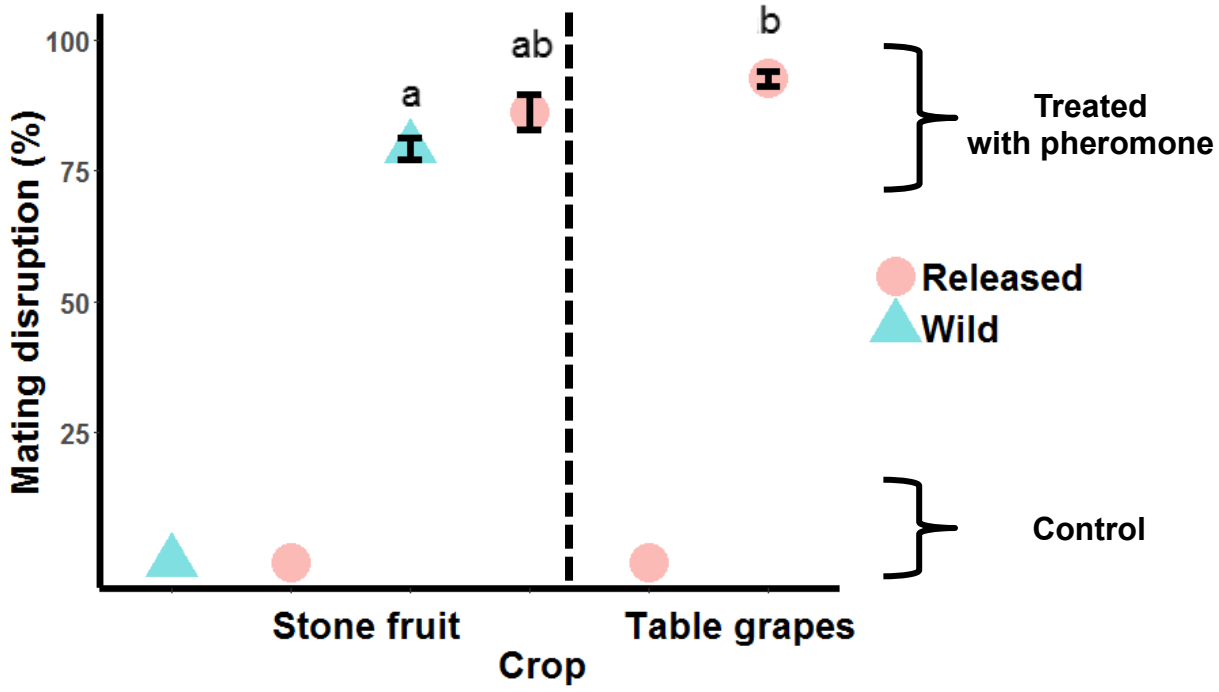

Fig. 4.7. Mean (± SE) mating disruption of *Thaumatotibia leucotreta* males in stone fruit and table grapes. The blue triangles represent the wild moths and the red dots represent the mating disruption of the sterile released moths. No wild moths were present in the table grape vineyards. Different letters above the error bars indicate significant differences ($p < 0.05$).

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4.4 Discussion

Reliable and reproducible results for mating disruption against FCM have historically proven to be difficult for two reasons. Firstly, due to little published work and secondly as testing has occurred over the long term, trap catches are influenced by several factors including temperature and pest density, making it hard to determine the effect of the pheromone. Hofmeyr et al. (1991) reported the first evidence of mating disruption being effective against FCM after their long-term study in citrus orchards. The current study offers the first experimental evidence that mating disruption is also effective against FCM in stone fruit and table grapes. In fact, a large percentage (13.5%) were recaptured of the total released moths, allowing the reliable calculation of mating disruption in both crops. In table grapes the addition of the pheromone worked particularly well to disorientate males and caused high (92%) levels of mating disruption. The mating disruption in stone fruit was slightly higher against sterile moths (86%), but did not significantly differ from the disruption of the wild (79%) moths.

The constraint on the experimental design of unknown pest densities can be alleviated by releasing a known number of individuals into the environment (McGhee et al., 2014). However, this approach also has its limitations, for instance previous mark-release-recapture studies on FCM recaptured less than 3% of males with their grid of traps (Visser et al., 2015). These low recaptures also cause similar limitations to the reliability of inferences that the traditional design has. Another limitation is the inability to reliably correlate temperature to recapture rates. Previous studies on Tortricidae have shown that different temperatures between releases may influence the recapture rates and the performance of insects (Chidawanyika and Terblanche, 2011). In the stone fruit control block releases, trap catch had a weak negative (r = -0.343) association with temperature and the lower temperatures recorded in the pre-treatment releases corresponded to fewer moths caught than in the post-treatment releases. However, though temperatures were similar between the pre- and post-treatment releases in table grapes, the number of moths caught in the pre-treatment control blocks were still fewer than the number caught in the post-treatment control blocks. This disparity indicates that there are other factors affecting the trapping efficiency of the moths and highlights the importance of not assuming causation from correlation. However, these limitations were improved with this design by increasing the number of release points (ensuring uniform distribution of moths), better trap placement and including a control to standardize moth quality (Williams et al., 2013).

Validation of the design showed that total distance away from the release point was not a reliable predictor of number recaptured, which is in contrast to Visser et al. (2015) who also

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marked and recaptured FCM. The trap layout probably contributed to the uniform recapture of the moths, as there were few differences between the numbers of individuals caught by the different positions in the grid. A constraint of high density trapping is the effect of trap interference, where central traps are masked by their neighbouring traps (Suckling et al., 2015). Trap interference recorded here was very low (< 1.3) suggesting that central traps were almost as efficient as border traps in the grid. An alternative explanation may be that the distance from release point and trap interference may be working in contrasting directions, thereby reducing their combined effect on recaptures. However, it may also be a result of the four release points, rather than a central release (Visser et al., 2015), that ensured the moths were evenly distributed throughout the blocks. Contrary to our expectation there was no preferential flight pattern of moths, as moths were equally likely to be caught in traps that were along or across the row from the release point. A possible explanation for this result is that the moths are flying above the crop and may indicate that FCM is a reasonably strong flyer, though direct tests should be conducted to confirm this theory. These additional factors that influence trap catches, highlighted the importance of including the paired identical release into our experimental design and ensured that trapping design did not bias our results for testing mating disruption.

Relevance of experiments on sterile organisms for deducing wild moths' behaviour may be questioned, as mass reared moths that are subsequently sterilised could have compromised performance in the field when compared to wild moths (Nepgen et al., 2015; Boersma and Carpenter, 2016). This begs the question of whether sterile moths' behavioural responses (e.g. ability follow pheromone plumes) correspond to that of wild moths. This may be answered by considering how the male moth locates a female's pheromone source, as the male moth must first detect the pheromone and then follow it to its source (Cardé, 2016). This behaviour is exploited as mate finding and trap finding require the same male attributes. This study found there was no difference in the number of wild and sterile moths caught in the different positions in our trapping grid. Furthermore, sterile and wild moths shared a trapping pattern (similar number of moths in different trap positions), indicating that sterile males are equally capable of finding mates/traps and justifies the use of sterile moths in such experiments.

A limitation of the design is that there is no direct measure of the success or failure of mating disruption. As the ultimate goal of mating disruption is the reduction of fruit damage, previous studies (Hofmeyr et al., 1991; Ochou et al., 2017) included damage assessments as their direct measure of mating disruption. Though feasible, damage may also only be a proxy of mating disruption especially against FCM, that is thought to move between fruit crops and therefore

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damage may be a result of mated females entering the treated area. A more direct measure of mating disruption could be achieved by counting the number of spermatophores within the female's bursa (Fadamiro and Baker, 2002). Therefore, as damage assessments have their own limitations and were impossible to conduct with this design due to the release of sterile moths, it is suggested that future studies should incorporate the counting of spermatophores within the released females' bursa, this will allow pest density estimation as well as a direct measure of mating disruption to be obtained.

Previous work on mating disruption against Lepidoptera have mostly reported success of the technique (Miller et al., 2006b; Miller and Gut, 2015). Some studies reported near total mating disruption (99.4%, Deland et al., 2004; 99.1%, Tcheslavskaia et al., 2005) with similar methods as used here. This indicates that the mating disruption calculated here (86% and 93%) can still be improved against FCM, especially as both these studies applied twice as much pheromone in their studies.

From this study, it is clear that mating disruption has potential as a control against FCM in stone fruit and table grapes. Though the tests shown here provide a good base for testing and calculating mating disruption, there are still a number of questions that need to be addressed. Whilst it was shown that the addition of mating disruption is effective, the mechanisms that causes FCM males to be disrupted are not fully understood. Miller et al. (2006), proposed that moths may be disrupted either competitively (male follows plume to its source) or non-competitively (male initially follows plume but later becomes disorientated as they approach the dispenser/source of synthetic pheromone) and that it is vital to understand this behavioural response for proper management of the pest. Another important question for management is discerning the optimal dose of pheromone to ensure high disruption with the lowest possible cost. Our validated design, helps us address these questions in Chapter 5 and allows us to delve deeper into the mechanistic disruption of FCM.

4.5 References

Agri-Intel, 2018. Accessed: Date:14/11/2018 Time:16:22 http://www.agri-intel.com/label-information/search-registration-information/

Allison, J.D., Cardé, R.T., 2016. Pheromone communication in moths: evolution, behavior, and application. Univ of California Press.

Stellenbosch University https://scholar.sun.ac.za

Angelini, A., Descoins, C., Lhoste, J., Trijau, J.P., Zagatti, P. 1981. Test of new formulations of synthetic attractants for sexual trapping of *Cryptophlebia leucotreta* Meyr. (Lepidoptera). Cot. Fib. Trop. 36, 259-264.

Boersma, N., Carpenter, J.E., 2016. Influence of holding temperature and irradiation on field performance of mass-reared *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). Florida Entomol. 99, 215-221.

Cardé, R.T., 2016. Moth navigation along pheromone plumes. In: Pheromone communication in moths: evolution, behavior and application. University of California Press, Berkeley, California, pp.173-189.

Chidawanyika, F., Terblanche, J.S., 2011. Costs and benefits of thermal acclimation for codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae): implications for pest control and the sterile insect release programme. Evol. Appl. 4, 534-544.

Deland, J.P., Judd, G.J.R., Roitberg, B.D., 1994. Disruption of pheromone communication in three sympatric leafroller (Lepidoptera: Tortricidae) pests of apple in British Columbia. Environ. Entomol. 23, 1084-1090.

Fadamiro, H.Y., Baker, T.C., 2002. Pheromone puffs suppress mating by *Plodia interpunctella* and *Sitotroga cerealella* in an infested corn store. Entomol. Exp. Appl. 102, 239-251.

Hofmeyr, J.H., Burger, B.V., 1995. Controlled-release pheromone dispenser for use in traps to monitor flight activity of false codling moth. J Chem. Ecol. 21, 355-363.

Hofmeyr, J.H., Burger, B.V., Calitz, F.J., 1991. Disruption of male orientation to female false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae), using synthetic sex pheromone. Phytophylactica, 23, 153-156.

Hofmeyr, J.H., Calitz, F.J., 1991. Field evaluation of synthetic sex attractants of the false codling moth, C*ryptophlebia leucotreta* (Lepridoptera: Tonricidae). Phytophylactica, 23, 149-152.

Hofmeyr, J.H., Hofmeyr, M., 2002. Evaluasie van die paringsontwrigter Isomate vir die kommersiële bestryding van valskoldingmot op nawelbome. CRI Ann. Res. Rep. 68-74.

Hofmeyr, J.H., Hofmeyr, M., Carpenter, J.E., Bloem, S., Slabbert, J.P., 2016. Sterile insect

Stellenbosch University https://scholar.sun.ac.za

releases for control of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae): an assessment on semi-commercial scale. African Entomol. 24, 80-89.

Hortgro, 2017. Peach and Nectarine FCM control guidelines. Accessed: [26 September 2017, www.hortgro-science.co.za/false-codling-month-control-guidelines-peaches-nectarines/].

Lance, D.R., Leonard, D.S., Mastro, V.C., Walters, M.L., 2016. Mating disruption as a suppression tactic in programs targeting regulated lepidopteran pests in US. J Chem. Ecol. 42, 590-605.

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Aerosol emitters disrupt codling moth, *Cydia pomonella*, competitively. Pest Manag. Sci. 70, 1859-1862.

McNeil, J.N., 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. Annu. Rev. Entomol. 36, 407-430.

Miller, J.R., Gut, L.J., 2015. Mating disruption for the 21st century: matching technology with mechanism. Environ. Entomol. 44, 427-453.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. J Chem. Ecol. 32, 2089-2114.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): case studies. J Chem. Ecol. 32, 2115-2143.

Moore, S.D., Kirkman, W., 2010. Evaluation of checkmate FCM-F for the control of false codling moth. CRI Ann. Res. Rep. 2009-10, 91-96.

Moore, S.D., Kirkman, W., 2011. Evaluation of checkmate FCM-F for the control of false codling moth. CRI Ann. Res. Rep. 2010-11, 91-96.

Moore, S.D., Hattingh, V., 2012. A review of current pre-harvest control options for false codling moth in citrus in Southern Africa. SA Fruit J. 11, 82-85.

Stellenbosch University https://scholar.sun.ac.za

Nepgen, E.S., Hill, M.P., Moore, S.D., 2015. The effect of long-distance transportation on the fitness of irradiated false codling moth (Lepidoptera: Tortricidae) for use in a sterile insect release program. J Econom. Entomol. doi: http://dx.doi.org/10.1093/jee/tov246

Newton, P.J., Mastro, V.C., 1989. Field evaluations of commercially available traps and synthetic sex pheromone lures of the false codling moth, *Cryptophlebia leucotreta* (Meyr.) (Lepidoptera: Tortricidae). Int. J. Pest Manag. 35, 100-104.

Newton, P.J., Thomas, C.D., Mastro, V.C., Schwalbe, C.P., 1993. Improved two-component blend of the synthetic female sex pheromone of *Cryptophlebia leucotreta*, and identification of an attractant for *C. peltastica*. Entomol. Exp. Appl. 66, 75-82.

Ochou, G.E.C., Kone, P.W.E., Didi, G.J.R., Kouakou, M., Bini, K.K.N., Mamadou, D., Ochou, O.G., 2017. Efficacité de la confusion sexuelle contre *Thaumatotibia* (= *Cryptophlebia*) *leucotreta* (Lepidoptera: Tortricidae) dans les exploitations cotonnières en Côte d'Ivoire. Int. J. Biol. Chem. Sci. 11, 1222-1235.

Persoons, C.J., Ritter, F.J., Nooyen, W.J., 1977. Sex pheromone of the false codling moth *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae). J Chem. Ecol. 3, 717-722.

Platt, J. R., 1964. Strong Inference. Science 146, 347-353.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reinke, M.D., Siegert, P.Y., McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Pheromone release rate determines whether sexual communication of Oriental fruit moth is disrupted competitively vs. non-competitively. Entomol. Exp. Appl. 150, 1-6.

Schoeman, P.S., De Beer, M., 2008. Control of the false codling moth with the Isomate mating disruption technology. SAAGA yearbook 31, 44-45.

Sievert, C., 2018. plotly for R. https://plotly-book.cpsievert.me

Stelinski, L.L., Gut, L.J., Pierzchala, A.V., Miller, J.R., 2004. Field observations quantifying attraction of four tortricid moths to high-dosage pheromone dispensers in untreated and pheromone-treated orchards. Entomol. Exp. Appl. 113, 187-196.

Suckling, D.M., 2015. Can we replace toxicants, achieve biosecurity, and generate market

Stellenbosch University https://scholar.sun.ac.za

position with semiochemicals? Front. Ecol. Evol. 3, 1-17.

Suckling, D.M., Conlong, D.E., Carpenter, J.E., Bloem, K.A., Rendon, P., Vreysen, M.J.B., 2017. Global range expansion of pest Lepidoptera requires socially acceptable solutions. Biol. Invasions 19, 1107-1119.

Suckling, D.M., Stringer, L.D., Kean, J.M., Lo, P.L., Bell, V., Walker, J.T., Twidle, A.M., Jiménez-Pérez, A., El-Sayed, A.M., 2015. Spatial analysis of mass trapping: how close is close enough? Pest Manag. Sci. 71, 1452-1461.

Tcheslavskaia, K.S., Thorpe, K.W., Brewster, C.C., Sharov, A.A., Leonard, D.S., Reardon, R.C., Mastro, V.C., Sellers, P., Roberts, E.A., 2005. Optimization of pheromone dosage for gypsy moth mating disruption. Entomol. Exp. Appl. 115, 355-361.

Visser, C.N., 2015. Determining the quality of mass reared male codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), by assessing flight performance under laboratory, semi-field and field conditions. (M.Sc., Stellenbosch University).

Warton, D.I., Hui, F.K., 2011. The arcsine is asinine: the analysis of proportions in ecology. Ecol. 92, 3-10.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Williams, D.T., Straw, N., Townsend, M., Wilkinson, A.S., Mullins, A., 2013. Monitoring oak processionary moth *Thaumetopoea processionea* L. using pheromone traps: the influence of pheromone lure source, trap design and height above the ground on capture rates. Agr. Forest Entomol. 15, 126-134.

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Chapter 5:

Mechanisms mediating false codling moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) mating disruption, using point-source pheromone dispensers

Abstract

False codling moth (FCM) is a polyphagous tortricid moth that is a priority agricultural pest, due to its direct crop damage and phytosanitary restrictions. Mating disruption shows potential as an environmentally friendly and sustainable control option against FCM in stone fruit, table grapes and citrus. However, it is unclear how the technique alters the mate-finding ability of FCM and what factors are important to ensure the control's success. Recent work has indicated that Lepidoptera are disrupted either competitively or non-competitively. No studies have yet been conducted on FCM to determine its disruptive mechanism. However, studies conducted on closely related species have proposed and critically evaluated a framework along with a dichotomous key for distinguishing between the two mechanisms. This provides a protocol for identifying the underlying disruption mechanism in other species. Here the dosage-response profile, the optimal dosage and optimal density was calculated from multiple mark-release-recapture (MRR) experiments using sterile FCM. Stone fruit and table grape blocks were treated with increasing levels of pheromone dispenser densities which allowed the level of disruption experienced (FCM caught in treated/untreated blocks) to be calculated. Mating disruption proved highly effective in stone fruit and table grapes against FCM, furthermore FCM follows a hybrid disruption profile and is disrupted competitively at low dosages and non-competitively at higher dosages. The shift to non-competitive disruption resulted in 99% disruption and was achieved at a dosage of 192 g/ha of active ingredient and remained effective with as little as 36 pheromone release sites/ha. Mating disruption is therefore highly recommended to form part of the current integrated pest management programme for FCM.

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5.1 Introduction

Mating disruption is the broadcasting of female pheromones within an agricultural system so as to decrease the reproduction of the pest insect (Miller and Gut, 2015). Mating disruption has been used since the 1970's and has been tested against many agricultural lepidopteran pests including Pyralidae, Noctuidae with around 200+ studies (Evenden, 2016) focussing on the control of Tortricidae (Cardé and Minks, 1995; Judd et al., 1997). Though there is variation in its effectiveness, with some species easily disrupted (Stelinski et al., 2008) and others requiring much higher doses (Miller et al., 2006b), the technique has been proven to be a highly effective method (Suckling, 2015). *Thaumatotibia leucotreta*, false codling moth (FCM) is one of the most economically important pests in Africa, as it is a polyphagous tortricid, with multiple generations per year (Malan et al., 2018) and no documented diapause to date (Terblanche et al., 2014). There is potential for the use of mating disruption against the adult stage of FCM in citrus, stone fruit and table grapes (Chapter 4; Hofmeyr et al., 1991). However, as yet it is unclear how the technique works against FCM and what mechanisms drive its success or failure.

Much speculation and experimental work has been conducted determining the disruption mechanisms involved for Lepidoptera (Cardé et al., 1998; Miller et al., 2006a; Allison and Cardé, 2016) and can be divided into two broad classes, namely, competitive disruption and non-competitive disruption (Miller and Gut, 2015). Competitive disruption includes competitive attraction (false trail following, confusion), induced allopatry (aggregation of males close to dispenser), and induced arrestment (reduced search) so its success is dependent on pest density as females compete with dispensers for male visitations (Miller and Gut, 2015). Non-competitive disruption's success is not linked to pest density and includes camouflage, sensory imbalance (desensitization or sensory blockage) (Cardé and Minks, 1995), shifted activity period (Mori and Eveden, 2013; Gerken and Champbell, 2018) and suppressed calling (Miller and Gut, 2015).

These broad classes of mating disruption mechanisms can be differentiated by following mathematical and graphical tools proposed by Miller et al. (2006a, b). Miller et al. (2006a) suggested that, when competitively disrupted, the catch will drop sharply initially with the addition of pheromone dispensers, but additional dispensers will have a diminishing net effect on catch. Therefore, the catch does not decrease linearly with additional dispensers, but reaches a plateau as dispenser density increases. Contrastingly, under non-competitive disruption, catch is expected to decrease linearly with additional dispensers. With these predictions in place,

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Miller et al. (2006b) reviewed the literature and used data from previous studies to assess the overall disruption mechanism at play for Lepidoptera. Of the 13 studies reviewed, the prediction of competitive disruption was supported by 11 of the studies, suggesting, with the current technology (dispensers), that competitive disruption is the most prevalent mechanism.

Recently, other studies have used the framework offered by Miller et al. (2006a, b) and revised by Miller et al. (2010) to determine the manner in which lepidopteran pests are disrupted, by various pheromone dispensers (McCormick et al., 2012; Reinke et al., 2014; McGhee et al., 2014). Reinke et al. (2014) provided the first experimental evidence that competitive and non-competitive disruption are not mutually exclusive. In their study, they showed that the disruption of oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) may shift from competitive to non-competitive disruption with high releasing point-source pheromone dispensers, while codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) continues to be disrupted competitively, even with high releasing aerosol pheromone emitters (McGhee et al., 2014).

To ensure adoption of mating disruption, clarity of the disruption mechanism involved is only part of the problem, another obstacle is the efficient or optimal application of the pheromone to reduce costs (Lapointe and Stelinski, 2011). Hand applied pheromone dispensers require the greatest input of labour and time, as between 500 - 1000 dispensers are applied per hectare depending on pest and crop treated (Witzgall et al., 2008). A popular method to reduce the application costs is to reduce the number of pheromone sources or pheromone sites/ ha. For instance, the use of puffers reduces the pheromone sites from 500 - 1000 uniformly distributed sites per hectare to as little as five with disruption remaining effective (+ 90% disruption, McGhee et al., 2014). Another approach is to leave sections of the orchard untreated (referred to as intentional gaps) thereby reducing the pheromone sites, dose and application costs (Tcheslavskaia et al., 2005; Lapointe and Stelinski, 2011).

The aim of this study was to reveal the mechanism employed by FCM to result in mating disruption and to identify the optimal application of the pheromone dispensers in stone fruit and table grapes. Dosage-response profiles, optimal dosage and optimal density were determined using the framework of Miller et al. (2006a, b, 2010) to show that 1) FCM has a hybrid disruption profile, 2) FCM is disrupted competitively at low dosages and non-competitively at high dosages, 3) optimal dosage is 192 g/ha, and 4) optimal density is 36+ pheromone sites.

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5.2 Material and methods

5.2.1 Source of insects

Thaumatotibia leucotreta were collected as 1-day-old sterile adults from a mass reared population maintained at XSIT (Pty) Ltd in Citrusdal, South Africa (see Chapter 4.2.1). The moths were transported in a cooler box to the stone fruit (33°54'26.9"S 18°47'42.7"E, Stellenbosch) or table grapes (33°10'09.0"S 18°59'20.6"E, Halfmanshof) to ensure the cold chain was not interrupted. Upon arrival at the experimental plot the moths were allowed to warm up for 10 min after which they were released.

5.2.2 Mark-release-recapture protocol

The data from Chapter 4 was used along with additional 34 releases, therefore data from 48 releases were analysed in this chapter. The release protocol described and tested in Chapter 4 was followed. Briefly, experiments were conducted in 0.5 ha blocks of either stone fruit (plum) (November 2016 - April 2017) or table grapes (November 2017 - January 2018). Each block had a grid of nine Delta traps loaded with the Chempac FCM lure (L7254, Chempac, Simondium, South Africa) and 200 sterile FCM (100 males and 100 females) were released at one of four sites per 0.5 ha block (Fig. 4.1). Each release had its own paired release/control and sterile males were recaptured every 24 h for five days. Treatments consisted of either no pheromone dispensers or a specific number of point-source polyethylene tube dispensers (Isomate FCM 240, lot no. FCM-50393, Shin-Etsu Chemical Co., Ltd., Japan) that are formulated commercially and loaded with 240 mg of active ingredients (166.8 mg of E-8-dodecenyl acetate + 70.8 mg of Z-8-dodecenyl acetate + 2.4 mg of E / Z-8-dodecenol). The release rate of these dispensers is reportedly 50 - 60 µg h^{-1} and considered as high releasing dispensers (Witzgall et al., 2008; Reinke et al., 2014), though this was not measured in this study. Unless stated otherwise, dispensers were hung uniformly throughout the experimental block in the top 0.5 m of the tree or vine (at least 1.8 m above the ground).

5.2.3 Dosage response

The dosages included 0, 200, 400, 600, 800, 1600 dispensers per ha, that is equivalent to 0, 48, 96, 144, 192 and 384 g of active ingredients/ha, respectively. All treatments were repeated at least four times (releases), except treatments of 0, 400 and 800 dispensers per ha, which were repeated eight times. Currently the recommended field dosage for stone fruit and table grapes is 600 dispensers/ha (Isomate™ FCM, Nulandis, South Africa).

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Optimal dosage

Mating disruption was calculated for each of the different dosages using formula 4.2 (Chapter 4) and following Miller et al. (2006b). The optimal dosage was calculated as the point where additional pheromone dispensers did not cause a significant increase in disruption. This will ensure that the additional pheromone justifies the additional cost and that the best disruption is achieved with the lowest financial input.

Competitive vs non-competitive disruption

In accordance with Miller et al. (2006a, b), Miller et al. (2010) and Miller and Gut (2015) the treatment means (± SE) were calculated for catch, 1/catch and dispenser density (number of point sources) multiplied by catch. The calculated data from the releases were then fitted to an untransformed plot (Fig. 5.1A), the Miller-Gut plot (Fig. 5.1B) and the Miller-de Lame plot (Fig. 5.1C). Each of the mechanisms has a distinctive theoretical dosage-response profile on each of the plots, which were used to differentiate between the two mechanisms in this study (Miller and Gut, 2015). The theoretical profiles were drawn for each of the mechanisms, the green line represents the predicted profile of competitive disruption, whereas the blue line represents the predicted profile of non-competitive disruption (Fig. 5.1). The line that best fitted the dosage-response profile in each scenario was chosen following Reinke et al. (2014), which compared the R^2 values of the lines. The catch referred to here is the standardized catch (formula 4.1, Chapter 4) which is the mean number of males caught per release relative to its paired control, divided by the number of traps, so that catch equals the relative number of males caught per trap per release. All calculations were done per ha. The data from table grape and stone fruit blocks were pooled as they did not significantly ($p > 0.05$) differ within treatment.

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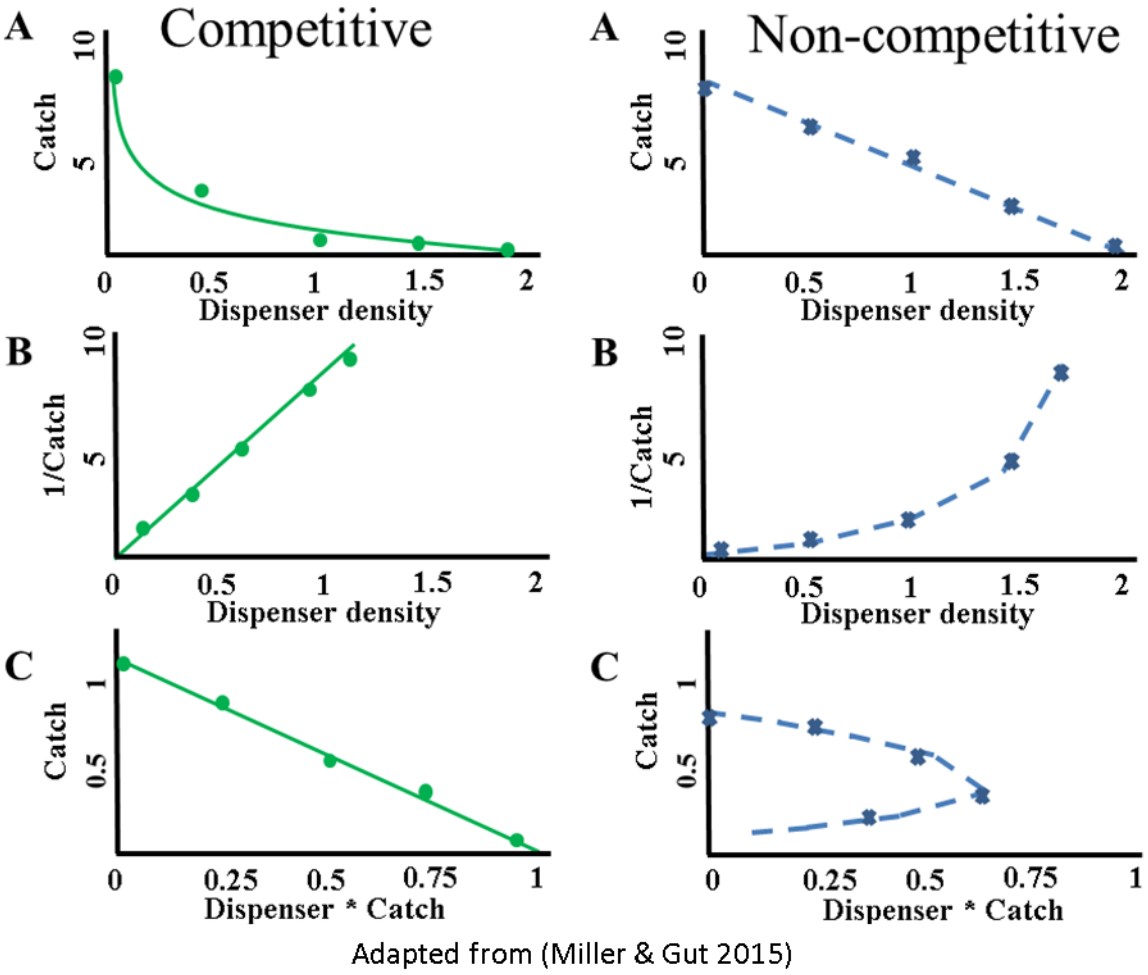

Fig. 5.1. The theoretical profiles drawn for each of the mechanisms. The solid green line represents the predicted profiles of competitive disruption, whereas the dotted blue line represents the predicted profiles of non-competitive disruption. Panel A) untransformed data, competitive disruption has a diminishing net effect of each additional dispenser. Panel B) Miller- Gut plot, panel C) Miller-de Lame plot (most stringent test of the three), competitive disruption has a negative slope and non-competitive will be best fit with a quadratic function.

5.2.4 Density response

Optimal density

Due to the use of point-source pheromone dispensers, there was an inherent problem with our design, namely that to increase the dosage I also had to increase the number of point sources. To tease apart the respective influences of dosage and the number of pheromone sources, an experiment was conducted with a constant dosage of 800 dispensers (192 g) per hectare. The 800 dispenser dosage was chosen for two reasons 1) calculated as the optimal dosage, and 2) shown in dosage experiment as point where disruption shifts to non-competitive disruption.

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Dispensers were not uniformly distributed throughout the experimental block, rather the dispensers were clustered into a certain number of pheromone release sites. The clusters were made by attaching a number of dispensers to a modified wire hanger (Fig. 5.2). Dispensers were spaced evenly along hanger to allow for proper functioning of the dispensers. Pheromone release site densities included 0, 9, 18 and 36, which were compared to 800 individual dispensers uniformly distributed throughout the block.

Fig. 5.2. A diagram of the modified wire hanger (left) with a number of pheromone dispensers attached and right) the modified wire hanger in a table grape vineyard that was used to test the density response whilst keeping the dosage constant.

Confirmation of non-competitive disruption

To confirm that the dosage equivalent to 800 dispensers/ha was in fact the point where disruption switches from competitive to non-competitive disruption as shown (section 5.3.1), the profile analysis was repeated on the density response results. The profile analysis was conducted as described above (section 5.2.3).

5.2.5 Statistical analyses

The statistical analysis was performed using Statistica 12 (Stat-Soft Inc., 2012). If the residuals were not normally distributed the data were log(X + 1)-transformed for further analysis. If the assumptions (normality and equality) of a one-way analysis of variance (ANOVA) were violated irrespective of transformation, the Kruskal-Wallis H-test and Mann-Whitney U test were used to separate the means at $p < 0.05$. When applicable, means were separated using the Fisher's LSD method. TableCurve 2D (version 5.01.02) (SYSTAT Inc, San Jose, California, USA) was used to fit expected dosage-response profiles and calculate the coefficient of determination (R^2) for linear and non-linear equations. RStudio version 1.0.143 and R version 3.5.1 (R, 2018), was used to illustrate the findings graphically, the packages used were ggplot2, plotly and forcats (Wickham, 2016; Sievert, 2018).

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5.3 Results

5.3.1 Dosage response

Optimal dosage

The mating disruption of all dosages were significantly ($p < 0.001$) higher than that of the control (Fig. 5.3). The lowest dose (200/ha) tested in stone fruit caused high (82.36% ± 4.19%) disruption and did not differ significantly ($df = 25$, $p = 0.848$) from the recommended field dosage (600/ha) for stone fruit. The level of disruption was slightly higher in table grapes than in stone fruit, but did not significantly differ for the 400/ha, ($p = 0.323$) or 800/ha ($p = 0.281$) treatments. There was an initial increase in mating disruption that remained constant (82 - 92%) up to the 600/ha dosage. However, the increase to 800/ha caused a significant increase of mating disruption (96 - 99%) in stone fruit (800/ha > 600/ha, $p = 0.007$) and table grapes (800/ha > 400/ha, $p = 0.045$). The highest dosage in table grapes (1600/ha) did not significantly ($p = 0.958$) differ from the 800/ha and caused less than 100% mating disruption. Increasing the number of pheromone dispensers above 800/ha did not cause an increase in the realized disruption, therefore the optimal dosage was 800 dispensers/ha.

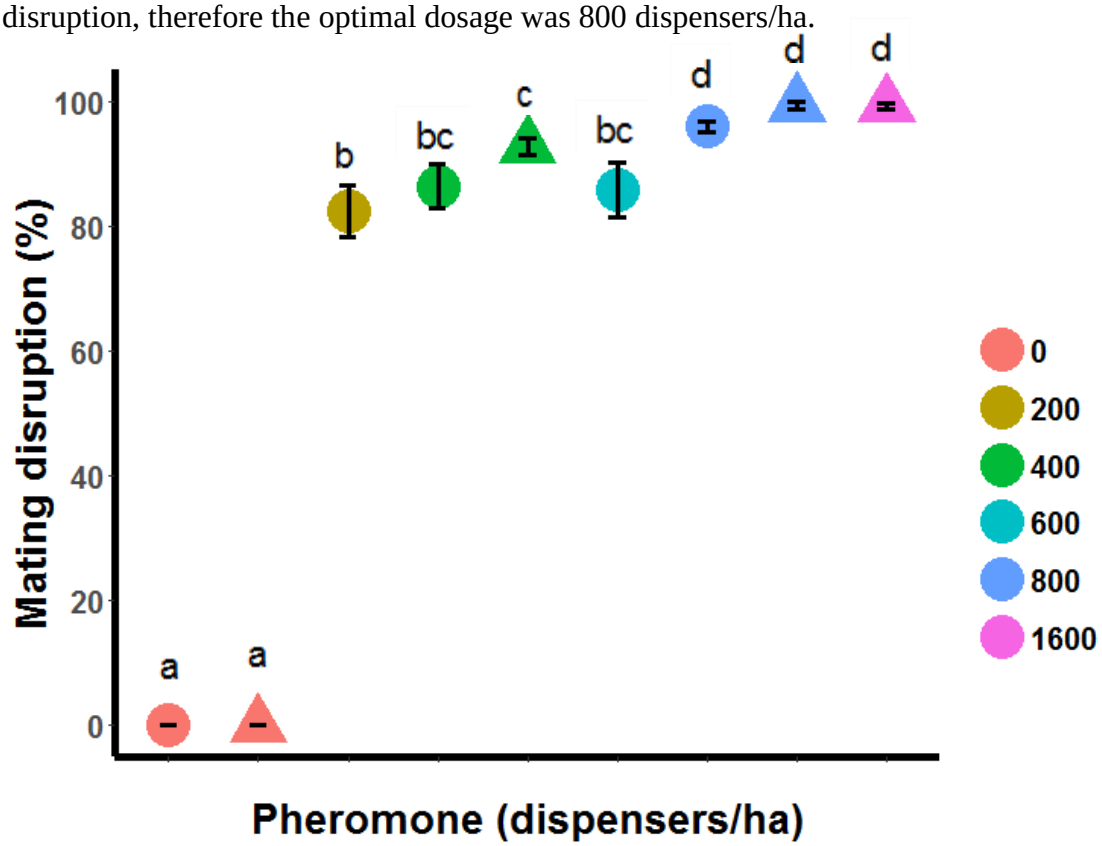

Fig. 5.3. Mean mating disruption (± SE) of *Thaumatotibia leucotreta* in stone fruit (dots) and table grapes (triangles) calculated as number of moths caught in treated block / number of males caught in the control block. Coloured points refer to the number of pheromone dispensers per hectare. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

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Competitive vs non-competitive disruption

The untransformed plot (Fig. 5.4) showed an initial sharp drop in trap catches from 0 to 200 dispensers per ha, after which trap catches plateau. The data most closely resembled a decaying exponential curve (R^2 = 0.783, solid black line) compared to the negative linear slope (R^2 = 0.397), illustrating the diminishing net effect (reduction of catch) of each additional dispenser. The 800 and 1600/ha dispenser (dark blue and pink points) were slightly lower than would be expected (see Fig. 5.1) under purely competitive disruption. However, most evidence for this plot supported the predictions of competitive disruption.

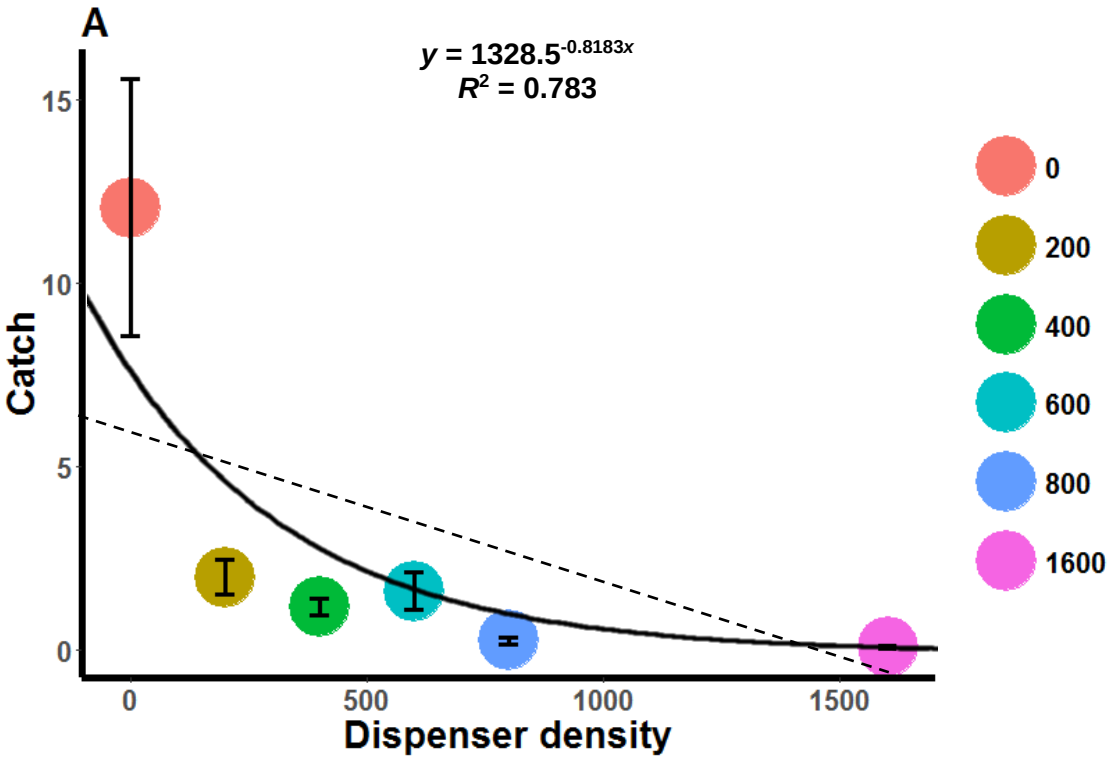

Fig. 5.4. Untransformed plot from dosage response results, that sets catch of *Thaumatotibia leucotreta* against dispenser density. The capital letter (A) in the top left corner indicates the plot which it refers to of the theoretical profile analyses process. The equation is for the line (solid line) of best fit. Different colours represent the number of Isomate™ dispensers/ha. The error bars indicate the standard error of the mean.

The inverse of catch is strongly correlated (r = 0.973, p = 0.001) with number of point sources (Fig. 5.5). A positive linear model (solid black line, R^2 = 0.947) better fit the data than the exponential model (R^2 = 0.771). The Miller-Gut plot therefore most closely follows the predictions of competitive disruption.

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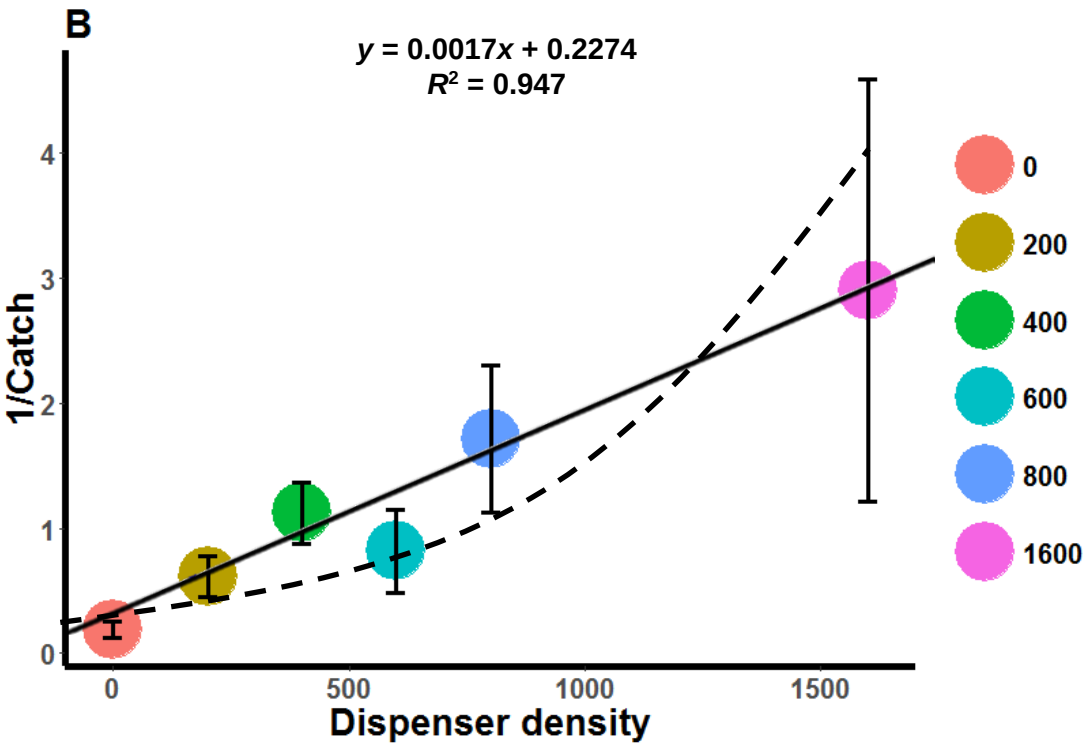

Fig. 5.5. Miller-Gut plot from dosage response results of *Thaumatotibia leucotreta* that sets the inverse of catch against dispenser density. The equation is for the line (solid line) of best fit. Different colours represent the number of Isomate™ dispensers/ha. The error bars indicate the standard error of the mean.

Contrastingly, when data are shown on the Miller-de Lame plot (Fig. 5.6) there is a distinctive re-curve at dosages higher than 600/ha, which provides strong evidence for non-competitive disruption. This is strengthened by the weak negative association (r = -0.422) and the quadratic function (R^2 = 0.624) that was the best fit for the data. However, the inflection point here is much lower than theoretically predicted (0.5 of max catch, Miller and Gut, 2015) under non-competitive disruption. The simultaneous consideration of these findings indicate that competitive disruption is prevalent at lower (< 600/ha) dosages, however, at the higher (800+ /ha) dosages non-competitive disruption is better supported.

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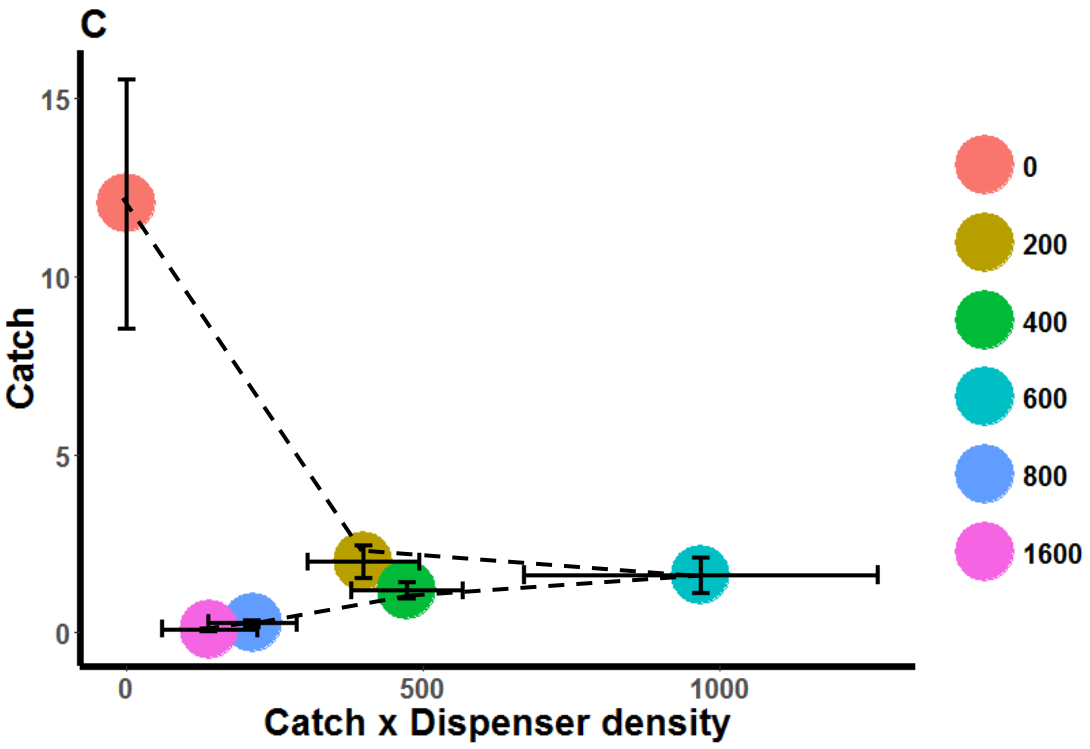

Fig. 5.6. Miller-de Lame plot from dosage response results, that sets catch calculated as number of male *Thaumatotibia leucotreta* moths per trap per release caught in treated block against catch multiplied by the dispenser density. The hand drawn dashed line illustrates the distinctive recurve predicted for non-competitive disruption. Different colours represent number of Isomate™ dispensers/ha. The error bars indicate the standard error of the mean.

5.3.2 Density response

Optimal density

There was a significant ($F_{4, 11} = 105.93$, $p < 0.001$) effect of number of pheromone sites (density) on the mating disruption in table grapes (Fig. 5.7), however, the control did not significantly ($p = 0.058$) differ from the lowest density (nine pheromone sites; 14.77% ± 8.97%). Using 18 pheromone sites (64% ± 17.04%) per hectare caused significantly ($p < 0.001$) higher mating disruption than nine pheromone sites and the control, but caused significantly lower disruption than 36 ($p = 0.01$) and 800 ($p < 0.001$). There was no significant difference in disruption between 36 pheromone sites (91.21% ± 5.21%) and 800 (98.81% ± 1.19%) uniformly distributed pheromone dispensers.

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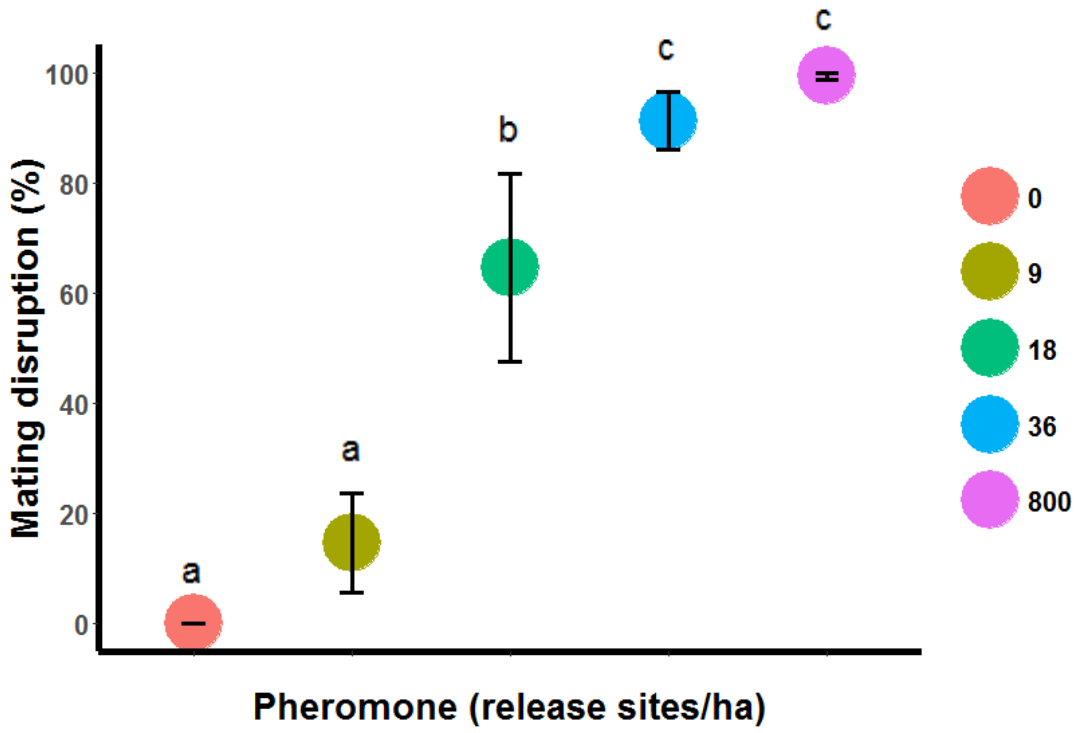

Fig. 5.7. Mean mating disruption (± SE) of *Thaumatotibia leucotreta* males in table grapes. Treated blocks had 800 Isomate™ dispensers/ha that were either clustered (9, 18, 36 sites/ha) or singly (800 sites/ha) distributed throughout vineyard. Different letters above the vertical bars indicate significant differences ($p < 0.05$).

Confirmation of non-competitive disruption

Catch decreased significantly, and had a strong negative (r = -0.964, p = 0.036) association, with increasing pheromone sites (Fig. 5.8A). The linear model (R^2 = 0.93) was a better fit than the exponential model (R^2 = 0.817). The linear model shows that additional release sites have an equal nett effect of disruption, furthermore it is graphically similar and follows the predictions of the non-competitive disruption profile (Fig. 5.8A, blue). The Miller-Gut plot (Fig. 5.8B) also provides evidence for non-competitive disruption as the exponential model (R^2 = 0.998) was a better fit than the linear model (R^2 = 0.873). There was a distinctive recurve present on the Miller-De Lame plot (Fig. 5.8C) making it graphically similar to the theoretical profile for non-competitive disruption (Fig. 5.8C, blue). Furthermore, it was better fit with a quadratic function (R^2 = 0.718) than with a negative linear model (R^2 = 0.034). The inflection

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point on the Miller-De Lame plot is similar to half of maximum catch (as predicted). All three plots offer evidence for non-competitive disruption for the density response data (Fig. 5.8).

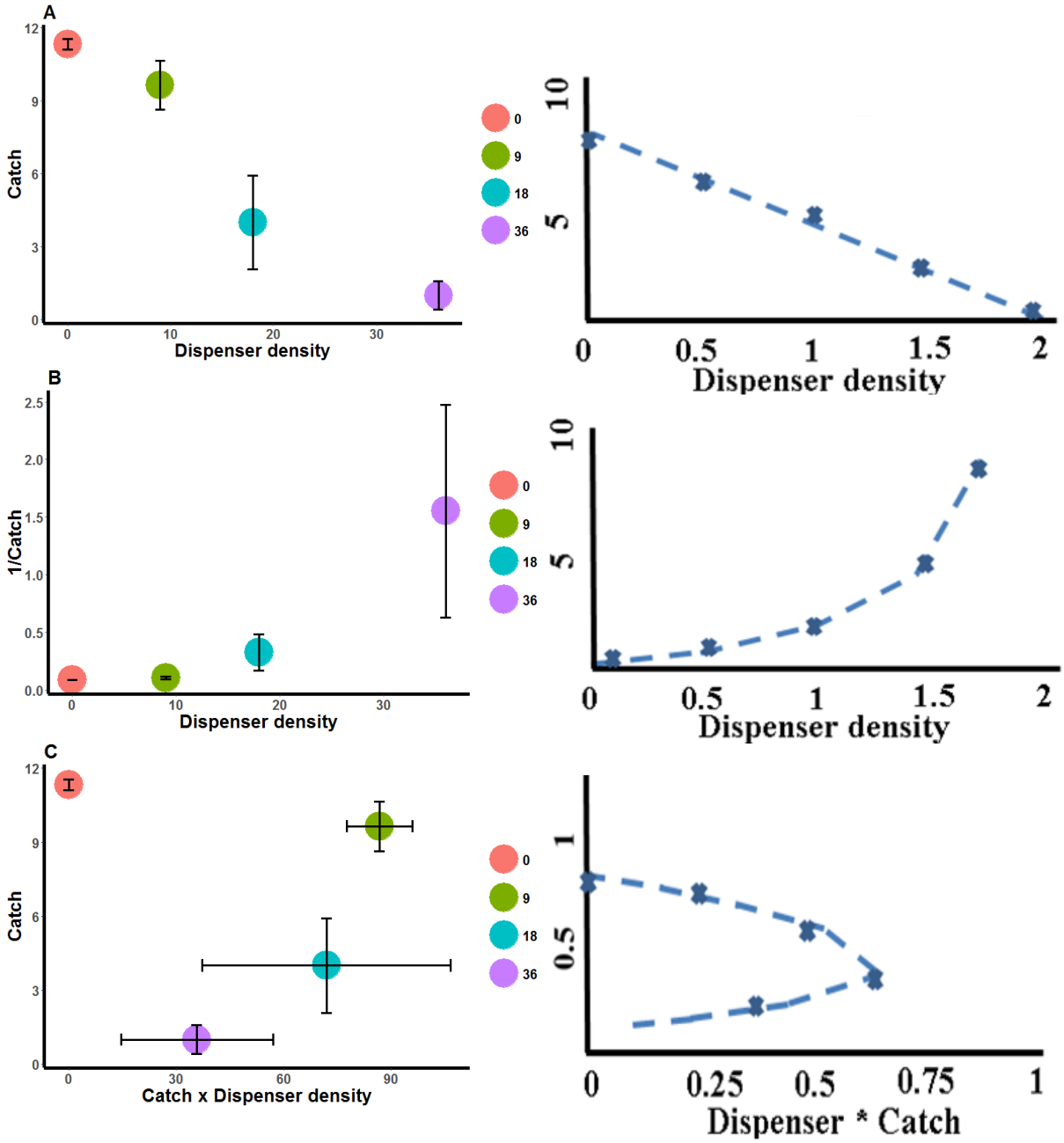

Fig. 5.8. The catch of *Thaumatotibia leucotreta* from the density response experiments in table grapes, A) Untransformed plot, B) Miller-Gut plot, C) Miller-de Lame plot. Different colours represent number of Isomate™ dispensers/ha. The error bars indicate the standard error of the mean. The plots in blue on the right represent the theoretical profiles for non-competitive disruption (adapted from Miller and Gut, 2015).

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5.4 Discussion

This study provides novel insights into the behavioural mechanisms of FCM under mating disruption. FCM's disruption profile (Fig. 5.6C) closely follows the predictions of the hybrid disruption profile (Fig. 6C of Miller and Gut, 2015), suggesting that FCM is likely disrupted competitively at low doses and non-competitively at high doses. Miller and Gut (2015) suggested that the hybrid profile may be as a result of some males being disrupted non-competitively and others impacted competitively, due to poor coverage of the pheromone. If this was true, the uniform distribution of the pheromone would be important to cause the shift to non-competitive disruption. However, from our density response trial, in which the pheromone was not uniformly distributed but rather released at few pheromone sites, it is clear that FCM was disrupted non-competitively. A similar result was found by Reinke et al. (2014) that showed it was possible to shift Oriental fruit moth disruption to a non-competitive response at higher dosages. In contrast, codling moth remains competitively disrupted even under very high dosages (Miller et al., 2010; McGhee et al., 2014). This indicates that even closely related species may respond differently in various scenarios to the addition of pheromone (Miller and Gut, 2015). In this study, FCM disruption shifted from competitive disruption to non-competitive disruption at 800 dispensers per hectare. This is a promising result as the ability to disrupt moths non-competitively has been suggested to contribute to of the efficiency of controlling the pest (Gut et al., 2004, Reinke et al., 2014; Miller and Gut, 2015) as disruption is not affected by the asymptotic effect that is prevalent with competitive disruption (Miller et al., 2006b; McGhee et al., 2014).

Mating disruption proved to be very effective (up to 99%) against FCM and confirms our findings from Chapter 4. Interestingly, the realized disruption remained constant from 200 dispensers/ha to 600/ha and only substantially improved at 800/ha. In fact, the disruption shown in Chapter 4 (86% stone fruit, 93% table grapes), was improved dramatically (+ 10% stone fruit and + 6% table grapes) by increasing the number of dispensers to 800/ha. This extra increase in disruption is suggested to be as a result of disruption shifting from competitive to non-competitive disruption.

The optimal dosage (800 dispensers per ha or their equivalent release rate) found in this study, has a two-fold result and the best option will depend on the practitioner. The first option is to increase the dosage, this will have a synergistic effect, causing a significant increase in disruption and additionally it will cause a shift to non-competitive disruption making it effective even under very high pest densities (Miller and Gut, 2015). The synergistic increase in

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disruption seen here may be as a result of altering calling behaviour of the female (Stelinksi et al., 2014) or delayed mating (Gerken and Campbel, 2018) that both occur under non-competitive disruption. The delayed mating decreases mating success thereby reducing fecundity causing a steady decline of the reproductive performance of the pest (Amaoh et al., 2018) and should therefore be explored further for FCM. However, if the practitioner does not deem the extra disruption worthwhile for the additional cost, it would be better to reduce the dosage, to 200 - 400 dispensers per ha as the disruption with these dosages does not differ from the current recommended (600/ha) dosage although new registration would be required. Another option to offset the cost of the additional dosage is by changing the method of application to include intentional gaps and have fewer pheromone sites (Lapointe et al., 2014).

Optimizing mating disruption by the reduction of pheromone sources or pheromone sites without the loss of disruption may prove to be difficult, especially with pests disrupted competitively (Witzgall et al., 2010). The reduction of pheromone sites by clustering pheromone dispensers simultaneously decreases the number of pheromone sites and increases the release rate per site (Miller et al., 2006b). Therefore, even though each pheromone site is causing a proportionally higher disruption, it may lead to reduced overall disruption. For instance, Suckling and Angerelli (1996), targeting light brown apple moth (*Epiphyas postvittana*), which has been shown to be disrupted competitively (Miller et al., 2006b), held 200 dispensers per ha constant, whilst varying number (0, 2, 18, 200) of clusters and reported 10% less disruption with 18 clusters opposed to 200 uniform dispensers, significantly reducing the overall treatment potency. However, here it was shown that disruption of FCM remained high (91%) with as few as 36 pheromone sites/ha and caused similar disruption as 800 uniformly distributed pheromone dispensers. I suggest that when the pest is disrupted non-competitively the number of pheromone sites may be reduced without the loss of disruption, as the dispensers do not compete with females for male visitations. Furthermore, I suggest that the potency of the overall treatment seen in the present study illustrates that incorporating intentional gaps (Tcheslavskaia et al., 2005), is likely to be effective against FCM.

Mating disruption is highly effective against FCM in stone fruit and table grapes. It is suggested that the release rate of the dispensers is measured using gas chromatography and that the equivalent active ingredients released by 800 dispensers per hour is calculated so that the exact amount of active ingredient required in the system to shift FCM to non-competitive disruption can be determined. Therefore, it is suggested that the active ingredients released per hour equivalent to 800 dispensers per hectare should be used against FCM and that with as few as

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36 pheromone sites/ha disruption will remain effective. Furthermore, due to FCM being disrupted non-competitively at higher pheromone dosages, it will remain an effective control strategy even with high pest densities. The adoption of this technique into the current integrated pest management programme is highly recommended.

5.5 References

Allison, J.D., Cardé, R.T., 2016. Pheromone communication in moths: evolution, behavior, and application. Univ of California Press.

Amoah, B.A., Mahroof, R.M., Gerken, A.R., Campbell, J.F., 2018. Effect of delayed mating on longevity and reproductive performance of *Lasioderma serricorne* (Coleoptera: Anobiidae). J. Econ. Entomol. doi: 10.1093/jee/toy336

Cardé, R.T., Minks, A.K., 1995. Control of moth pests by mating disruption: successes and constraints. Annu. Rev. Entomol. 40, 559-585.

Cardé, R.T., Staten, R.T., Mafra-Neto, A., 1998. Behaviour of pink bollworm males near high-dose, point sources of pheromone in field wind tunnels: insights into mechanisms of mating disruption. Entomol. Exp. Appl. 89, 35-46.

Evenden, M., 2016. Mating disruption of moth pests in integrated pest management a mechanistic approach. In: Allison, J.D., Cardé, R.T. (eds). Pheromone Communication in Moths: Evolution, Behavior, and Application. Univ of California Press, Oakland, CA, pp. 365-393.

Gerken, A.R., Campbell, J.F., 2018. Life history changes in *Trogoderma variabile* and *T. inclusum* due to mating delay with implications for mating disruption as a management tactic. Ecol. Evol. 8, 2428-2439.

Gut, L., Stelinski, L.L., Thomson, D.R., Miller, J.R., 2004. Behaviourmodifying chemicals: prospects and constraints in IPM. In: Integrated Pest Management: Potential, Constraints and Challenges (ed. by Koul, O., Dhaliwal, D.S., Cuperus, G.W.). CABI Publishing, Cambridge, MA, USA, pp. 73-122.

Hofmeyr, J.H., Burger, B.V., Calitz, F.J., 1991. Disruption of male orientation to female false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae), using synthetic sex pheromone. Phytophylactica 23, 153-156.

Stellenbosch University https://scholar.sun.ac.za

Judd, G.J.R., Gardiner, M.G.T., Thomson, D.R., 1997. Control of codling moth in organically-managed apple orchards by combining pheromone-mediated mating disruption, post-harvest fruit removal and tree banding. Entomol. Exp. Appl . 83, 137–146.

Lapointe, S.L., Stelinski, L.L., 2011. An applicator for high viscosity semiochemical products and intentional treatment gaps for mating disruption of *Phyllocnistis citrella*. Entomol. Exp. Appl. 141, 145-153.

Lapointe, S.L., Stelinski, L.L., Keathley, C.P., Mafra-Neto, A., 2014. Intentional coverage gaps reduce cost of mating disruption for *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in citrus. J. Econ. Entomol. 107, 718-726.

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

McCormick, A.L.C., Karlsson, M., Ochoa, C.F.B., Proffit, M., Bengtsson, M., Zuluaga, M.V., Fukumoto, T., Oehlschlager, C., Prado, A.M.C., Witzgall, P., 2012. Mating disruption of Guatemalan potato moth *Tecia solanivora* by attractive and non-attractive pheromone blends. J. Chem. Ecol. 38, 63-70.

McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Aerosol emitters disrupt codling moth, *Cydia pomonella*, competitively. Pest Manag. Sci. 70, 1859-1862.

Miller, J.R., Gut, L.J., 2015. Mating disruption for the 21st century: matching technology with mechanism. Environ. Entomol. 44, 427-453.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. J. Chem. Ecol. 32, 2089-2114.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): case studies. J. Chem. Ecol. 32, 2115-2143.

Miller, J.R., McGhee, P.S., Siegert, P.Y., Adams, C.G., Huang, J., Grieshop, M.J., Gut, L.J., 2010. General principles of attraction and competitive attraction as revealed by large-cage studies of moths responding to sex pheromone. PNAS 107, 22-27.

Stellenbosch University https://scholar.sun.ac.za

Mori, B.A., Evenden, M.L., 2013. When mating disruption does not disrupt mating: fitness consequences of delayed mating in moths. Entomol. Exp. Appl. 146, 50-65.

Reinke, M.D., Siegert, P.Y., McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Pheromone release rate determines whether sexual communication of Oriental fruit moth is disrupted competitively vs. non-competitively. Entomol. Exp. Appl. 150, 1-6.

Stelinski, L.L., Miller, J.R., Rogers, M.E., 2008. Mating disruption of citrus leafminer mediated by a noncompetitive mechanism at a remarkably low pheromone release rate. J. Chem. Ecol. 34, 1107-1113.

Stelinski, L., Holdcraft, R., Rodriguez-Saona, C., 2014. Female moth calling and flight behavior are altered hours following pheromone autodetection: possible implications for practical management with mating disruption. Insects 5, 459-473.

Sievert, C., 2018. plotly for R. https://plotly-book.cpsievert.me

Suckling, D.M., 2015. Can we replace toxicants, achieve biosecurity, and generate market position with semiochemicals? Front. Ecol. Evol. 3, 1-17.

Suckling, D.M., Angerilli, N.P.D., 1996. Point source distribution affects pheromone spike frequency and communication disruption of *Epiphyas postvittana* (Lepidoptera: Tortricidae). Environ. Entomol. 25, 101-108.

Tcheslavskaia, K., Brewster, C., Thorpe, K., Sharov, A., Leonard, D., Roberts, A., 2005. Effects of intentional gaps in spray coverage on the efficacy of gypsy moth mating disruption. J. Appl. Entomol. 129, 475-480.

Terblanche, J.S., de Jager, Z., Boardman, L., Addison, P., 2014. Physiological traits suggest limited diapause response in false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae). J. Appl. Entomol. 138, 683-691.

Witzgall, P., Stelinski, L., Gut, L., Thomson, D., 2008. Codling moth management and chemical ecology. Annu. Rev. Entomol. 53, 503-522.

Witzgall, P., Kirsch, P., Cork, A., 2010. Sex pheromones and their impact on pest management. J. Chem. Ecol. 36, 80-100.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Stellenbosch University https://scholar.sun.ac.za

Chapter 6:
General Discussion

Integrated pest management (IPM) was born from the knowledge that no "silver bullets" exist, therefore this management method strives to better serve the long term by utilising multiple techniques and to tailor the control to specific species. False codling moth (FCM), *Thaumatotibia* (= *Cryptophlebia*) *leucotreta* (Meyrick) was first reported in citrus by Fuller (1901), since then much research has been conducted on its biology, ecology and control. Despite this, FCM remains a priority pest in South Africa causing direct crop damage and posing phytosanitary restrictions. FCM's persistence in the agricultural system is likely due to its highly polyphagous nature (over 70 hosts), rapid life cycle and cryptic immature stages (Chapter 1). This coupled with the mobility of adults could allow FCM to utilise many different niches, making the choice of an appropriate control technique difficult (Chapter 1).

Understanding the ecology and the life cycle of the target insect pest is of utmost importance for the successful implementation of IPM (Botrell and Smith, 1982). This information can be used to determine the best stage of the life cycle to target and then investigate the potency of various control techniques to attack that stage. Chapter 1 suggested that the use of entomopathogens be investigated against the immature stage of FCM, whilst the addition of synthetic female pheromone could be used to disrupt males' mate-finding behaviour and thus limit the number of individuals in subsequent generations.

6.1 Synopsis of Findings

To realise the full potential entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) against FCM it is important to determine what local species are present in our agricultural soils. The use of a local species as a biopesticide would be advantageous, due to their better performance at warm temperatures and as they can be used without introducing exotic species into our soils that may have other negative impacts (Malan et al., 2018). Another advantage of a local species is that they have the proven ability to survive under conventional management techniques; in fact more than a third of the areas surveyed in this study tested positive for entomopathogens. Four different EPN species were isolated, cultured and identified from the soil survey, which included the first South African detection of *Oscheius microvilli*, the second

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report of *Heterorhabditis indica*, while other species isolated include *H. zealandica* and *H. bacteriophora*. Several EPF strains were also recovered from the soil survey, which included *Metarhizium anisopliae* and *Beauveria bassiana*.

The species recovered from the local soils proved to be virulent against the immature stages of FCM. This study is the first to report the susceptibility of FCM eggs to EPNs, this novel finding may have potential as a control option. Firstly, reduction of new entries into the population will reduce the speed with which the population grows (Simberloff, 2009) and secondly as the larvae are killed before they leave the egg, therefore no damage will be made to the fruit ensuring they remain viable for marketing and export. Above ground application of EPNs may prove difficult, though the use of adjuvants do help limit desiccation and may improve the performance of the EPNs (Shapiro-Ilan et al., 2010; Platt, 2019). To reduce the desiccation stress, another option could be to use symbiotic bacteria or supernatants (also virulent against the eggs, Chapter 2) of the EPNs as a post-harvest control, this will further reduce the chance of larvae boring into the fruit once packed and becoming phytosanitary concerns.

The below ground stages (larvae and pupae) were both susceptible to EPNs, however the EPNs caused only low mortality of the pupae and other techniques should be investigated against this stage. A possible solution could be to use EPNs in combination with EPF to control the pupae, this combination has been shown to work well (additive and synergistic effect) against other pests as the entomopathogens have similar ecological niches and modes of action (Batalla-Carrera et al., 2013; Bueno-Pallero et al., 2018). Both the EPNs and EPF strains were tested against the larval stage of FCM and proved highly virulent. EPNs were especially potent with *Steinernema yirgalemense* repeatedly causing 100% mortality in all laboratory trials and was therefore selected for further testing in the field to fully understand its benefit as a part of an IPM programme.

The use of EPNs for field trials and commercial application requires large quantities of nematodes. In this study EPNs, currently cultured *in vitro* by Stellenbosch University were used, however, before field trials could commence the virulence of *in vitro* cultured EPNs were assessed. As *in vitro* cultured EPNs have not been tested against FCM before, comparisons were made between *in vitro* and *in vivo* cultures. It was expected that the *in vitro* virulence would be lower than *in vivo* cultured EPNs as *in vivo* more closely resembles the natural process and has been shown to produce higher quality nematodes (Shapiro-Ilan and Gaugler, 2002; Ferreira et al., 2014). However, the laboratory tests revealed that the EPNs cultured *in vitro* for the field trials had similar virulence to the *in vivo* cultured EPNs. This novel finding is likely

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due to the high quality of the symbiotic bacteria that are cultured for the *in vitro* EPNs ensuring the EPNs are in optimal condition (Dunn et al., 2018).

The field trials mirrored the laboratory results in providing similar control of FCM to previous field trials that used *in vivo* cultured EPNs (Malan and Moore, 2016) and caused mortality of more than two thirds of the FCM larvae after 48 h. The EPNs also managed to persist (as expected) and provided control for longer than four weeks. This was two weeks longer than reported by previous studies. The potency of the EPNs was further realised by the low concentration 20 IJs/cm^2 remaining effective against the larvae. As shown in the laboratory in Chapter 2, *S. yirgalemense* proved to be the most effective species against the larvae in the field, however, this only became evident three weeks after the EPN was applied. In this study *S. yirgalemense* is thought to have persisted longer due to its inactivity (as observed when stored) allowing to retain its virulence longer. This highlights the importance of testing not only the immediate effect, but also the persistence of the biological agent as differences may be seen at different time points. The promising results shown in this study of the entomopathogens, especially the EPNs against the immature stages of FCM, encourages the use of the biologicals as part of the IPM system.

The potential of mating disruption was explored against FCM to reduce population growth (viable eggs), by limiting reproductive events. However, though mating disruption has been proven to be an effective and sustainable method worldwide (Miller and Gut, 2015; Suckling, 2015), limited work has been conducted in South Africa especially against indigenous pests (cf. Hofmeyr et al., 1991). It was therefore prudent to first establish and evaluate an appropriate experimental design that would lead to reliable results. In Chapter 4, several features of the experimental design proved to be beneficial. Firstly, the nine trap layout in the trapping grid which ensured a higher percentage of recaptured moths than in previous studies on FCM (Visser et al., 2015) and other Tortricidae such as codling moth, (McGhee et al., 2014). Secondly, the four release points ensured the moths were equally distributed throughout the experimental block. Lastly, the inclusion of the paired control allowed only one variable to be changed at a time (pheromone present or not) and facilitates relevant comparisons between the different treatments. Using the evaluated experimental design, this study provides the first evidence that mating disruption does disorientate (80 – 90% trap reduction) FCM males in both stone fruit and table grapes. However, the mating disruption calculated in this study was lower than achieved in previous studies against other Tortricidae (Stelinski et al., 2008; Miller et al., 2010; Reinke et al., 2014). This highlighted that mating disruption can be improved against FCM.

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To improve mating disruption against FCM several factors were investigated, firstly chapter 4 had shown that mating disruption does disorientate male FCM, although how FCM males were disrupted remained unclear. With the use of the quantitative tools proposed and evaluated by Miller et al. (2006a, b), Chapter 5 proved that FCM is disrupted competitively at low dosages, however if the dosage is increased to the equivalent of 800 dispensers of active ingredient (AI) per hectare (AI) it shifts to non-competitive disruption. The shift to non-competitive disruption is still evident at 800 dispensers/ha even when as few as 36 pheromone release sites were used. The shift to non-competitive disruption with the higher dosage, caused the mating disruption to increase to 96% in stone fruit and 99% in table grapes. This is similar to the disruption that is achieved against the Oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera:Tortricidae) that is considered a good target for mating disruption (Reinke et al., 2014; Miller and Gut, 2015), especially with high releasing pheromone dispensers.

Though the benefit of increasing the dosage is clear, the additional cost will probably not appeal to practitioners. However, these additional costs may be offset by how the pheromone is applied (as discussed in Chapter 5). For instance, labour costs may be saved by reducing the pheromone release sites to 36 instead of the 800 per hectare. Another option is to use the FCM hybrid disruption profile to our advantage, by keeping dosages low, FCM will be disrupted competitively (i.e. success density dependent) and then use other techniques to increase the pheromone in the system. A recent approach is the use of mass reared insects to increase the number of point sources in the system (Suckling et al., 2011), in the case of FCM two options can be utilised to achieve this. Firstly, sterile FCM females may be released into the landscape that can serve as mobile pheromone dispensers that may lead to a synergistic relationship between mating disruption and the sterile insect technique. A second novel technique that has been tested by Suckling et al. (2011) and shows great (82 - 95 % disruption, but only lasts 4 days) promise is impregnating mass reared sterile fruit flies with the moth pheromone and releasing them into the landscape, this technique has the advantage of being even more environmentally friendly and sustainable (no use of plastic dispensers) and targeting two pests simultaneously. Lastly, the high mating disruption with the few pheromone sites shown in this study, suggests that new technology such as aerosol emitters (also referred to as “puffers”) could prove very effective against FCM. These aerosol emitters or “puffers” can be programmed to dispense the pheromone only whilst moths are calling (reducing waste of pheromone release in the day by conventional dispensers) and at a high release rate (McGhee et al., 2014). An additional benefit of this technology is that the stocking density is very low (5 - 20/ha) which is a valuable attribute when using the control over large areas or even for area

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wide control. Unfortunately, this technology is not yet commercially available in South Africa for FCM, but the evidence from this study may encourage the product to become commercially available in future.

6.2 Future directions

The benefit of IPM to manage insecticide resistance should be explored against FCM. Insects may become resistant to insecticides due to individual tolerances, repeated use of a single approach or the sub-lethal application of the insecticide. However, by including alternate controls such as entomopathogens or mating disruption (Caprio and Suckling, 1995), the tolerant insects are, as likely to be removed from the population as the less tolerant individuals, thereby increasing the time to resistance build-up. In fact, the addition of mating disruption may even exclude the resistant individuals entirely from the gene pool, as resistance to insecticides has been shown to decrease calling ability in Lepidoptera (McNeil, 1991). Suggesting that insecticides and mating disruption may have a synergistic relationship, and should be further investigated for FCM.

The ultimate goal of mating disruption is the reduction of crop damage; therefore, it is suggested that the dosages shown here to disorientate FCM males are tested under natural conditions. These experiments should incorporate damage assessments and some measurement of female emigration so that mating disruption can be measured directly and so further improve our understanding of mating disruption in commercial orchards.

Now that it has been shown that FCM has a hybrid disruption profile this information can be used to our advantage, for instance, non-competitive disruption remains effective even under very high pest densities (99% mating disruption, 400 males/ha, Chapter 5). However, though FCM was shown to be disrupted non-competitively at higher doses, the exact way in which this may manifest is not yet clear and should be studied in future. Previous studies on other lepidopterans found evidence for; sensory imbalance (desensitization or sensory blockage) (Cardé and Minks, 1995), shifted activity period (Mori and Eveden, 2013; Gerken and Campbell, 2018) and suppressed calling (Miller and Gut, 2015). The results found in this study will provide future researchers with a solid foundation from which to start their investigations.

The potential of FCM to move in and out of different crops (as discussed in Chapter 1), may reduce the realised benefit of mating disruption, as previously mated females may enter the orchard/vineyard and deposit viable eggs on the fruit. This will result in damage still occurring

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even though mating disruption is working optimally. To combat this many studies advocate the use of mating disruption only over large areas, however, another option is by treating the edge of the orchard/vineyard with a repellent. It is suggested that moth repellents synthesised from plant volatiles should be identified, as has been done for other Lepidoptera (see De Moraes et al., 2001), and tested by qualitative studies in semi-field and field environments. If compounds exist to repel FCM females it is likely that their use, along with the use of the control techniques evaluated in this study, should entirely exclude the pest from agricultural crops.

6.3 Concluding remarks

A challenge for the effective control of insect pests is to tailor the IPM programme to suit the specific insect. Investigating which control techniques are suitable or not for specific life stages is an integral part of the problem. From this study, valuable knowledge on the susceptibility of the immature life stages of FCM to entomopathogens (Chapters 2 and 3) has been gained, and showed that mating disruption is a valuable control option (Chapters 4 and 5) to disrupt the mate finding ability of FCM males. It is clear from this study that with the integration of these techniques FCM can be effectively controlled at each stage of the life cycle, ensuring low population growth that may lead to the successful suppression of this infamous pest.

6.4 References

Batalla-Carrera, L., Morton, A., Santamaria, S., García-del-Pino, F., 2013. Isolation and virulence of entomopathogenic fungi against larvae of hazelnut weevil *Curculio nucum* (Coleoptera, Curculionidae) and the effects of combining *Metarhizium anisopliae* with entomopathogenic nematodes in the laboratory. Biocontrol Sci. Techn 23, 101-125.

Bottrell, D.G., Smith, R.F., 1982. Integrated pest management. Environ. Sci. Technol. 16, 282-288.

Bueno-Pallero, F.Á., Blanco-Pérez, R., Dionísio, L., Campos-Herrera, R., 2018. Simultaneous exposure of nematophagous fungi, entomopathogenic nematodes and entomopathogenic fungi can modulate belowground insect pest control. J. Invertebr. Pathol. 154, 85-94.

Caprio, M.A., Suckling, D.M., 1995. Mating disruption reduces the risk of resistance development to transgenic apple orchards: simulations of the lightbrown apple moth. In: Proceedings of the Forty Eighth New Zealand Plant Protection Conference, Angus Inn, Hastings, New Zealand, pp. 52-58.

Stellenbosch University https://scholar.sun.ac.za

Cardé, R.T., Minks, A.K., 1995. Control of moth pests by mating disruption: successes and constraints. Annu. Rev. Entomol. 40, 559-585.

De Moraes, C.M., Mescher, M.C., Tumlinson, J.H., 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature, 410, 577.

Dunn, M.D., Malan, A.P., 2018. Production optimization of a novel entomopathogenic nematode, *Steinernema jeffreyense*, in shake flasks. Appl. Microbiol. Tech. (Submitted).

Ferreira, T., Addison. M.F., Malan, A.P., 2014. In vitro liquid culture of a South African isolate of *Heterorhabditis zealandica* for the control of insect pests. African Entomol. 22, 80-92.

Fuller, C., 1901. The Natal codling moth, *Carpocapsa* sp. First report of the Government Entomologist. Natal Depart. Agri. Rep. 48-51.

Gerken, A.R., Campbell, J.F., 2018. Life history changes in *Trogoderma variabile* and *T. inclusum* due to mating delay with implications for mating disruption as a management tactic. Ecol. Evol. 8, 2428-2439.

Hofmeyr, J.H., Burger, B.V., Calitz, F.J., 1991. Disruption of male orientation to female false codling moth, *Cryptophlebia leucotreta* (Lepidoptera: Tortricidae), using synthetic sex pheromone. Phytophylactica 23, 153-156.

Malan, A.P., Moore, S.D., 2016. Evaluation of local entomopathogenic nematodes for the control of false codling moth, *Thaumatotibia leucotreta* (Meyrick, 1913), in a citrus orchard in South Africa. Afr. Entomol. 24, 489-501. https://doi.org/10.4001/003.024.0489

Malan, A.P., von Diest, J.I., Moore, S.D., Addison, P., 2018. Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. African Entomol. 26, 14-29.

McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Aerosol emitters disrupt codling moth, *Cydia pomonella*, competitively. Pest Manag. Sci. 70, 1859-1862.

McNeil, J.N., 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. Ann. Rev. Entomol. 36, 407-430.

Miller, J.R., Gut, L.J., 2015. Mating disruption for the 21st century: matching technology with mechanism. Environ. Entomol. 44, 427-453.

Stellenbosch University https://scholar.sun.ac.za

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. J. Chem. Ecol. 32, 2089-2114.

Miller, J.R., Gut, L.J., De Lame, F.M., Stelinski, L.L., 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): case studies. J. Chem. Ecol. 32, 2115-2143.

Miller, J.R., McGhee, P.S., Siegert, P.Y., Adams, C.G., Huang, J., Grieshop, M.J., Gut, L.J., 2010. General principles of attraction and competitive attraction as revealed by large-cage studies of moths responding to sex pheromone. PNAS 107, 22-27.

Mori, B.A., Evenden, M.L., 2013. When mating disruption does not disrupt mating: fitness consequences of delayed mating in moths. Entomol. Exp. Appl. 146, 50-65.

Platt, T., Stokwe, N.F., Malan, A.P., 2019. Foliar application of *Steinernema yirgalemense* to control *Planococcus ficus*: assessing adjuvants to improve efficacy. S. Afr. J. Enol. Vitic. 40. doi: http://dx.doi.org/10.21548/40-1-2920

Reinke, M.D., Siegert, P.Y., McGhee, P.S., Gut, L.J., Miller, J.R., 2014. Pheromone release rate determines whether sexual communication of Oriental fruit moth is disrupted competitively vs. non-competitively. Entomol. Exp. Appl. 150, 1-6.

Shapiro-Ilan, D.I., Cottrell, T.E., Mizell III, R.F., Horton, D.L., Behle, R.W., Dunlap, C.A., 2010. Efficacy of *Steinernema carpocapsae* for control of the lesser peachtree borer, *Synanthedon pictipes*: Improved aboveground suppression with a novel gel application. Biol. Control 54, 23-28.

Shapiro-Ilan, D.I., Gaugler, R., 2002. Production technology for entomopathogenic nematodes and their bacterial symbionts. J Ind. Microbiol. Biotechnol. 28, 137-146.

Simberloff, D., 2009. The role of propagule pressure in biological invasions. Annu. Rev. Ecol. Evol. Syst. 40, 81-102.

Stelinski, L.L., Miller, J.R., Rogers, M.E., 2008. Mating disruption of citrus leafminer mediated by a noncompetitive mechanism at a remarkably low pheromone release rate. J. Chem. Ecol. 34, 1107-1113.

Suckling, D.M., 2015. Can we replace toxicants, achieve biosecurity, and generate market position with semiochemicals? Front. Ecol. Evol. 3, 1-17.

Stellenbosch University https://scholar.sun.ac.za

Suckling, D.M., Woods, B., Mitchell, V.J., Twidle, A., Lacey, I., Jang, E.B., Wallace, A.R., 2011. Mobile mating disruption of light-brown apple moths using pheromone-treated sterile Mediterranean fruit flies. Pest Manage. Sci. 67, 1004-1014.

Visser, C.N., 2015. Determining the quality of mass reared male codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), by assessing flight performance under laboratory, semi-field and field conditions. (M.Sc., Stellenbosch University).