Thaumatotibia leucotreta (Meyrick) (Lepidoptera: Tortricidae) population ecology in citrus orchards: the influence of orchard age

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ABSTRACT

Anecdotal reports in the South African citrus industry claim higher populations of false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae), in orchards during the first three to five harvesting years of citrus planted in virgin soil, after which, FCM numbers seem to decrease and remain consistent. Various laboratory studies and field surveys were conducted to determine if, and why juvenile orchards (four to eight years old) experience higher FCM infestation than mature orchards (nine years and older). In laboratory trials, Washington Navel oranges and Nova Mandarins from juvenile trees were shown to be significantly more susceptible to FCM damage and significantly more attractive for oviposition in both choice and no-choice trials, than fruit from mature trees. Although fruit from juvenile Cambria Navel trees were significantly more attractive than mature orchards for oviposition, they were not more susceptible to FCM damage. In contrast, fruit from juvenile and mature Midnight Valencia orchards were equally attractive for oviposition, but fruit from juvenile trees were significantly more susceptible to FCM damage than fruit from mature trees. Artificial diets were augmented with powder from fruit from juvenile or mature Washington Navel orchards at 5%, 10%, 15% or 30%. Higher larval survival of 76%, 63%, 50% and 34%, respectively, was recorded on diets containing fruit powder from the juvenile trees than on diets containing fruit powder from the mature trees, at 69%, 57%, 44% and 27% larval survival, respectively. Bioassays were conducted to determine if differences in plant chemistry between fruit from juvenile and mature trees will have an impact on the susceptibility FCM to entomopathogenic nematodes (EPN), entomopathogenic fungi (EPF) and Cryptophlebia leucotreta granulovirus (CrleGV). No significant differences in the susceptibility of larvae reared on diets containing 15% fruit powder from juvenile and mature trees to EPN and EPF were recorded. Mortality of neonate larvae was significantly lower when placed on diets containing 15% fruit powder from mature trees (45% mortality) than diets containing 15% fruit powder from juvenile trees (61% mortality), after larvae ingested the lowest virus concentration tested, being 2 $x10^4$ OBs/ml. Data collected from field surveys showed significantly lower egg parasitism, virus infection of larvae and EPF occurrence in juvenile orchards than mature orchards. Egg parasitism was between 11% and 54% higher in mature orchards than juvenile orchards, with the exception of Mandarins during 2015, where egg parasitism was slightly higher in juvenile orchards, but not significantly so. A significantly higher proportion of larvae retrieved from mature

orchards (7% of larvae) were infected with CrleGV than larvae retrieved from juvenile orchards (4% of larvae). A significantly higher occurrence of EPF was recorded in non-bearing and mature orchards, with 40% and 37% occurrence respectively, than in juvenile orchards, with 25% occurrence recorded. EPF occurrence in juvenile orchards increased significantly by 16% to 32% from the first to the third year of sampling. In contrast to results recorded in laboratory trials, similar or higher pest pressure in juvenile orchards than mature orchards did not always result in significantly higher levels of FCM damage under field conditions. FCM damage in juvenile orchards may have been lower than expected, as greater extremes of temperature and lower humidity were recorded in juvenile orchards, which would increase larval mortality. Results of this study showed that juvenile and mature orchards are significantly different and should be managed differently.

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CHAPTER 1

General Introduction

1.1 Introduction

Landscape complexity improves plant and animal species richness (Atauri & De Lucio 2001, Moser *et al.* 2002), while the decrease of natural diversity in monocultures is closely linked to pest outbreaks (Altieri 1994). Natural enemy populations in monocultures become supressed because of the reduced availability of alternate hosts (Samways 2005). Natural enemies are also known to be more sensitive to pesticide applications than some pest species as they are less cryptic and more mobile (Samways 2005). Anecdotal reports in the citrus industry have observed higher populations of false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) during the first three to five harvesting years of citrus planted in virgin soil, after which, FCM numbers seem to decrease and remain consistent (D. Gerber, pers. comm.). This population increase of FCM in young citrus orchards is in contrast to what has been observed for codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) populations in young apple orchards. Codling moth population increase in young apple orchards is severely limited by a lack of protective pupation sites (Wearing & Skilling 1975), which is not the case for FCM, as larvae pupate in the soil and only in extremely rare cases in fruit (Newton 1998).

Since the first citrus tree was introduced to South Africa in the 1650s from St Helena (CGA Annual Report 2007), the citrus industry has grown extensively with approximately 21 million trees planted over approximately 64 510 ha of land (Bedford 1998, CGA Key Industry Statistics 2015). Fuller was the first to describe FCM as a citrus pest in 1901, after discovering infested citrus fruit in the Kwa-Zulu Natal area (Schwartz 1981, Newton 1998). Since being described, FCM has spread to all seven citrus growing regions (Western Cape, Eastern Cape, Northers Cape, KwaZulu-natal, North-West, Limpopo and Mpumalanga) in South Africa and is

considered one of the most damaging and economically important citrus pests in South Africa (Moore 2002). Surveys conducted by Newton *et al*. (1986), showed that FCM was responsible for 20% to 30% of fruit drop in the Nelspruit and Rustenburg areas and responsible for up to 90% of fruit drop in the Citrusdal area. Moore (2002) reported that crop loss due to FCM equated to more than ZAR 100 million annually to the South African Citrus industry. However, more recent reports show that FCM control has since improved considerably due to the diligent use of a range of available integrated control options (Moore & Hattingh 2012, Barnes *et al*. 2015, Moore *et al*. 2015). When managed and integrated correctly the control options currently available have shown the ability to reduce FCM infestation by 97% or more (Moore *et al.* 2015).

Problem Statement

The influence of orchard age on not only FCM, but crop pests in general has not been determined. Since the microclimate, tree structure, management systems and soil quality change as an orchard ages, the ecological processes within the system also change. This body of work will give insight into what ecological changes are occurring. The knowledge gained will then be utilised to assess if any changes in pest management are required for orchards of different ages.

1.2 Insect Pest Ecology

Insects play a key role in ecosystems and relative to their abundance, the impact they have on the ecosystem is disproportionately large (Power *et al*. 1996). Insect herbivores play an essential role in the energy flow of natural food webs, as energy obtained from primary producers is assimilated more efficiently by insect herbivores than vertebrates and consequently supply more energy to their natural enemies (Price 1984). MacFadyen (1957) describes the concept of a niche as “that set of ecological conditions under which a species can exploit a source of energy effectively enough to be able to reproduce and colonise further in such set

conditions". The success of a certain insect pest species will depend greatly on its ability to fully exploit their niche (Root 1967). Each individual species will display its own unique niche exploitation pattern which explains its breeding ability, abundance and distribution of the species within these limits (Price 1984). The niche exploitation pattern will also depend greatly on the relative stability and sustainability of the environment (Root 1967). The stability of an agro-ecosystem depends not only on trophic level diversity, but also on the density-dependent nature of each trophic level (Southwood & Way 1970).

Biodiversity provides critical ecosystem services in agricultural systems (Altieri 1999). Modern agriculture is responsible for the simplification of landscapes by aggregation and enlargement of fields (Altieri *et al.* 2009). Agro-ecosystems lack the capacity to uphold pest regulation and soil fertility when deprived of basic functional and regulating components (Altieri 1999). Species richness is affected by the structure, configuration and composition of different landscape elements (Weibull *et al.* 2003). Specialised insect herbivore species tend to show higher abundance in monocultures when compared to polycultures (Altieri 1999). Plant diversity disrupts the orientation of specialist herbivores to their preferred host (Horn 2000). In contrast, monocultures cause uniformity in vegetation (Strong *et al.* 1984) and consequently, the niche area available to an individual insect species adapted to those specific environmental conditions becomes greater, allowing species population levels to explode (Dent 2000). Intra- and interspecific competition is experienced after much higher population densities in monocultures than in polycultures (Van Emden 1965). Monocultures are also unfavourable environments for natural enemies as resources critical for sustaining the various life stages such as alternative prey hosts, refuge and breeding sites, pollen and nectar are limited (Altieri *et al.* 2009). The lack of predation by natural enemies contributes considerably to higher insect pest population levels (Weiss 2002). Insect pest population dynamics can be complex and are often not fully understood (Root 1967). The ability of an insect species to develop into a pest in certain areas will depend on a variety of biotic and abiotic factors (Luckmann & Metcalf 1994).

Agricultural systems can be compared to an early-successional habitat in which the continued coexistence of herbivore, natural enemy and plant is disrupted

(Price *et al.* 1980). Semi-permanent ecosystems such as citrus orchards are characterized by higher structural diversity and suffer fewer disturbances than annual cropping systems (Horn 2000). Orchards are more stable than crop fields, which are disturbed annually when crops are harvested and new crops planted, but less stable than forests for example where disturbances are minimal (Pekár 2003). Disturbances in orchards are mostly limited to mowing weeds and pesticide applications (Pekár 2003). Plant diversity is higher in orchards than in annual cropping systems, as their associated design components are conserved and managed within the orchard boundary (Simon *et al.* 2010). Windbreaks and plant covers associated with orchards improve plant diversity; windbreaks also serve as physical barriers that reduce pesticide drift from adjacent orchards (Simon *et al.* 2010). Brown & Adler (1989) compared the diversity of the phytophagous arthropod community on managed, "organic" and abandoned apple orchards to determine the influence of orchard management on arthropod diversity. Abandoned orchards showed the greatest species diversity, followed by "organic" orchards and the least diversity was observed in managed orchards. The above mentioned study also showed that cultivar and orchard age did not have a significant effect on arthropod diversity. However, Pekár (2003) showed the arthropod community of juvenile apple orchards (1 – 4 years) differed from established orchards (15 – 20 years). Spider numbers increased as the juvenile orchards aged, while population numbers were constant in established orchards. Juvenile orchards were also shown to have significantly lower species diversity of spiders than established orchards. Goble *et al.* (2010) conducted a survey in the Eastern Cape Province in South Africa to determine the influence of orchard management on the abundance of entomopathogenic fungi. Results of the study showed a higher occurrence of entomopathogenic fungi (EPF) in soil samples collected from refugia than soil samples collected from organically and conventionally managed farms. However, when the occurrence of EPF from soil samples collected from organically and conventionally managed farms were compared, there was no significant difference recorded.

Changes in the abundance, diversity and behaviour of arthropods and micro-organisms in citrus orchards as the system ages have not been determined. Parasitoids for example are more exposed to wind and dust in younger orchards.

Dust can increase grooming and reduce foraging, oviposition and lengths of visits on dusty foliage (Van Driesche & Bellows 1996). The reduced ability of parasitoids to locate hosts and reproduce could in part explain the higher numbers of FCM infestation reported in juvenile citrus orchards. Furthermore it is uncertain how long it takes FCM to colonise a new orchard and how long it takes their natural enemies to find them. Newton (1998), described FCM as a poor disperser. Moore *et al.* (2004) determined the persistence of Cryptophlebia leucotreta granulovirus (CrleGV) treatments when applied to single trees compared to block treatments. Fruit infestation was reduced by an average of 70% for 17 weeks in block treatments (0.15 ha) compared to only 53% for seven weeks in single tree treatments. These results indicate that FCM recolonizes treated trees from adjacent untreated trees almost immediately after the treatment has been broken down while treated blocks will only be recolonized ten weeks after. Another field study conducted by Stotter *et al.* (2014) found decreasing FCM numbers with increasing distance from infested orchards with the exception of areas that contained high densities of alternate hosts. FCM population peaks occurred at the same time in citrus orchards and alternate hosts, indicating that moths did not migrate between alternate hosts and nearby citrus orchards. The poor dispersal ability of FCM is further supported by a genetic study conducted by Timm *et al.* (2010) who found genetically recognizable FCM populations, which can be separated from each other by less than a kilometre, suggesting very limited gene flow. As mentioned above, Goble *et al.* (2010) showed that orchard management decreases the occurrence of entomopathogenic fungi, but it is still to be determined how EPF and entomopathogenic nematodes (EPN) occurrence and diversity changes as the orchard ages. In addition to the impact that chemical applications will have on EPN and EPF occurrence, trees will also grow larger, which will change the microclimate of the orchard.

1.3 Plant insect interactions

Herbivorous insects utilise plant volatiles to locate and identify suitable host plants (Bengtsson *et al.* 2006). Insects possess specialized olfactory receptor neurons which enable them to discern plant signals and segregate food sources and

oviposition sites from other background chemicals in the environment (Dethier 1982, Menken *et al.* 1992, Bernays 2001, Mustaparta 2002). Some insect species have even shown the ability to inspect potential oviposition sites for signs of intra- and interspecific competition before deciding if the oviposition site is a favourable habitat for their offspring (Huth & Pellmyr 1999, Cope & Fox 2003). According to Minkenberg *et al.* (1992), insect reproduction and fitness is directly influenced by oviposition and foraging behaviour. Oviposition behaviour along with host suitability determines potential host use and drives the natural selection of host preferences (Futuyma & Keese 1992). Although positive correlations between oviposition preferences and larval performance have been documented for some insect species (Singer & Thomas 1988, Kouki 1993), other species prefer laying eggs on plant species that are not suitable for larval survival and fitness (Zalucki & Kitching 1982, Thompson 1988, Berdegué *et al.* 1998). Most immature stages of phytophagous insects and especially Lepidoptera larvae have limited mobility and therefore adult oviposition choice will have a significant influence on their survival (Renwick 1989). A negative interaction between adult oviposition choice and the survival and development of their offspring can be explained partially by variations in the relationship between host choice and larval performance under different ecological conditions and selection pressures (Thompson 1988).

Thompson (1988) examined four hypotheses of selection pressures that could influence oviposition selection and progeny performance: 1) *the time hypothesis*. Females may oviposit on plants recently introduced to their environment that are unsuitable for the development of larvae or nymphs. Over time selection pressures will either shift oviposition choice away from unsuitable novel hosts or improve the ability of immature life stages to survive on the host. 2) *Patch dynamic hypothesis.* Females may choose host quantity over quality for oviposition. 3) *The parasite/grazer hypothesis.* Parasites are phytophagous insects that complete development on a single plant while grazers are able to develop on multiple hosts. Grazers are thought to be less likely to develop strong oviposition to host plant relationships than parasites. Possibly, grazer insect species may oviposit on hosts that provide superior egg survival; hatchlings may then move to hosts that are more suitable for growth and development. 4) *Enemy-free space h*ypothesis. Host plants that provide greater protection against natural enemies may be preferred to host

plants of superior nutritional quality that provide less shelter. Negative correlations between oviposition preferences and larval performance have been exploited for use in agriculture. Trap crops are sometimes planted as cultural control methods for certain pest species. Efficient trap crops are more attractive to the adult stage of a pest species as either oviposition site or food source than the crop produced and are not suitable hosts for the immature stages of the pest acting as a sink rather than a source for successive generations (Badenes-Perez *et al*. 2004).

Little is known about the exact cues FCM uses to locate oviposition sites. To date, despite having a wide host range and its utilization of citrus being fairly recent, no trap crops have been identified for FCM. Reed (1974) investigated the possibility of using maize plants to attract FCM away from cotton fields, but the trial was unsuccessful. Love *et al.* (2014) determined that some Navel orange cultivars are more attractive oviposition sites for FCM than others. Results of both choice and no-choice tests showed that Newhall and Fukumoto Navels were significantly more attractive for oviposition than Fischer Navels. Fischer Navels were also shown to be less susceptible to larval penetration. The precise reason why some citrus cultivars are less attractive for oviposition than others is unknown. Once female moths have reached the inside of the tree framework they will respond to visual and / or olfactory stimuli to locate fruit for oviposition sites. The oviposition preference of female Mediterranean fruit fly, *Ceratitis capitata* Weidemann (Diptera: Tephritidae) for example, has been shown to be greatly influenced the type and quantity of essential oils produced by different citrus types (Ioannu *et al*. 2012). Soutar *et al*. (2015) conducted Y-tube choice experiments to determine the attractiveness of commercially available citrus volatiles to gravid female FCM. Although only a fraction of the total number of volatiles produced by citrus fruit were tested, the study gave some insight as to which volatiles might be preferred. Four chemicals, d-limonene, ocimene, β -caryophyllene and naphthalene were compared, both individually and as part of various blends. A blend of naphthalene, ocimene and β-caryophyllene was shown to be the most attractive. Newton (1989) determined that FCM prefer to lay eggs on damaged fruit. Limonene, α-pinene, sabinene, β-myrcene, acetaldehyde, ethanol, ethylene and CO_2 have been identified as the main volatile compounds emitted by wounded citrus fruit (Eckert *et al.* 1994). According to Newton (1989), the increased oviposition preference of FCM females to injured fruit may indicate that

olfactory stimuli may play a more import role than visual stimuli when locating oviposition sites. It is possible that FCM is able to detect nutrient and volatile differences between fruit from juvenile and established orchards, if such differences exist, but this is still to be determined.

Plants have shown the ability to protect themselves from herbivores, either chemically by unbalancing nutrients, producing digestibility reducers and toxins, or physically by trichomes and tissue toughness (Price *et al.* 1980, Cortesero *et al.* 2000, How & Jander 2008). Sub-lethal effects of digestibility reducers and poor nutrient quality, supress herbivore numbers by reducing fecundity, impairing growth and weakening disease resistance (Price *et al.* 1980, Cortesero *et al.* 2000, How & Jander 2008). Chan *et al.* (1978) determined the effect of tannin (extracted from cotton) in artificial diet on the larval development rate of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) and found that the addition of 0.2% tannin reduced larval development by 84%. The results of their study are supported by a dosage effect study conducted by Nomura & Itioka (2002), who found that larval development of the common cutworm, *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) was proportional to the amount of tannin ingested. Reduced growth rate increases the probability of insect herbivores being discovered by their natural enemies (Tanada & Fuxa 1987). However, Clancy & Price (1987), determined that larger leaf-galling sawfly, *Pontania sp.*, with faster development rates, are more prone to parasitism by ectoparasitoids, possibly because of their higher nutritional value. Although tannins in red oak reduce gypsy moth, *Lymantria dispar* (Linnaeus) (Lepidoptera: Erebidae), fecundity (Campbell & Sloan 1978), they also reduce susceptibility to nucleopolyhedrovirus (NPV), possibly by preventing entry through the peritrophic membrane by binding to virus particles in the gut (Keating *et al.* 1990). A study done by Feeny (1970) showed that the availability of nitrogen and not carbohydrates in oak leaves to be the most limiting growth factor for the winter moth, *Operophtera brumata* (Linnaeus) (Lepidoptera: Geometridae). An earlier study conducted by Feeny & Bostock (1968) showed larval growth rate, pupal weight and fecundity to decrease during late summer because of reduced availability of nitrogen in oak leaves. In addition to reduced growth rate and fecundity, consuming foods with lower nutritional value may also increase the probability of insect herbivores to obtain pathogen infections, as they will have to consume higher food quantities per

unit time to obtain the nutrients they require (Boots 2000). Although ontogenetic physical and chemical defence changes in citrus trees have not been determined, a study conducted by Khalid *et al.* (2012) showed that tree age has a significant influence on citrus rind quality. Three year old Kinnow mandarin trees were shown to have higher rag mass, rind thickness, percentage rind mass, ascorbic acid, pH, non-reducing sugars, rind manganese and iron content, while 18 year old trees contained higher reducing sugars, total soluble solids and were more acidic.

A review by Boege & Marquis (2005) found that ontogenetic changes in plant resistance and tolerance to diseases and herbivory are rather common in nature. According to Boege *et al.* (2007), these shifts in resistance and tolerance are due to changes in plant architecture and resource availability. Until maturity, plants prioritise growth above other metabolic activities such as producing chemicals for defence purposes (Tiffin 2002, Weiner 2004). Plant chemical defences may remain constrained until plant growth slows down and the optimal resource-foraging capacity is reached (Boege 2005). In addition to changes in plant defence due to resource availability, various studies have also shown that plants are able to become more resistant to herbivory after previous exposure. Studies on many different plants have shown that induced resistance is more effective after repeated attacks by herbivores after multiple seasons (Karban & Myers 1989, Karban & Niiho 1995). According to Howe & Jander (2008), plants are also able to distinguish between attacks from insects with different lifestyles and feeding behaviours. Similar to pathogen induced immunity, plants are able to recognise exogenous molecules from insect secretions which enable them to optimise defence strategies (Kessler *et al.* 2004).

1.4 Natural enemy interactions

Schmidt *et al.* (2003) illustrated the importance of natural enemies in agricultural systems. Results of their study on cereal aphids, *Metopolophium dirhodum* (Walker) (Hemiptera: Sternorrhyncha), *Sitobion avenae* (Fabricius) (Hemiptera: Sternorrhyncha) and *Rhopalosiphum padi* (Linnaeus) (Hemiptera: Sternorrhyncha) showed lower numbers of predators and parasitoids to increase aphid numbers by 18% and 70% respectively. In fields were lower numbers of both

predators and parasitoids were recorded, aphid numbers increased by 172%. According to Lim (1986), a lack of parasitoids and natural enemies in general is a major cause of diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Plutellidae) outbreaks in many parts of the world. The importance of natural enemies is further illustrated by mite outbreaks, for example, occurring due to the non-target effects of pesticide applications on their natural enemies (Penman & Chapman 1988, Beers *et al.* 2005). A study conducted by Grout & Richards (1992) showed 10.7% fruit damage caused by citrus thrips, *Scirtothrips aurantii* (Faure) (Thysanoptera: Thripidae) in citrus orchards where predatory mite numbers demised after a methiocarp treatment compared to only 0.2% damage observed in untreated orchards.

The impact of generalist vertebrate enemies on insect pest populations should also be considered. Generalist natural enemies are believed to play an important role in controlling low-density pest populations, thus preventing pest outbreaks (Elkinton & Liebhold 1990, Perfecto *et al.* 2004). Campbell & Sloan (1976) found vertebrate predation of late instar larvae and pupae of gypsy moth to be much greater than predation caused by invertebrates. Their results are supported in a later study by Elkinton *et al.* (1996), which shows a correlation between increased gypsy moth numbers and decreased numbers of the white-footed mouse, *Peromyscus leucopus* (Rafinesque) (Rodentia: Cricetidae). Marquis & Whelan (1994), determined that insectivorous birds indirectly improve plant growth by feeding on leaf-chewing insects. In their study on white oak, *Quercus alba* (Linnaeus) (Fagales: Fagaceae), saplings were caged to prevent birds from consuming insects. The number of insect recorded on saplings in cages were double the number recorded on control plants. Leaf area loss was 12% higher in caged saplings than in control plants. Results of a similar study conducted by Tremblay *et al.* (2001) showed significantly higher cutworm and weevil numbers in cornfields where birds were excluded. In addition to decreasing insect numbers by directly feeding on them, birds may also play a role in reducing insect numbers by spreading diseases (Briggs & Godfray 1995).

Insect diseases are present at either enzootic or epizootic levels in pest populations (Shapiro – Ilan *et al.* 2012). Enzootic host-pathogen systems can shift to become epizootic if favourable changes in the environment occur or if there are

changes in host susceptibility or pathogen virulence (Fuxa & Tanda 1987). According to Steinhaus (1958), disease is a density dependent factor. A study by Dwyer (1991), showed that transmission of the NPV virus of Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough) (Lepidoptera: Lymantriidae) was significantly dependent on the densities of both infected and healthy hosts. The host density dependence of EPF epizootics have also been illustrated in various studies (Carruthers *et al.* 1985, Hajek 1997, Devi *et al.* 2003). Opoku-Debrah *et al.* (2013) used overcrowding as an induction method for latent infections of CrleGV in FCM and recovered five new isolates of the virus. Latent infections of insect pathogens are primarily associated with viruses (Kaya & Vega 2012). However, Dwyer & Elkinton (1993) showed that host density may not play such an important role in epizootics of NPV on gypsy moth. The model used to predict virus epizootics performed poorly in study sites where the season began with low host densities. Epizootics observed at these low densities were more severe than the model predicted, possibly due to density-related adjustments in larval behaviour. Chouvenc & Su (2012), showed that even though environmental conditions in termite mounds are favourable for the dispersion of entomopathogens, epizootics of entomopathogenic fungi (and possibly any other entomopathogen) are not possible, due to multilevel disease resistance mechanisms within a termite colony. Similar disease resistance mechanisms such as nest hygiene (Pereira & Stimac 1992, Siebeneicher *et al*. 1992), antibiotic secretion (Blum *et al.* 1958), grooming (Siebeneicher *et al*. 1992), avoidance (Marikovsky 1962), and dispersal of infected individuals from the colony (Evans 1982) have also been observed in ant colonies. A study by Nielsen *et al*. (2009) has shown that ants are even able to protect aphids from their obligate fungal pathogens. In the abovementioned study, ants swiftly removed fungal-infected aphid cadavers from the aphid colony they were tending to. In addition, ants showed the ability to detect and remove infective conidia from living aphids. Insect diseases can also spread by means of carrier insects. Numerous studies reviewed by Whitfield & Asgari (2003) illustrate the evolution of endosymbiosis between polydnaviruses and their wasp carriers. EPF have also been shown to disperse by means of various carriers such as collembolans (Dromph 2001), ants (Bird *et al.* 2004), mites (Schabel 1982) and coccinellids (Pell & Vandenberg 2002, Roy *et al.* 2001).

The efficacy of a given natural enemy will depend on its ability to locate its prey as well as the ability of its prey to detect and avoid contact with its enemy. Plant volatiles may serve as synomones which guide insect predators to detect an appropriate foraging habitat (Dicke *et al.* 1990). Some egg parasitoids respond to synomones emitted by plants when feeding or oviposition activities of insect herbivores occur (Colazza *et al.* 2004, Mumm *et al.* 2003). According to Hagen *et al.* (1999), coccinellids primarily rely on vision to locate foraging habitats. Plant height, architecture and morphology have been shown to influence their searching behaviour (Kareiva & Sahakian 1990, Hodek 1993). Once predators have located an appropriate foraging habitat, predators will utilize allelochemicals associated with their prey to locate food sources (Dicke *et al.* 1990). Insects have developed various mechanisms to detect and avoid predators. Crickets, mantids and locusts, for example are able to detect ultrasonic pulses emitted by bats during prey searching, which enables them to flee before being discovered (Hoy 1992). When distressed, aphids secrete an alarm pheromone, which warns nearby aphids of danger, urging them to disperse (Nault *et al.* 1976). According to Meyling & Pell (2006), "insects can assess their environment based on cues related to mortality risks to themselves or their offspring." Meyling & Pell (2006), conducted choice tests to determine the behaviour of *Anthocoris nemorum* (Linnaeus) (Heteroptera: Anthocoridae), a generalist predator when exposed to *Beauveria bassiana* (Balsamo) Vuillemin. *Anthocoris nemorum*, showed the ability to detect and avoid leaf surfaces inoculated with *B. bassiana* and consequently oviposition was significantly higher on control leaves. Females were observed to retreat when they encountered infected cadavers and were very reluctant to crawl onto infected leaves when compelled to do so. Some insect herbivores have developed cryptic or disruptive colouration to avoid detection by predators (Edmunds 1990) or in contrast aposematism, warning predators that they are either poisonous or distasteful (Guilford 1990). Aposematism is further exploited in Müllerian and Batesian mimicry. In the case of Müllerian mimicry, two toxic or distasteful species evolve to resemble one another by sharing a warning-colour pattern (Kapan 2001). Shared warning signals teach predators to avoid similar coloured species more rapidly causing less damage to both species (Holmgren & Equist 1999). Batesian mimicry is a form of protective mimicry where a harmless or palatable species imitates the warning colour patterns of a harmful or

unpalatable species and therefore predators similarly avoid those species (Mandal 2012).

FCM has developed various strategies to protect itself from natural enemies. Adult moths display cryptic and disruptive colouration, which impair the ability of predators to locate them. Once inside fruit, larvae are also fairly protected from natural enemies, with the exception of larval parasitoids such as *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) which is capable of infecting the first two instars (Sishuba 2003, Gendall 2007). Female *A. bishopi* have long ovipositors, nearly the length of its body, measuring approximately 4 mm (Sishuba 2003, Gendall 2007), which enables them to reach larvae just as they penetrate the fruit rind (Glendall, 2007). Goble *et al.* (2010) found FCM to be less susceptible to EPF than *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) and fruit flies (*Ceratitis spp.*), possibly because the silk cocoon that covers pupae prevent fungal spores from coming into direct contact with the insect. FCM pupae are also very well camouflaged and resemble the orchard floor.

1.5 False codling moth

1.5.1 Classification and taxonomy

FCM was originally named the Natal codling moth after being discovered in Kwa-Zulu Natal by Fuller (1901) (Newton 1998). Fuller (1901) classified the Natal codling moth under the genus *Carpocapsa* (Newton 1998), due its similarity to the codling moth, *Cydia pomonella* (Lepidoptera, Tortricidae); a cosmopolitan pest of pome fruit (Catling & Aschenborn 1978). FCM was later found in the northern areas of South Africa and referred to as the orange codling moth (Newton 1998). The taxonomic classification of FCM has been changed several times since its original discovery (Newton 1998). Meyrick (1912) was first to describe FCM taxonomically as *Argyroploce leucotreta* (Eucosmidae, Olethreutidae) (Van den Berg 2001). In 1958 Clark moved it to the genus *Cryptophlebia* and 41 years later Komai placed it into the

genus where it currently remains, *Thaumatotibia* (Venette *et al.* 2003). The classification of FCM as it currently stands can be seen in Table 1.1.

Table 1.1 Classification of FCM (Stibick 2010).

Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Tortricidae
Tribe	Grapholitini
Genus	*Thaumatotibia*
Species	*leucotreta*
Synonym	*Cryptophlebia leucotreta*
Common name	False codling moth

1.5.2 Biology and morphology

FCM eggs (Fig. 1.1 A) are small, measuring approximately 0.6 mm in width and 0.8 mm in length (Van den Berg 2001). Female moths usually lay eggs singly on fruit, but injured fruit may attract multiple egg laying (Newton 1989). FCM eggs are translucent in colour directly after being laid and become red as the larvae develop to dark brown just before hatching occurs, usually between 6 – 12 days after oviposition (Georgala 1969, Daiber 1979a, Newton 1998).

Neonate larvae are approximately 1.5 mm in length (Van den Berg 2001); they enter fruit through the navel ends of navel oranges or cracks and wounds in the fruit (Stotter 2009) or bore into the rind leaving behind burrows of approximately 1 mm in diameter (Newton 1998). Most neonate larvae do not survive as they are very fragile and especially sensitive to cold temperatures and although rare, cannibalism towards eggs and other larvae has been reported (Newton 1998). The first three larval instars are cream with a dark brown head; larvae (Fig. 1.1 B) turn light pink during the 4th instar and are pink-red just before spinning a cocoon (Georgala 1969,

Newton 1998, Van den Berg 2001). The exact larval instar can be determined by measuring the width of the head capsule as indicated in Table 1.2 below. Usually only one larva develops per fruit (Catling & Aschenborn 1974). After approximately 15-67 days, depending on the season, larval development is completed (Stofberg 1954); larvae then leave the fruit, drop to the ground and pupate in the soil (Newton 1998, Van den Berg 2001).

Table 1.2 The average head capsule width (Daiber 1979b) and ranges (Hofmeyr *et al.* 2016) of FCM larval instars.

Larval instar	Average (mm)	Head capsule range (mm)
1	0.21	0.00 - 0.28
2	0.37	0.29 - 0.46
3	0.61	0.47 - 0.77
4	0.94	0.78 - 1.16
5	1.37	1.17

A mature larvae spins a cocoon that consists of silken threads, which bind to sand particles and soil debris (Stofberg 1954, Newton 1998). Two pupal stages occur within the cocoon, the pre pupal and the pupal stage (Fig. 1.1 C). Prepupae are light beige and turn dark brown after 2 - 27 days during the pupal stage, which lasts 11 – 39 days (depending on temperature) before adult FCM emerge (Daiber 1979c, Stibick 2010).

Adult moths (Fig. 1.1 D) have mottled dark black-brown to grey wings which span 16 - 20 mm (Newton 1998, Van den Berg 2001). Males are distinguished from females by their generally smaller size, black anal tufts, lengthened hairs on their hind tibia and a scent organ located near each hind wing (Newton 1998, Van den Berg 2001). Mating occurs within 2 – 3 days after emerging from pupae. The adult lifespan generally lasts 1 – 3 weeks, during which time polyandrous females can lay up to 450 eggs (Annecke & Moran 1982, Stibick 2010). Up to six overlapping generations may occur each year, depending on various environmental factors (Stibick 2010).

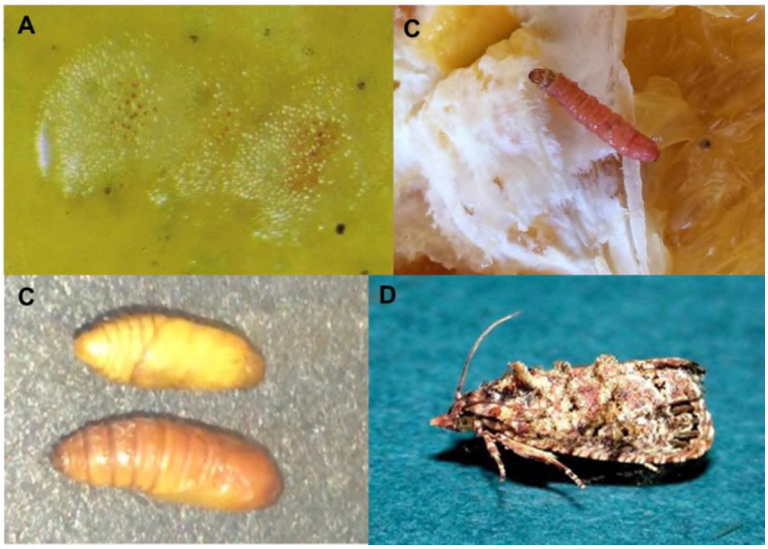

Figure 1.1: FCM life stages. (A) eggs (Photo credit: River Bioscience, http://www.riverbioscience.co.za/eggs.html), (B) final instar larvae; (C) pupae; (D) adult (Photo credit: W. Kirkman).

1.5.3 Distribution and host range

FCM is considered to be indigenous and endemic to Africa, primarily in sub-tropical and tropical areas south of the Sahara (Schwartz 1981). Records indicate FCM occurrence in Senegal, Congo, Ivory Coast, Nigeria, Uganda, Togo, Kenya, Somalia, South Africa, Burkina Faso, Madagascar and Mauritius (Hill 1975, Wysoki 1986, Newton 1998). In South Africa, FCM is widely distributed and inhabits all seven citrus producing regions in the country (Newton 1998). Although FCM is considered a serious pest in all seven citrus producing regions, the impact of the pest varies under different climatic conditions; pest pressure in known to be much less in the drier regions in the far north of South Africa (Newton 1998, Moore & Hattingh 2012, Moore 2017).

FCM has a broad host range and has been recorded on over 80 different plant species, which include commercially produced crops such as stone fruit, pome fruit, macadamias, litchis, cotton, citrus and avocados (Van den Berg 2001, Venette *et al.* 2003, Kirkman 2007, Kirkman & Moore 2007, Stibick 2010). However, EPPO (2013) conducted a thorough review of the relevant literature and found that the host status of many of the plants listes was open to debate. Furthermore, Eppo (2013) could not find the original source or substantiating data for many of the hosts

reported. They found that the validity of sevral host listed was questionable and outright refuted 36 of the host spescies listed, thus, substantially reducing the host list for FCM. The latest survey of FCM occurrence on host plants from South Africa was conducted by Kirkman & Moore (2007) who recorded FCM on approximately 24 cultivated and 50 wild plant species. Citrus is the preferred host of FCM in South Africa (Annecke & Moran 1982), especially Navel oranges which are known to be highly susceptible to FCM compared to other citrus types (Newton 1998). The broad host range of FCM is problematic as it enables population numbers to persist even after citrus trees have been harvested (Van den Berg 2001). However, a study conducted by Kirkman & Moore (2007) reported a generally low occurrence of alternative hosts near citrus orchards. Furthermore, FCM has also been shown to be a poor disperser (Newton 1998, Moore *et al.* 2004, Timm *et al.* 2010. Stotter *et al.* 2014). Therefore, alternative hosts are only problematic if they occur adjacent to citrus orchards at high densities.

1.5.4 Economic importance and damage

FCM larvae damage fruit by cryptic internal feeding (Fig. 1.2), which causes fruit to ripen prematurely; immature fruit of no more than 15 - 20 cm in diameter may drop as soon as November (Newton 1998). Larval penetration holes may also cause post-harvest losses due to secondary infection by bacteria and fungi, which cause fruit decay (Kirkman & Moore, 2007). In addition to direct crop losses, the phytosanitary status of FCM, in all export markets outside Africa (Venette *et al.* 2003, Stibick 2010, Moore 2017), may cause consignments to be rejected if any live FCM larvae are found, resulting in great financial losses (Moore 2002, Moore 2017). Although strict fruit monitoring systems are in place, larval penetration marks on fruit are usually only visible a few days after larval penetration (Georgala 1969), which could result in infested fruit which have been harvested shortly after infestation to be packed into cartons destined for export markets (Moore 2002).

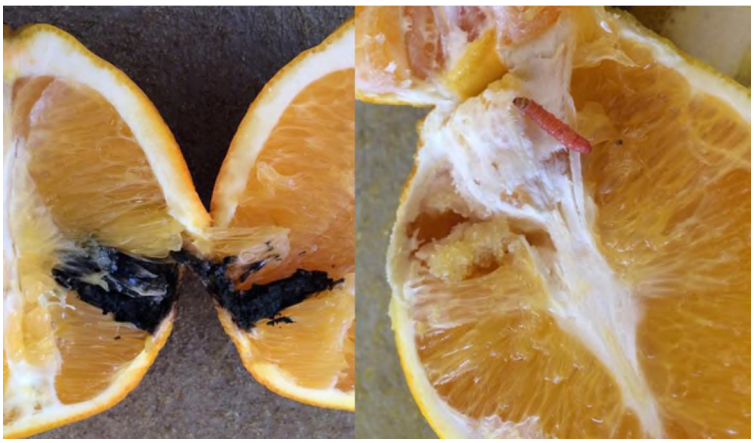

Figure 1.2: Damage caused by FCM larvae on citrus.

1.5.5 Control methods

Reliance on insecticides to achieve sustainable management of insect pest populations generally fails because the target pest usually develops resistance to insecticides that are overexploited (Hofmeyr & Pringle 1998, Dent 1995, Norris *et al.* 2003). International pesticide regulations for fruit export further pressurises crop producers to use alternative methods for pesticide control (Urquhart 1999). Biological control methods, on the other hand, are often aimed at only one pest species and one life stage and are dependent on various environmental factors to be effective. Therefore an Integrated Pest Management (IPM) approach is essential for efficient and sustainable FCM control. IPM can be defined as making use of multiple control methods aimed at harming pest populations whilst causing minimal disruption to the environment and natural enemies (Urquhart 1999). In addition to chemical and biological control, IPM programmes for FCM control in citrus orchards may also include behavioural control methods such as mating disruption and attract and kill or cultural control methods such as orchard sanitation and stripping orchards of any remaining fruit after harvest. FCM populations may also be supressed by using the sterile insect technique (SIT).

1.5.5.1 Monitoring

Efficient pest management relies on monitoring pest population numbers in order to decide if pest control is required (Dent 2002). FCM population numbers can

be monitored in various ways. FCM pheromone traps have been proven to be a very effective monitoring method (Moore 2017). Three types of pheromone dispensers are currently available and registered; the Lorelei, FCM PheroLure and Chempac FCM Lure (Moore 2017). Pheromone dispensers are placed in delta traps with sticky floors to capture male FCM that are lured in. The economic treatment threshold for chemical control of FCM is 10 moths per trap per week (Moore *et al.* 2008). However this economic threshold is no longer applicable, since FCM's pest status has become a phytosanitary concern rather than an economic one (Moore 2017). Therefore, Moore (2017) developed guidelines for fruit drop surveys where fruit are collected from selected trees and dissected to determine FCM infestation. Scouting for eggs on fruit is another monitoring option, but it is rather time-consuming, as FCM eggs are small and translucent, making them difficult to observe (Kirkman 2007).

1.5.5.2 Chemical control

The use of conventional insecticides for crop protection is the most common pest control method, mostly because they are easily available, fast acting, reliable (Rodriguez-Saona & Stelinski 2009), persistent and most of them control a broad range of pests species (Haynes 1988). The broad host range and persistence of insecticides such as pyrethroids, is however detrimental to natural enemies and the environment. Insecticides for FCM control are aimed at eggs and neonate larvae, before they bore into fruit where chemicals cannot reach them (Reed 1974, Newton 1998). Since the first chemicals were registered for FCM control in the early 1980s (Moore 2002), some have been phased out because of environmental and health risks associated with the various chemicals. Chemicals that have not been phased out are used carefully because of strict residue restrictions imposed by overseas markets (Inceoglu *et al*. 2001).

Chemicals currently registered for FCM control are cypermethrin, fenpropathrin, triflumuron, triflubenzuron, methyl parathion, spinetoram, chlorantraniliprole and methoxyfenozide (Hattingh & Hardman 2014). Fenproathrin and cypermethrin are both pyrethroids with a broad host range which can be detrimental to natural enemies and are therefore not ideal for use in an IPM approach (Moore *et al*. 2004). International residue requirements permit the use of

methyl parathion, (an organophosphate that kills larvae on contact) no later than 50% petal drop, which makes it an impractical control option for FCM, which consequently is never used (Hattingh & Hardman 2014). Triflumuron and triflubenzuron are chitin synthesis inhibitors with a more specific mode of action aimed at inhibiting the embryonic development of larvae inside FCM eggs (Newton 1998, Kirkman 2007, Moore & Hattingh 2012, Moore 2017). Chitin inhibitors are however only effective if eggs are laid on the surface of treated fruit that already possess a treatment residue (Moore 2017). FCM resistance to these chemicals has been reported (Hofmeyr & Pringle 1998, Moore 2002) and they have been shown to be detrimental to the egg parasitoid *T. cryptophlebiae* (Hatting & Tate 1997; cited by Moore 2002) and predatory beetles, which can result in secondary outbreaks of citrus red mite, oriental mite and mealybugs (Moore 2017). In order to address concerns with regards to the negative effect of pesticide residues on human health and the environment, modern insecticides such as spinetoram, chlorantraniliprole and methoxyfenozide have been developed to have environmentally friendly ecotoxicological profiles and shorter lasting residues, which make them more compatible with IPM (Urquhart 1999, Moore & Hattingh 2012).

1.5.5.3 Biological control

Various biological control options have been identified and developed to control FCM in citrus orchards, which include a variety of parasitoids, predators and entomopathogens. Parasitoids are the natural enemies most commonly used for insect control (Mattiacci *et al*. 1999, Van Driesche & Bellows 1996), of which the majority belong to the order Hymenoptera (Newton 1998). Although larval parasitoids are the most common, the only parasitoid currently commercially available for FCM control is the egg parasitoid *Trichogrammatoidea cryptophlebia* (Nagaraja) (Hymenoptera: Trichogrammatidae) (Moore & Hattingh 2012). The virus, CrleGV is commercially available and has been used with great success (Moore *et al.* 2004, Moore *et al.* 2015). Recent studies have been conducted to assess the ability of entomopathogenic fungi EPF and EPN to control the soil inhabiting life stages of FCM which include late fifth instar in search of pupation sites, prepupae, pupae and emerging adults (Malan *et al*. 2011). Developing EPN and EPF products will greatly

improve FCM control, as no other control method is currently targeted at controlling the soil inhabiting life stages of FCM.

1.5.5.3.1 Parasitoids

Moore (2002) lists three tachnid and 14 hymenopterans parasitoid species of FCM. Of these 17 parasitoid species, the egg parasitoid, *T. cryptophlebia*, and the larval parasitoid, *A. bishopi*, show the most potential for FCM control (Newton 1998, Carpenter *et al*. 2004). Larval parasitoids such as *A. bishopi* play an important role in FCM control as they are able to parasitize larvae after they have bored into fruit, where pesticides cannot reach them (Gendall 2007), while egg parasitoids such as *T. cryptophlebia*, control FCM before they are able to bore into fruit, thus preventing fruit damage (Newton 1998).

Sishuba (2003) conducted surveys in the Gamtoos and Sundays River Valleys and found parasitism of FCM larvae by *A. bishopi* to be density dependent. The highest parasitism rates of 38% in Sundays river Valley and 34% in Gamtoos River Valley were observed during December when FCM infestation levels were at their highest. At present, *A. bishopi* is not available commercially, because this species has proven to be difficult to mass-rear due to fungal and viral contamination and low productivity (Gendall 2007). However, Zimba (2016) has recently developed a rearing protocol for *A. bishopi* that shows improved potential for commercial use.

T. cryptophlebia has been shown to achieve up to 60% control of FCM (Newton & Odendaal 1990). However, to achieve satisfactory control, approximately 100 000 parasitoids have to be released per hectare per season from the beginning of October (Moore & Hattingh 2012, Moore 2017). Presently, *T. cryptophlebia* is mass reared by Vital Bugs (Letsitele, South Africa) and commercially available for augmentative inundative control (Moore & Hattingh 2012).

1.5.5.3.2 Predators

Predators are generalist natural enemies, which also include vertebrates such as small mammals, birds and lizards. Although ants are known to cause outbreaks of scale insects, psyllids and aphids, Bownes *et al.* (2014) showed the brown house ant, *Pheidole megacephala* (Fabricius) (Hymenoptera: Formicidae), and the pugnacious ant, *Anoplolepis custodiens* (Smith) (Hymenoptera: Formicidae), to prey on FCM pupae. Orchards in which chemicals were applied for ant control had a higher number of surviving pupae than untreated orchards. Bownes *et al.* (2014) therefore recommended using more localised ant control methods, such as ant bands, to prevent ants from reaching the tree canopy whilst still allowing them to forage the orchard floor for FCM pupae. Mites, *Pediculoides* sp. (Prostigmata: Pyemotidae), and bugs, *Orius insduosus* (Say) (Hemiptera: Anthocoridae) and *Rhynocoris albopunctatus* (Nyiira). (Hemiptera: Reduviidae) have also been reported to prey on FCM. The influence of vertebrates on FCM numbers is unknown.

1.5.5.3.3 Entomopathogenic nematodes

EPN are fatal insect pathogens which are used to control a broad range of economically important insect pest species (Grewal *et al*. 2005). Nematodes used for insect control are primarily from the two families Steinernematidae and Heterorhabditidae, which are associated with symbiotic bacteria from the genera, *Xenorhabdus* and *Photorhabdus*, respectively (Griffin *et al*. 2005, Jagdale *et al*. 2009). Both steinernematids and heterorhabditids have a free-living non-feeding stage known as dauer or infective juveniles (IJs) that are able to actively seek and infect hosts (Glazer & Lewis 2000). IJs either enter their hosts directly by penetrating through the insect's cuticle or through natural openings such as the anus, mouth or spiracles (Shapiro-Ilan 2009). Heterorhabditids have a dorsal tooth which enables them to directly penetrate the host's cuticle (Peters 1996). Once inside the host's haemocoel, IJs secrete bacteria, which kill the host within 24 - 48 h by means of either septicaemia or toxaemia; nematodes then feed on bacteria cells and broken down host tissue (Adams & Nguyen 2002, Dowds & Peters 2002). EPN are highly combatable with IPM programmes, as they have proven to be tolerant to most pesticides (Rovesti & Deseö 1990, Grewal *et al.* 2005, Van Niekerk & Malan 2014)

and have no known harmful effects on mammals and the environment (Gaugler & Boush 1979). Although EPN are primarily aimed at controlling soil-dwelling insects, they have proven to control some foliar pests as well (Arthurs *et al*. 2004, Van Niekerk & Malan 2012). Since nematodes require a water film to maintain mobility and ensure survival, their efficacy is greatly limited by soil moisture content (Wright *et al*. 2005). However, the addition of adjuvants that suppress desiccation have been shown to improve the ability of EPN to control foliar pests significantly (Schroer & Ehlers 2005, Van Niekerk & Malan 2015). Other abiotic factors that will have an influence on their survival and infectivity include soil type and aeration (Lacey & Georgis 2012), temperature (Lacey *et al*. 2005) and exposure to ultraviolet (UV) radiation (Gaugler & Boush 1978, Gaugler *et al*. 1992).

The use of EPN for FCM control has only been investigated recently. Malan *et al*. (2011) conducted bioassays to determine the susceptibility of FCM to six nematode species isolated from citrus orchards throughout South Africa. The last instar of FCM was shown to be highly susceptible to all six species tested, with the highest control of 100% obtained by *Steinernema yirgalemense* (Nguyen). Pupae were however shown to be less susceptible than the pre-pupal or wandering final instar larvae. More recently, Manrakhan *et al*. (2014) determined that naturally occurring EPN have a significant impact on FCM population levels. The study was conducted in a citrus orchard in Nelspruit, South Africa and showed 59% lower fruit infestation by FCM in a citrus block where EPN were conserved compared to a citrus block where a nematicide (cadusafos) was applied. Field trials conducted by Malan & Moore (2016) showed that application of *Heterorhabditis bacteriophora* (Poinar) and *H. zealandica* achieved more than 80% control of FCM in citrus orchards. River Bioscience, South Africa, was the first company to supply a commercial EPN product in South Africa, Cryptonem™. Cryptonem™ is produced and imported from e-Nema in Germany and contains *H. bacteriophora*.

1.5.5.3.4 Entomopathogenic fungi

Soil-inhabiting entomopathogenic fungi play an important role in the natural suppression of some soil-inhabiting insect populations, of which most fungal species belong to the order Hypocreales and Entomophthorales (Meyling & Eilenberg 2007,

Quesada-Moraga *et al.* 2007). The first mention of applying EPF as control agents was recorded 125 years ago when Metchnikoff proposed the use of *Metarhizium anisopliae* (Metschnikoff) Sorokin to control the grain beetle, *Anisoplia austriaca* (Herbst) (Coleoptera: Scarabaeidae) (Lord 2005). Presently more than 12 species of EPF have been developed into approximately 170 products, which control a wide variety of pest species (Vega *et al.* 2009, Jackson *et al.* 2010). EPF used commercially are all Hypocreales, which are easier to mass produce than Entomophthorales (Dolinski & Lacey 2007).

EPF infect insects by producing conidia and asexual spores, which stick to the host's cuticle; the spores then germinate and produce hyphae, which penetrate through the cuticle and invade the host's circulatory system (haemolymph) from where infections spreads to the rest of the body by blastospores (Inglis *et al.* 2001). Infected individuals usually die after 3 – 7 days, due to organ contamination, limited nutrient availability or toxic compounds produced by some fungi (Inglis *et al.* 2001). EPF will then spread further by hyphal growth from the insect cadaver, which produces conidia, which then gradually disperse through the ecosystem by wind or water movement (Inglis *et al.* 2001, Shah & Pell 2003). Similar to EPN, soil moisture is the most limiting environmental factor for EPF efficiency; relative humidity levels lower than 90% can prevent spore germination, host infection and sporulation of the fungus (Hesketh *et al.* 2010).

EPF are favourable additions to IPM programmes, as they are not hazardous to the environment because they leave no toxic residues which could contaminate crops, riparian habitats or ground water, they pose minimal risk to vertebrates and have a low impact on non-target arthropods (Inglis *et al.* 2001, Zimmermann 2007a,b). Although various species of EPF have shown potential for FCM control, particularly *Metarhizium* sp. and *B. bassiana,* the only commercially formulated products available is BroadBand® (BASF Crop Protection, Halfway House, South Africa) and Eco-Bb® (Plant Health Products, Pietermaritzburg, South Africa) which contains *B. bassiana* as active ingredient. However, these products have only been registered for foliar treatments.

Goble *et al*. (2011) collected soil samples from citrus farms and surrounding natural vegetation (refugia) in the Eastern Cape, South Africa to isolate and identify naturally occurring EPF species. Sixty-two isolates from four genera were recovered of which 21 were selected to test their potential for FCM control. Thirteen of the 21 EPF isolates significantly reduced FCM adult emergence to below 20%. Coombes (2012) re-screened 12 of the original isolates collected by Goble *et al*. (2011). Exposure-time response and concentration dose bioassays were conducted and the three isolates with the highest potential for FCM control were identified; one was an isolate of *B. bassiana* and the other two were *M. anisopliae* var. *anisopliae*. The field persistence of the three selected isolates were then compared to two commercially produced isolates of *B. bassiana* Eco-Bb® strain R444 (Plant Health Products, South Africa) and *M. anisopliae* ICIPE 69 (Real IPM, Kenya). All three non-commercial isolates persisted better than the two commercial isolates with the two *M. anisopliae* isolates achieving the highest persistence overall (Coombes *et al*. 2013). Coombes (2015) recently investigated the potential of the three non-commercial EPF isolates for commercial use and production. One *M. anisopliae* isolate was excluded from further study because of its poor performance at a lower field application rate of 1×10^{14} spores/ha compared to the other two isolates. The highest reduction in FCM infestation of between 33.85% and 81.72% was achieved by the non-commercial *B. bassiana* isolate, whilst the remaining non-commercial *M. anisopliae* isolate reduced FCM infestation by 28.32% - 63.02%.

1.5.5.3.5 Viruses

CrleGV attacks FCM and occurs naturally in sub-Saharan Africa (Moore 2002, Kirkman 2007). The development of this virus as a commercially available product for FCM is very beneficial for IPM programmes, as it is compatible with many of the chemicals used in citrus production and the virus is very specific and of low risk to non-target arthropods (Moore 2002, Kirkman 2007, Moore & Hattingh 2012, Moore 2017). Three products with CrleGV as their active ingredient are currently registered for FCM control, Cryptogran® (River Bioscience, South Africa), Cryptex® and Gratham® (both Andermatt-Biocontrol AG, Switzerland), (Moore & Hattingh 2012, Opoku-Debrah *et al*. 2013, Moore 2017). The above mentioned products are applied

as a full cover spray. After neonate larvae ingest virus particles along with contaminated fruit tissue, the virus is absorbed in the gut by microvilli from which it spreads to the rest of the insect's body causing morbidity, flaccidity, appetite loss and finally death (Moore 2002, Opoku-Debrah *et al*. 2013). Moore *et al*. (2015) reported that since 2000 more than 50 field trials have been conducted to determine control achieved against FCM. The results of 13 representative field trials were selected which showed CrleGV to achieve between 30 and 90% control. Field trials conducted by Moore *et al.* (2004) showed CrleGV able to achieve 70% control for up to 17 week. Opoku-Debrah *et al*. (2013) discovered five new isolates of the virus that could be used if FCM were ever to develop resistance to the isolate currently in use.

1.5.5.4 Cultural control

Orchard sanitation is the main cultural control method implemented by citrus producers. This control method involves weekly collections of dropped fruit from underneath the tree canopy (Newton 1998). Collected fruit are then drenched in water for a week or buried in a 30 cm deep trench to ensure that any FCM larvae inside fruit are destroyed (Georgala 1969). Alternatively, larvae can be destroyed by pulping fruit with a hammer mill and then leaving the pulp to dry in the sun (Schwartz 1974). Orchard sanitation has been shown to reduce FCM infestation by 40 – 75% (Newton 1998, Moore & Kirkman 2009). Georgala (1969) originally recommended orchard sanitation to commence in December, but Schwartz (1974) later suggested that orchard sanitation should commence earlier to achieve the best results. Any fruit left on trees after harvest should also be removed to reduce FCM numbers during the following season (Moore 2017).

1.5.5.5 Mating disruption

Mating disruption involves saturating the orchard with synthetic female moth pheromones, which misleads male moths into following false pheromone trials, thus delaying or preventing the ability of males to find females for mating (Minks & Cardé 1988, Moore 2002, Moore 2017). Four mating disruption products are currently registered for FCM control i.e CheckMate® FCM-F (Suterra, United States of

America), Isomate® (Pacific Biocintrol Corporation, United States of America), Splat® (River bioscience, South Africa) and Xmate® (Insect Science, South Africa). When applying mating disruption it is important to keep in mind that pheromone-baited traps will become inefficient (Stotter 2009).

1.5.5.6 Attract and Kill

Similar to mating disruption, the attract and kill method lures males to follow a false pheromone trial with the exception that the pheromone source also contains an insecticide which kills males on contact. The only attract and kill product currently registered for FCM control in South Africa is Last Call FCM™ (Insect Science, South Africa). Efficacy trials have shown this product to be less effective than mating disruption products and is therefore only recommended for use in areas with low pest pressure (Moore & Hattingh 2012, Moore 2017).

1.5.5.7 Sterile insect technique

SIT is a non-disruptive, host specific control method which is highly compatible with an IPM programme. This control measure involves the mass-rearing and sterilization of the target pest by non-lethal levels of gamma radiation and then releasing over-flooding numbers of sterile males into orchards. The gamma radiation causes the chromosomes of reproductive cells to divide incorrectly, thus producing abnormal gametes (Bloem *et al.* 2010); wild female moths that mate with sterile males will then produce non-viable, infertile eggs (Moore & Hattingh 2012, Moore 2017). Trials to evaluate the potential of SIT to control FCM were first initiated in Citrusdal, Western Cape, South Africa during 2002 (Hofmeyr *et al.* 2015). During a three year trial period, SIT showed the ability to reduce pre-harvest crop losses by 93% and export fruit rejection by 38% (Hofmeyr *et al.* 2015). Carpenter *et al.* (2004) determined the compatibility SIT with *T. cryptophlebiae* for FCM control. Although *T. cryptophlebiae* prefers eggs from non-irradiated males as hosts, parasitoid larvae are able to develop in sterile eggs. The success of SIT requires crop producers to work together, as SIT has to be applied on an area-wide basis to have optimum impact (Moore 2017). Hofmeyr & Hofmeyr (2004) also recommended a ratio of

sterile to wild males of at least 10:1 in order for SIT to be efficient. The efficacy of SIT will also depend on pest population factors such as sterile male fitness, females only mating once and low initial pest population levels (Bloem *et al.* 2001, Moore 2002). SIT has been commercialised by XSIT (Pty) Ltd in 2007 (Moore 2017) and is presently applied in the Citrusdal and Hex River areas of the Western Cape, some areas of the Eastern Cape and the Northern Cape. Reports from Citrusdal have shown that IPM programmes that use SIT as the backbone of the programme are able to decrease moth catches by 99%, fruit infestation by 96% and export rejection by 89% (Barnes *et al.* 2015).

1.5.5.8 Post harvest control

Myburgh (1965) was the first to investigate the possibility of using cold sterilisation and radiation for the post-harvest control of FCM. Gamma radiation inhibits the development of immature life stages to moths at exposures between 10 and 120 Kr at a radiation intensity of 80 r per minute (Myburgh 1963). Hofmeyr & Hofmeyr (2005) found that irradiating FCM larvae at 200 Gy resulted in 100% mortality in artificial diet. Although gamma radiation shows potential for the post-harvest control of FCM, it is not implemented commercially in South Africa (Moore 2002, Kirkman 2007).

To date cold sterilisation has shown to be the most efficient post-harvest control method for FCM (Boardman 2012). Myburgh (1965) found that FCM eggs are highly susceptible to cold temperatures, while larvae were more resistant, followed by pupae, which is the most cold tolerant life stage. Exposing FCM larvae in artificial diet to a temperature of -0.5 °C for 21 days resulted in zero survival (Myburgh, 1965). Van Der Geest *et al.* (1991) found that exposing FCM eggs and larvae to temperatures below 10° C significantly reduced development. Adult FCM have been shown to be less tolerant to low temperatures with high mortality achieved when exposed to temperatures below 0 °C for periods of up to 10 hours (Stotter & Terblanche 2009). Boardman *et al.* (2012) exposed FCM larvae to very low temperatures of -14 °C to -18 °C for one hour and found that larvae were unable to recover, resulting in 100% mortality. Cold sterilisation is used commercially but it is very expensive, therefore its use is only justified when sending fruit to markets which

regulate FCM as a phytosanitary organism, making such treatments mandatory, and if these markets are sufficiently profitable. Such markets are Japan, China and the United States of America (SA-DAFF 2015). Currently phytosanitary procedures of the above mentioned markets usually require citrus to be exposed to a temperature of -0.55 °C for 22 days. Regrettably, this procedure is also detrimental to fruit quality, especially easy peeling varieties and white grapefruit (Lafuente *et al*. 2003).

Recent cold treatment studies conducted by Moore *et al*. (2016a, b, 2017) suggest that a systems approach that applies partial cold treatment as the final step in the system could replace current cold treatment protocols. Partial cold treatments will be less detrimental to fruit quality, more affordable and easier to implement logistically. Moore *et al*. (2016b) evaluated the efficacy of various cold treatments and found cold treatments at 2 °C for a duration of 18 days to be the most efficient for inclusion as a step in a systems approach. The above mentioned cold treatment achieved 99.94% control. The majority of surviving larvae were unable to develop into moths and if they did reach adulthood many of them were unable to reproduce. After taking this into consideration the actual control achieved increased to 99.9%.

1.6 Aim of the study

Understanding pest biology and its interaction with natural enemies and the environment is essential to developing efficient and effective control programmes (Faust 2008). The current knowledge on FCM ecology is limited. Many studies have been aimed at evaluating and improving various control methods but only a few have attempted to understand the tritrophic interactions between FCM, natural enemies and the environment. The influence of orchard age on crop pests in general has also not been determined. An improved understanding of the environmental and orchard management changes that occur as orchards age would be highly advantageous in order to improve not only FCM control but possibly the control of other fruit pests as well. This study will enable us to assess the influence of these changes on pests and their natural enemies. Orchard management practices can then be adjusted to improve natural enemy population numbers and supress pest insect numbers.

The aims of the study were to determine 1) the influence of juvenility on citrus fruit nutritional composition, FCM oviposition preference, fruit infestation, larval growth rate, pupal mass and susceptibility of FCM to CrleGV, EPF and EPN (Chapter 2 and 3), 2) the above ground influence of orchard age on FCM occurrence and infestation, mortality of FCM due to virus infections and parasitism (Chapter 4) and 3) the sub-terrestrial influence of orchard age on the diversity and occurrence of EPF and EPN (Chapter 4 and 5).

CHAPTER 2

False codling moth oviposition preferences and host susceptibility: the influence of orchard age

2.1 Introduction

There are anecdotal reports in the South African citrus industry of higher false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) infestation in juvenile orchards (age four to eight years) compared to mature orchards (nine years and older) (D, Gerber, pers comm). A possible reason for higher FCM infestations in juvenile orchards could be physiological differences between fruit from trees of different ages. A study by Love *et al.* (2014) has shown that all citrus cultivars are not equally susceptible to FCM, even within variety. The abovementioned study showed that differences in oviposition preference and susceptibility to FCM exist between different citrus cultivars and should be taken into consideration when deciding which cultivars to plant. In addition to cultivar differences, studies have shown that the rootstock used will also have an influence on fruit physiology (Fallahi & Rodney 1992, Castle 1995). A study conducted by Fallahi & Rodney (1992), for example, showed significantly higher soluble solid concentrations (SSC) in Fairchild Mandarin fruit from trees planted on Carrizo citrange rootstocks than fruit from trees planted on Volkameriana lemon and rough lemon rootstocks. According to Khalid *et al.* (2012), tree age is one of the most important factors affecting rind quality of citrus fruits. In their study, Khalid *et al.* (2012) compared the fruit quality of three, six, 18 and 35 year old Kinnow Mandarin trees. The results of Khalid *et al.* (2012), showed that reducing sugars, acidity and total soluble solids (TSS) were higher in fruit from 18 year old trees, whereas fruit from three year old trees were shown to have higher rag mass, rind thickness, percentage rind mass, ascorbic acid, pH, non-reducing sugars, rind manganese and iron content.

Boege & Marquis (2005) hypothesised that a plant's resistance to attack by herbivores is likely to change as the plant matures due to changes in plant architecture and resource allocation. As trees mature and grow, the shoot to root ratio increases while metabolic activity slows down (Farnsworth 2004). Shifts in defence cost and benefit may occur due to changes in resource availability (Bergelson 1994, Hochwender *et al.* 2000, Stowe *et al.* 2000). After germination, resources are limited and plants prioritise growth above other metabolic activities (Tiffin 2002, Weiner 2004). The availability of resources required for chemical defence may be constrained until the plant's resource foraging ability is optimal (Boege & Marquis 2005). Various studies have shown that invertebrate herbivores prefer to forage on juvenile plants rather than mature plants (Price *et al.* 1987, Fritz *et al*. 2001, Del Val & Dirzo 2003, Boege *et al.* 2007).

In addition to passive changes in plant defence due to shifts in resource allocation, FCM numbers may be lower in mature orchards, because of tree defences becoming more advanced in response to previous exposure to FCM. Multiple studies on various plant species, reviewed by Karban & Myers (1989) and Karban & Niiho (1995), have shown that plant defence responses become more effective after repeated attacks by herbivores over multiple seasons compared to plants which have only been attacked by the same species of herbivore for only one season. Some plants have even shown the ability to distinguish between attacks from different insect species by recognising insect proteins excreted during feeding, which allow them to develop defence mechanisms that are more species specific (Howe & Jander 2008, Erb *et al*. 2012). According to Karban & Myers (1989), these studies show that induced plant defences should be regarded as a graded response rather than a system which can be switched on or off as needed.

The aim of the study was therefore to determine if fruit from juvenile trees were more susceptible to FCM larval penetration and development than fruit from mature trees, as well to determine if juvenile tree fruit were preferred by gravid FCM for oviposition above mature tree fruit.

2.2 Materials and Methods

2.2.1 Fruit collection

Fruit from juvenile (four to eight years old) and mature (nine years and older) trees were collected from various orchards in the Sundays River Valley, Eastern Cape, South Africa (Table 2.1). Fruit were collected approximately one week before harvest and used within 24 h after being picked. Before being picked, fruit were inspected to insure that they were not compromised by disease, insect damage or infestation, or mould.

Table 2.1. Details of orchards from which fruit were harvested for laboratory trials.

Farm	Orchard number	Cultivar and variety	Rootstock	Year planted	Plant and row spacing (meters)
Douglasdale	52, 55	Washington Navel	Carizzo citrange	2011	3 x 6
Douglasdale	83, 84	Washington Navel	Carizzo citrange	2003	3 x 6
Halaron	34	Washington Navel	Carizzo citrange	2013	3 x 6
Miskruier	50	Washington Navel	Carizzo citrange	2005	3 x 6
Kleinplaas	2	Nova Mandarin	Corizzo citrange	1998	2 x 5.5
Kleinplaas	22	Nova Mandarin	Corizzo citrange	2012	3 x 5
Buffelsbos	707, 710	Nova Mandarin	Corizzo citrange	2013	3 x 6
Woodridge	320, 322	Nova Mandarin	Corizzo citrange	1999	2 x 5
Miskruier	7, 11	Cambria Navel	Carizzo citrange	2013	3 x 6
Habata	20, 23	Cambria Navel	Carizzo citrange	2012	2 x 6
Habata	6, 11	Cambria Navel	Carizzo citrange	2005	3 x 5.5
Falcon Ridge	9, 12	Cambria Navels	Carizzo citrange	2005	3 x 5.5
Kleinplaas	20, 23	Midnight Valencia	Carizzo citrange	2012	3 x 5
Kleinplaas	14, 17	Midnight Valencia	Carizzo citrange	2007	3 x 5
Miskruier	69	Midnight Valencia	Carizzo citrange	2013	2 x 6
Miskruier	51	Midnight Valencia	Carizzo citrange	2005	2 x 6
Riverbend	19	Midnight Valencia	Carizzo citrange	2000	2 x 5
Riverbend	506	Midnight Valencia	Rough lemon	2013	3 x 6

2.2.2 Internal fruit quality tests

Standard internal fruit quality tests were performed to determine fruit acid content (g per 100 ml citric acid), brix (sugar percentage), the ratio of brix/acid, juice percentage and peel mass (g) (Anonymous 1946, Anonymous 1999). Six fruit samples, consisting of six fruit each were tested per tree maturity and cultivar combination. The size, peel thickness and colour of each fruit were recorded before juicing. A colour maturity chart was used to determine fruit colour (Anonymous

1995). To determine fruit peel mass and juice percentage, a sample of six fruit per sample was taken and each fruit was weighed before and after juice extraction. The juice extracted from the sample was then mixed before determining the acid and brix percentage. Brix percentage was determined by the use of a refractometer. Acid contend was calculated by titrating the juice with sodium hydroxide. Six samples were tested per tree maturity and cultivar combination (Anonymous 1999).

2.2.3 Nutritional content of Washington Navel oranges

To determine differences in the nutritional composition of fruit from juvenile and mature Washington Navel trees from the same farm, Douglasdale, during 2015 (Table 2.1), 20 segments of equal weight (55 g) were cut from 20 individual fruit for each maturity group to make up one test sample. The fruit segments were then dried in an oven at 60°C. The dried fruit samples were then sent to CAL Laboratories (Roodepoort, South Africa) to be analysed for ash, lignin, protein, fat and calcium content. Fresh Washington Navel oranges collected from two farms and three orchards per maturity group during 2017 (Table 2.1) were sent to Bemlab (Strand, South Africa), to be analysed for nitrogen, phosphorus, potassium, calcium, magnesium, sodium, manganese, iron, copper, zinc, boron, silica and moisture content. Two fruit samples were sent per orchard (six samples per maturity group).

2.2.4 Washington Navel volatile profile

Six fruit from juvenile and mature Washington Navel trees respectively from the same farm (Douglasdale, Table 2.1) were collected during 2015 to compare the volatile profile of fruit from juvenile and mature trees. The volatile profile of each sample was collected by exposing a 100 µm polydimethyl siloxane (PDMS) non-bonded fibre (Supelco Co., Bellefonte, United States of America) to a 2 cm^2 piece of fruit in a 2 L glass container for 30 min at 20°C. Gas chromatography (GC)/mass spectrophotometry (MS) separation was done using a HP6890 series gas chromatograph (Agilent) equipped with a Zebron DB 5 (30 m, 0.25 mm internal diameter, 0.25 µm film thicknesses) capillary column and coupled to an HP 5973 mass selective detector (MSD). The temperature of the MSD was set at 230 °C and the quadrapole set at 150°C. Chemicals absorbed by the PDMS fibre were injected

directly into the injector of the GC/MS by thermal desorption (TD) at 250 °C for 2 min. Splitless injection was used with a 1 µL volume. Helium was used as a carrier gas at a constant flow rate of 0.05 $ml.s^{-1}$. Prior to injection the oven was heated to 50°C, after injection the temperature increased at a rate of 0.167°$C.s^{-1}$ until 130°C, followed by a 0.333°$C.s^{-1}$ increase to 210 °C which was held for 10 minutes Component peaks were identified using the NIST10/HPPest/Wiley275 mass spectral libraries.

2.2.5 False codling moth cultures

Egg sheets and pupae were obtained from a commercial culture held at River Bioscience, Addo, South Africa. FCM cultures were reared on a diet which was formulated by Moore *et al.* (2014) and consists of maize meal, wheat germ, milk powder, brewers' yeast, nipagin and ascorbic acid.

2.2.6 Oviposition preference of adult female FCM

To separate the sexes, pupae were placed individually into 40 ml plastic pharmaceutical vials (Omnisurge, South Africa) and allowed to eclose. After emerging, moths were sexed. Male moths were identified by the presence of an anal tuft and dense black tufts of scales on the hind tibia, both of which are not present on female moths (Catling & Aschenborn 1978, Newton 1998). Virgin females and males were paired together in the same vial within 24 h after eclosing. After pairing moths, a food source of cotton wool moistened with 10% sugar water was added to each vial. Trials were conducted the following day after moth pairs were allowed to copulate overnight at a temperature of approximately 25°C.

Trials were conducted in large mesh oviposition cages (150 x 70 x 70 cm) (Fig. 2.1) at a temperature of approximately 25 °C in a laboratory with a large window. Four gravid females were released per trial just before dusk to allow time for oviposition to occur overnight. The time used has been shown to be sufficient, as Love *et al.* (2014) determined that only 4 h under nocturnal conditions are required to allow oviposition to occur on the majority of the fruit. Fruit were inspected the following morning and the number of eggs per fruit was recorded. Choice trials were

conducted using 10 fruit from mature and 10 fruit from juvenile trees of the same cultivar. Fruit were marked as mature or juvenile before being placed randomly into oviposition cages. No choice trials were conducted in the same way as mentioned above except, only 10 fruit from the same cultivar and age group was used.

During 2016, six choice tests were performed on Washington Navel oranges from Douglasdale Farm (Table 2.1) on separate dates. Three choice and no choice tests were done during 2017 to determine FCM oviposition preferences between fruit from mature and juvenile trees for Nova Mandarin, Washington Navel, Cambria Navel and Midnight Valencia oranges respectively. Nova Mandarin, Washington Navel and Cambria Navel oranges were collected from two different farms and three different mature and juvenile orchards respectively (Table 2.1). Oranges used for the Midnight Valencia trials were collected from three different farms (Table 2.1) which each had both mature and juvenile Midnight Valencia orchards. Each replicate per cultivar was conducted with fruit from a different orchard.

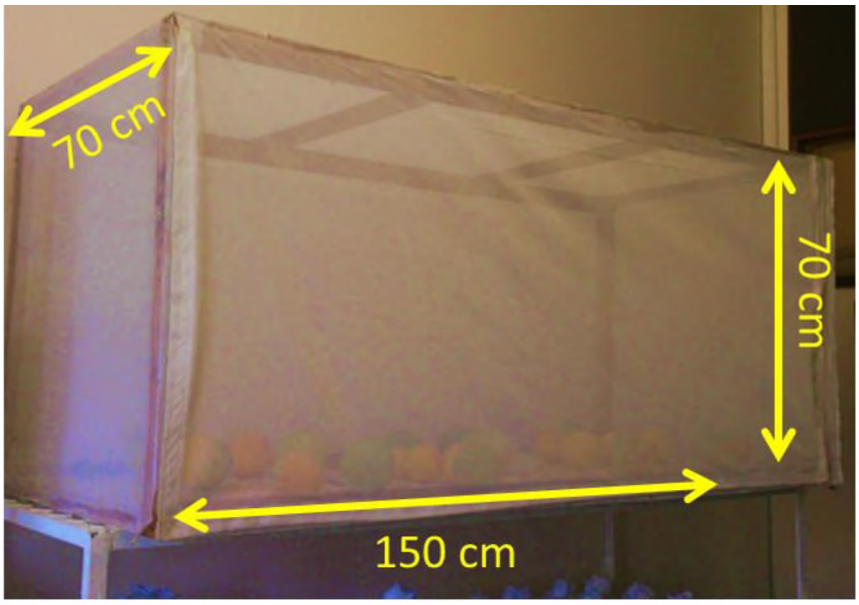

Figure 2.1: Large mesh cages, 150 cm x 70 cm x 70cm, used for oviposition trials.

2.2.7 Fruit susceptibility to FCM

Sheets containing FCM eggs were placed into glass jars, sealed with a mesh lid to allow airflow and kept at 25°C. Fruit used in experiments were collected less than 24 hours before needed. Once hatching had occurred, the mesh lid of the jar

was replaced with a metal lid from which neonate larvae were more easily removed. To ensure neonate fitness, only larvae that were able to crawl up onto the lid were used. Neonate larvae were carefully removed from the lid using a 000 paintbrush and gently placed onto the surface of the fruit. Four neonate larvae were placed onto each fruit. Thirty fruit from mature and juvenile trees respectively were infested per replicate. The fruit were then kept at 25 °C for 20 days to allow larvae to penetrate fruit and develop. Fruit that showed signs of decay before 20 days had passed were removed and dissected to determine and record FCM infestation. After 20 days, oranges were inspected externally for any signs of penetration marks and then dissected carefully to search for larvae or signs of larval damage. The instar and number of larvae retrieved from fruit were recorded.

During 2015, three replicates were performed on Washington Navel oranges from Douglasdale Farm (Table 2.1) to compare FCM infestation between fruit from juvenile and mature trees. The experiment in its entirety was repeated during 2016 as infestation levels were low due to high neonate mortality caused by low humidity (< 40% RH) in the room where trials were conducted. Each replicate was conducted by using neonate FCM from different egg batches. During 2017, the same tests were conducted on Nova Mandarin, Washington Navel, Cambria Navel and Midnight Valencia oranges. Fruit from Nova Mandarin, Washington Navel and Cambria Navel oranges were collected from two different farms and three different mature and juvenile orchards respectively (Table 2.1). Oranges used for the Midnight Valencia trials were collected from three different farms (Table 2.1) which each had both mature and juvenile Midnight Valencia orchards. Each replicate per cultivar was done with fruit from a different orchard.

2.2.8 Developmental rate, weight and fecundity of FCM reared on artificial diet

Artificial diets simulating the nutrient composition of fruit from juvenile and mature trees respectively were prepared to compare FCM growth rate, larval and pupal weight and fecundity. The use of an artificial diet was necessary as larvae (especially 5th instar) were often damaged during fruit dissection which would skew the data collected from fruit infestation trials. Fecundity trials could also not be

conducted as only a limited number of 5th instar larvae (which eventually pupate) could be retrieved from fruit infestation trials.

Fruit nutritional analyses conducted by Bemlab showed that fruit from both mature and juvenile trees consisted of approximately 15% dry weight (section 2.3.2). To prepare the diets, Washington Navel oranges from Douglasdale Farm (Table 2.1) were collected during 2015 from mature and juvenile trees respectively, cut into thin slices and dried in an oven at 60 ºC. The dried fruit were then ground to a powder with the use of a coffee grinder. Fifteen grams of fruit powder from juvenile and mature trees respectively were then added to 35 g maize meal. Nipagin (0.32 g) and ascorbic acid (0.14 g) were added to the diet to prevent microbial contamination. Fifty ml of distilled water was then added to the dry content and mixed thoroughly. It was necessary to add maize meal to the diet, as the diet was too moist for larval survival when 85% water was added to 15% fruit powder only. After preparation, jars were sealed with a mesh lid to allow airflow and baked in an oven at 180ºC for 25 minutes. A control diet was also prepared which contained 50 g maize meal only.

One densely laid egg sheet (2.5 cm x 2.5 cm) was then added to each jar and eggs were left at 25 ºC to hatch and develop. Egg sheets were surface sterilised before being added to the jars by dipping them in 30% of a stock formalin solution for three seconds. After 20 days the sex and weight of 50 randomly chosen larvae and 25 pupae were determined. Pupae were sexed using a dissection microscope. Female pupae were identified by the presence of a genital slit on the lower end of the ventral side of the abdomen while males had a kidney shaped bump (Fig. 2.2). Male larvae were differentiated from female larvae by the presence of a black dot on the lower dorsal side of the abdomen, visible from the 3rd to 5th instar (Fig. 2.2). The number of larvae and instar from each jar were also recorded. Each jar contained between 95 and 163 larvae.

Pupae were collected and kept individually in plastic pharmaceutical vials (40 ml) to separate the sexes. After emerging, moths were sexed as described in section 2.2.6 and paired. Moth pairs were placed in 9 mm diameter Petri dishes to allow mating and oviposition. A food source as described in section 2.2.6 was added to each Petri dish. Once the female moth died, the number of eggs in each Petri was

recorded. Moths were used within 24 h after eclosion. The experiment in its entirety was repeated three times on separate occasions.

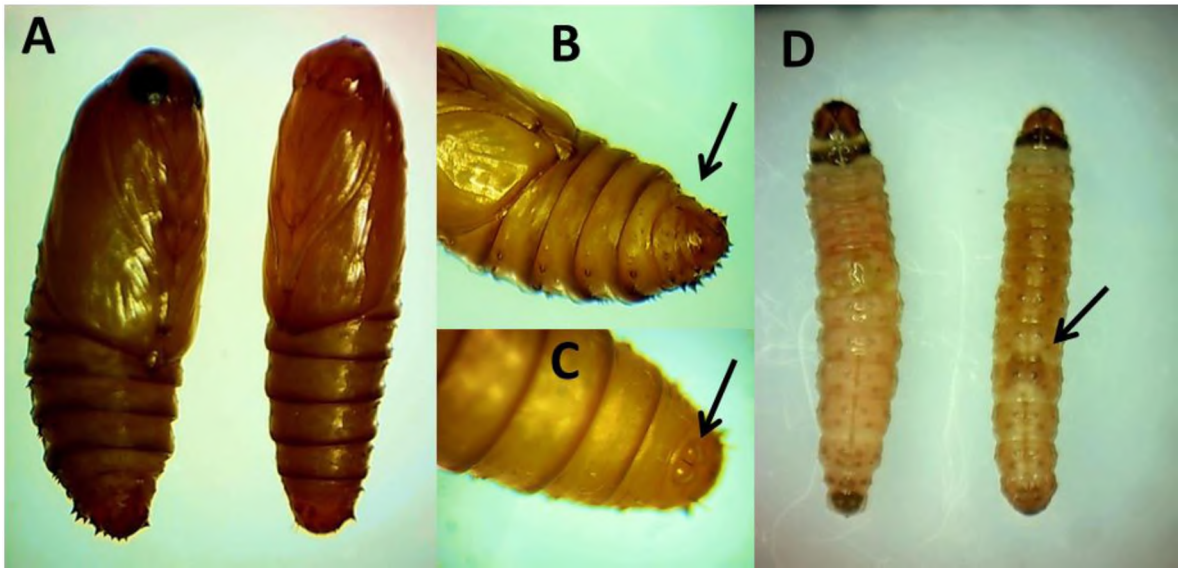

Figure 2.2: Sexing FCM pupae and larvae. (A) Female and male pupae identified by the presence of (B) a genital slit (see arrow) on females and (C) a kidney shaped bump on males (see arrow). (D) Male larvae differentiated from female larvae by the presence of a black dot on the lower dorsal side of the abdomen (see arrow).

2.2.9 Fruit powder dose bioassay

Bioassays were conducted in 25 cell bioassay trays (Nunc™, New York, United States of America). The various diets containing five, 10, 15 and 30% dried fruit powder from mature and juvenile Washington Navel trees from Douglasdale Farm (Table 2.1), harvested during 2015, were prepared as described in section 2.2.8. A thin layer of diet (approximately 1 cm thick) was spread onto square baking trays. Baking trays were sealed with a layer of silver foil and baked in an oven at 180ºC for 25 min. After cooling, the diet was cut up into small 1.5 cm x 1.5 cm sections and placed into bioassay cells. Fifty neonate larvae were treated per fruit powder dose and tree maturity combination (two bioassay trays per dose with 25 larvae per tray) and assays were replicated three times. Therefore, 150 larvae were treated with each dose. One neonate larva was placed into each cell. Trays were sealed with three sheets of paper towel to prevent larvae from escaping and kept at 25 ºC. Survival of larvae was determined after eight days.

2.3 Statistical analysis

All statistical analyses were performed using Statistica version 13.2, 2017 (Statsoft S. Africa Research (Pty) Ltd, Johannesburg, South Africa). Fruit quality, nutritional content and volatile emissions of fruit from mature and juvenile trees were compared using a t-test. The oviposition preference data in no-choice tests and mean number of larvae per fruit collected from fruit in susceptibility trials were found to not be normal. Therefore, a nonparametric Mann-Whitney U test was used to determine significance between fruit from juvenile and mature trees. A chi-square test which compared the absence and presence of eggs on fruit (observed vs. expected) was used to compare oviposition data in choice-tests. A chi-square was which compared the absence and presence of larvae in fruit (observed vs. expected) was also used to compare susceptibility of fruit from mature and juvenile trees to FCM. All other data were analysed using ANOVA. All post-hoc comparisons of means were done using Fisher's LSD test. Significant differences were determined on a 95% probability level.

2.4 Results

2.4.1 Internal fruit quality tests

Mean rind thickness of fruit from juvenile trees was significantly higher than fruit from mature trees for all cultivars tested with the exception of Cambria Navel oranges (Table 2.2). During 2016 and 2017, Washington Navel oranges from juvenile trees had significantly lower sugar and acid content than fruit from mature trees. No significant quality differences were recorded between fruit from juvenile and mature Cambria Navel trees. Fruit from juvenile Midnight Valencia trees had a significantly lower juice percentage and significantly higher sugar content, brix to acid ratio and fruit size than fruit from mature trees.

Table 2.2 Internal quality test results indicating potential quality differences between fruit from mature and juvenile trees. Significant P-values are presented in bold.

Washington Navel 2016							
	Mean mature	**Mean juvenile**	**t-value**	**df**	**P-value**	**SE mature**	**SE juvenile**
Peel mass (g)	789.67 a	1116.8 b	-4.27	10	**0.002**	58.45	61.79
Juice (%)	52.00 a	48.00 b	3.29	10	**0.008**	1.22	0.82
Brix (sugar %)	9.55 a	8.45 b	6.78	10	**<0.001**	0.11	0.12
Acid content (g/100 ml citric acid)	0.94 a	0.67 b	4.26	10	**0.002**	1.22	0.22
Ratio (Brix/acid)	10.36 a	12.56 b	-3.31	10	**0.008**	0.58	0.31
Washington Navel 2017							
	Mean mature	**Mean juvenile**	**t-value**	**df**	**P-value**	**SE mature**	**SE juvenile**
Peel mass (g)	595.17 a	611.17 a	0.58	10	0.574	17.24	21.49
Juice (%)	52.00 a	60.00 b	-5.12	10	**<0.001**	0.82	1.22
Brix (sugar %)	12.25 a	10.00 b	12.48	10	**<0.001**	0.16	0.09
Acid content (g/100 ml citric acid)	1.06 a	0.88 b	3.95	10	**0.003**	0.01	0.04
Ratio (Brix/acid)	11.58 a	11.58 a	-0.02	10	0.984	0.20	0.67
Colour	3.64 a	5.25 b	3.26	70	**0.002**	0.01	0.02
Fruit size / count	80.25 a	87.8 a	0.72	70	0.473	0.08	0.28
Rind thickness (mm)	3.49 a	4.35 b	4.32	70	**<0.001**	0.02	0.16
Nova Mandarin 2017							
	Mean mature	**Mean juvenile**	**t-value**	**df**	**P-value**	**SE mature**	**SE juvenile**
Peel mass (g)	250 a	344.83 b	-2.36	10	**0.040**	**1.09**	0.12
Juice (%)	63.60 a	60.60 a	0.91	10	0.383	0.65	3.21
Brix (sugar %)	12.62 a	11.30 a	1.74	10	0.112	0.32	0.67
Acid content (g/100 ml citric acid)	1.01 a	0.97 a	0.37	10	0.716	0.04	0.07
Ratio (Brix/acid)	12.64 a	11.66 a	1.09	10	0.301	0.73	0.52
Colour	2.00 a	2.14 a	0.47	70	0.640	0.20	0.17
Fruit size / count	70.58 a	70.53 a	-0.05	70	0.960	0.92	0.62
Rind thickness (mm)	1.12 a	2.36 b	4.46	70	**<0.001**	0.18	0.21
Cambria Navel 2017							
	Mean mature	**Mean juvenile**	**t-value**	**df**	**P-value**	**SE mature**	**SE juvenile**
Peel mass (g)	562.00 a	595.17 a	-0.67	10	0.518	44.65	21.49
Juice (%)	58.00 a	59.00 a	-0.76	10	0.465	1.22	0.82
Brix (sugar %)	11.87 a	12.32 a	-0.61	10	0.554	0.34	0.65
Acid content (g/100 ml citric acid)	0.91 a	0.86 a	1.41	10	0.189	0.07	0.44
Ratio (Brix/acid)	13.12 a	14.31 a	-1.23	10	0.246	0.56	0.80
Colour	3.89 a	4.39 a	1.27	70	0.207	0.23	0.32
Fruit size / count	76.64 a	78.97 a	1.61	70	0.111	1.07	0.97
Rind thickness (mm)	3.01 a	3.47 a	1.79	70	0.077	0.18	0.18
Midnight Valencia 2017							
	Mean	**Mean**	**t-value**	**df**	**P-value**	**SE**	**SE**

	mature	juvenile				mature	juvenile
Peel mass (g)	366.5 b	463.33 a	-4.17	10	**0.002**	10.90	20.48
Juice (%)	64.50 a	59.20 b	4.54	10	**0.001**	0.01	0.01
Brix (sugar %)	9.95 b	11.57 a	-4.55	10	**0.001**	0.20	0.29
Acid content (g/100 ml citric acid)	1.31 a	1.27 a	0.48	10	0.644	0.59	1.65
Ratio (Brix/acid)	7.60 b	9.32 a	-2.23	10	**0.049**	0.24	0.73
Colour	2.17 a	2.11 a	-0.19	70	0.848	0.23	0.18
Fruit size / count	72.78 b	75.33 a	2.00	70	**0.049**	0.77	1.01
Rind thickness (mm)	3.13 b	9.90 a	2.93	70	**0.005**	0.15	0.22

Values are compared for each row only. Different letters following values denote significant differences (comparison of means, $P < 0.05$).

2.4.2 Nutritional content of Washington Navel oranges

The pooled ash content of 20 fruit segments collected during 2015 from 20 Washington Navel oranges from mature and juvenile trees respectively was considerably higher in juvenile tree fruit (3.32%) than mature tree fruit (0.26%). The protein content of fruit from juvenile trees was also slightly higher (6.26%) than mature tree fruit (4.92%). No significant differences between the macro- and micro-nutrient composition and moisture content of fruit from juvenile and mature Washington Navel trees were recorded for samples tested during 2017 (Table 2.3).

Table 2.3 Nutritional content of fruit from mature and juvenile Washington Navel orchards collected during 2017.

Nutrient	Mean juvenile	Mean mature	t-value	df	P-value	SE juvenile	SE mature
N (mg/100g)	147.50	153.33	0.51	10	0.619	4.69	10.38
P (mg/100g)	20.13	20.81	0.32	10	0.757	1.32	1.69
K (mg/100g)	155.50	171.67	1.42	10	0.181	3.81	10.57
Ca (mg/100g)	59.45	68.68	0.77	10	0.457	10.62	5.45
Mg(mg/100g)	12.68	12.42	0.28	10	0.783	0.84	0.42
Na (mg/kg)	30.93	31.77	0.28	10	0.785	2.11	2.09
Mn (mg/kg)	0.73	0.56	2.05	10	0.067	0.08	0.02
Fe (mg/kg)	3.17	2.93	0.31	10	0.761	0.62	0.42
Cu (mg/kg)	0.85	0.78	1.45	10	0.177	0.03	0.03
Zn (mg/kg)	0.67	0.62	0.27	10	0.794	0.15	0.11
B (mg/kg)	3.45	3.48	0.17	10	0.865	0.06	0.07
Si (mg/kg)	18.60	18.79	0.06	10	0.955	2.84	1.78
Moisture (%)	85.30	84.90	0.85	10	0.413	0.45	0.13

2.4.3 Washington Navel volatile profile

Only significantly different peak heights of volatile compounds are reported in Table 2.4. All volatile peak heights that were significantly different were higher in fruit from juvenile trees than in fruit from mature trees. Mean peak heights of volatile compounds were between 32% and 69% higher in fruit from juvenile trees than fruit from mature trees (Fig. 2.3).

Table 2.4 Peak heights of volatile compounds emitted by fruit from mature and juvenile Washington Navel trees collected during 2015.

Volatile (pico-amps)	Mean juvenile	Mean mature	t-value	P-value	SE juvenile	SE mature
Linalool	16005278	10068669	-4.02	0.002	690044	1340443
Nonanal	4350972	2523398	-2.68	0.023	528926	453506
Liminene oxide, cis	263744	84890	-3.02	0.013	24316	55275
Decanal	20567307	14171548	-2.74	0.021	1132099	2095088
Undecanal	2365066	1059301	-4.13	0.002	192719	259076
Cyclosativene	1080781	652738	-4.87	0.001	67622	58916
Caryophyllene	8449051	5192581	-3.76	0.004	71952	513707
β-Copaene	10864981	6314074	-6.15	<0.001	501058	565823
α.-Guaiene	300688	130522	-2.75	0.020	20815	59609
Humelene	1440707	993335	-2.98	0.014	103064	113333
Germacrene D	4065665	2751592	-3.25	0.009	297790	285214

All volatile compounds reported had significantly different peak heights. (comparison of means, $P < 0.05$).

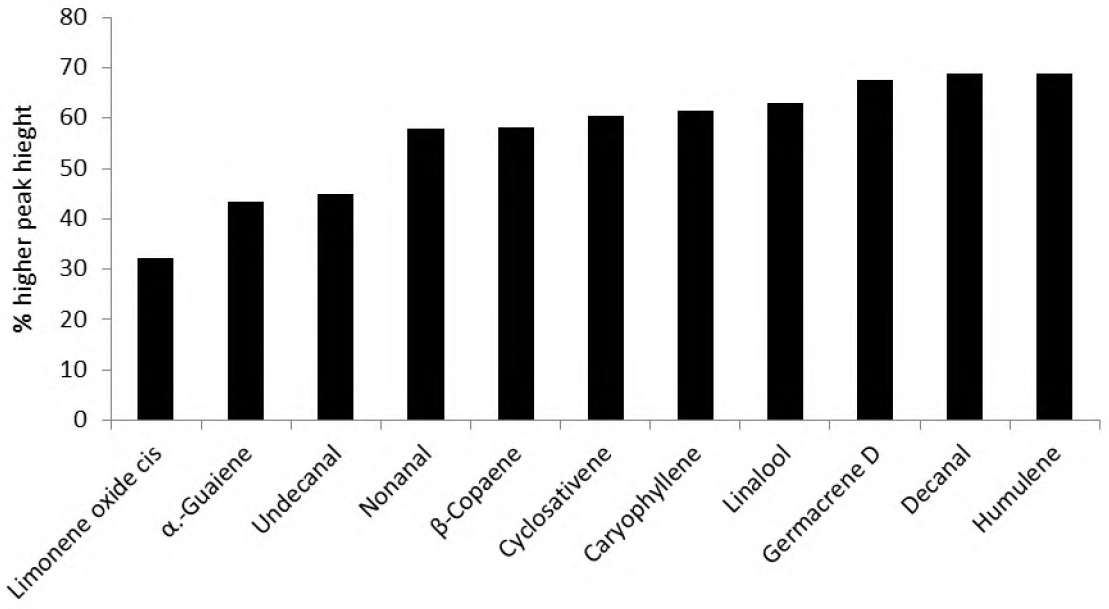

Figure 2.3: Percentage higher mean peak height (pico amp) of volatile compounds emitted by fruit from juvenile Washington Navel trees compared to volatile compounds emitted by fruit from mature trees from the same farm.

2.4.4 Oviposition preference of adult female FCM

Fruit collected from juvenile trees were significantly more preferred for oviposition in both choice and no-choice tests for all cultivars tested with the exception of Midnight Valencia oranges (Table 2.5, Fig. 2.4). The results of this trial are presented in both table and graph format to allow a visual presentation of the data while still reporting χ^2, P and Z-values.

Table 2.5 Mean number of FCM eggs oviposited in choice trials. Means (± standard errors) are presented. Significant P-values are presented in bold.

Choice trials				
Cultivar and year	**Mean eggs juvenile**	**Mean eggs mature**	χ^2	**P-value**
Washington 2016	16.05 (2.07) a	4.17 (3.46) b	226	**<0.001**
Washington 2017	11.50 (2.51) a	3.90 (0.91) b	59.9	**<0.001**
Nova 2017	2.20 (0.40) a	1.07 (0.24) b	6.08	**0.014**
Cambria 2017	12.33 (3.27) a	2.63 (0.74) b	105	**<0.001**
Midnight 2017	8.50 (2.33) a	8.83 (3.28) a	0.962	0.756

Values are compared for each row only. Different letters following values denote significant differences (comparison of means ranks, $P < 0.05$).

Table 2.6 Mean number of FCM eggs oviposited in no-choice trials. Means (± standard errors) are presented. Significant P-values are presented in bold.

No-choice trials				
Cultivar and year			Z-value	P-value
Washington 2017	12.83 (3.08) a	5.43 (0.22) b	2.42	**0.016**
Nova 2017	8.30 (0.20) a	3.53(0.51) b	2.86	**0.004**
Cambria 2017	26.43 (3.45) a	12.73 (2.36) b	4.43	**0.002**
Midnight 2017	7.00 (2.45) a	9.30 (3.32) a	-0.06	0.953

Values are compared for each row only. Different letters following values denote significant differences (comparison of means ranks, $P < 0.05$).

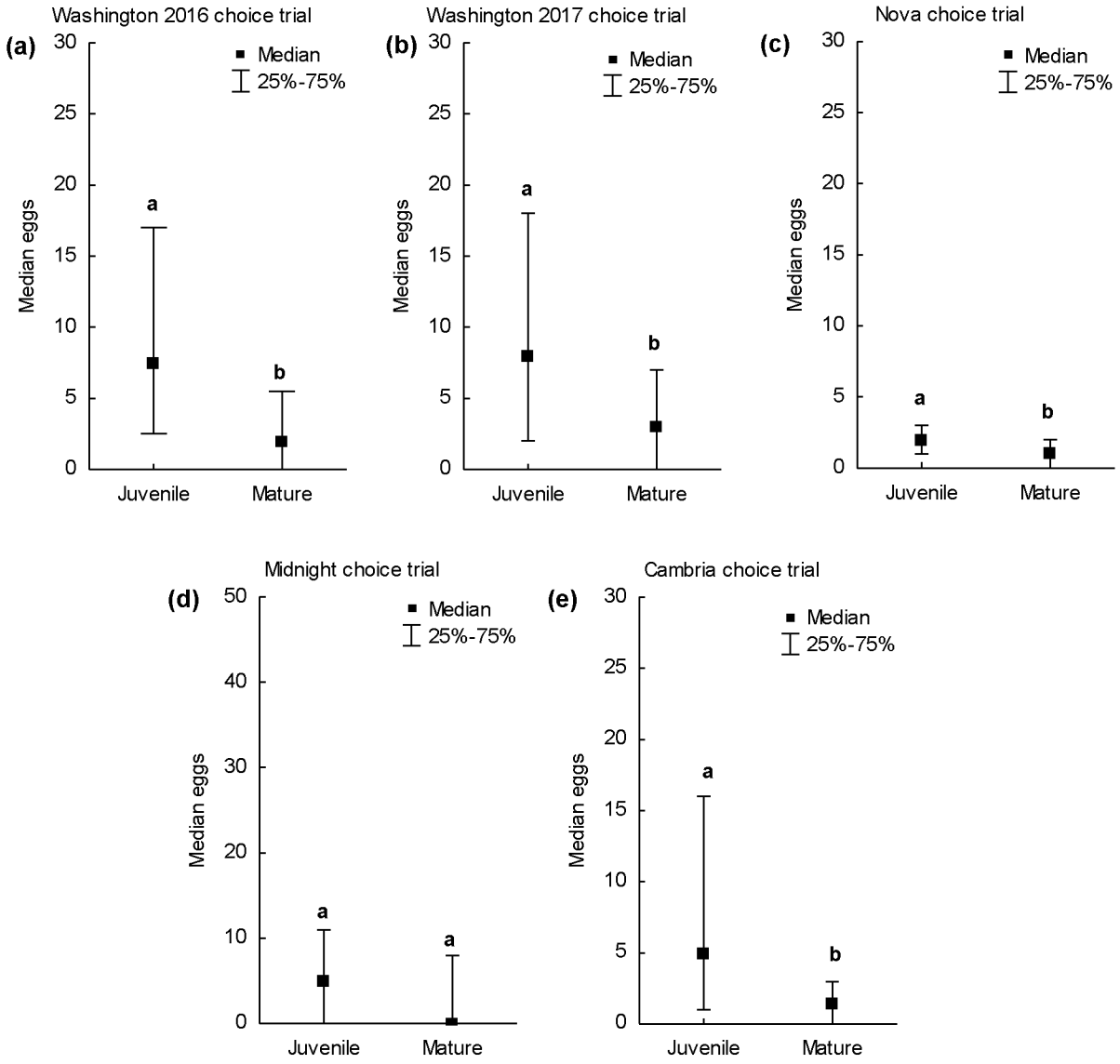

Figure 2.4: Oviposition preference of female FCM in choice trials on fruit from mature and juvenile (a) Washington Navel trees harvested during 2016 (n = 60), (b) Washington Navel oranges harvested during 2017 (n = 30), (c) Nova Mandarins (n = 30), (d) Midnight Valencia oranges and (e) Cambria Navel oranges (n = 30). Different letters denote significant differences (comparison of mean ranks, $P < 0.05$).

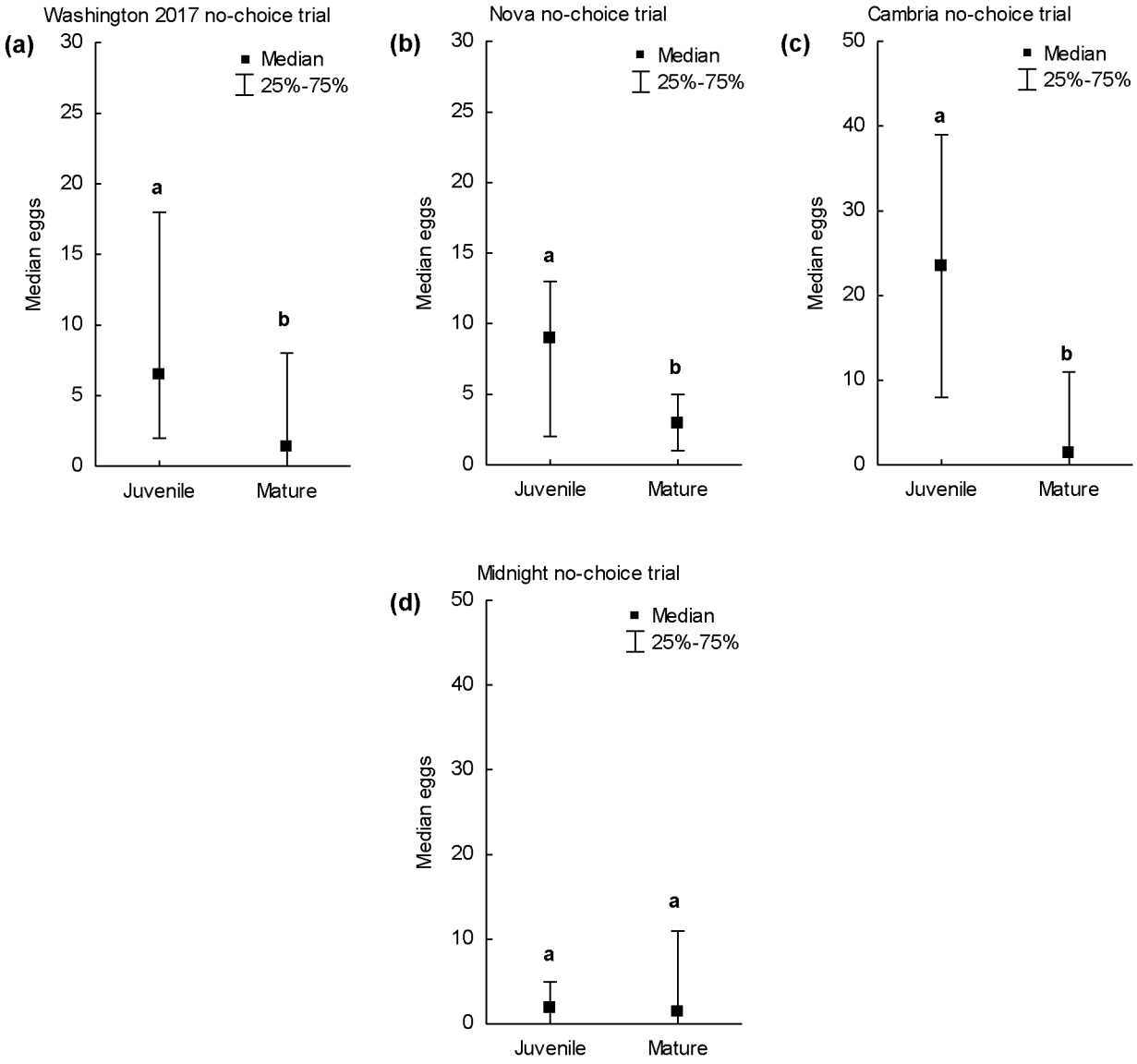

Figure 2.5: Oviposition preference of female FCM in no-choice trials on fruit from mature and juvenile (a) Washington Navel oranges harvested during 2017 (n = 30), (b) Nova Mandarins (n = 30), (c) Cambria Navel oranges and (d) Midnight Valencia oranges (n = 30). Different letters denote significant differences (comparison of mean ranks, $P < 0.05$).

2.4.5 Fruit susceptibility to FCM

During 2015, Washington Navel oranges from juvenile trees were significantly more susceptible to FCM attack (24.67 %) (Fig. 2.6) than fruit from mature trees (12.33 %) (= 4.49, P = 0.034). No significant difference in FCM damage (χ^2 = 3.06, P = 0.053, χ^2 = 3.28, P = 0.070) was recorded in Washington Navels from juvenile

trees during 2016 (58.78%) and 2017 (47.78%) compared to fruit from mature trees, with 41.11% and 34.44% damage respectively. Fruit from both juvenile Nova Mandarin (77.78% damage) and Midnight Valencia (85.56% damage) trees were significantly more susceptible to FCM attack than fruit from mature trees with 46.67% and 58.89% damage respectively ($\chi^2 = 18.53$, $P < 0.001$, $\chi^2 = 13.73$, $P < 0.001$). Fruit from juvenile and mature Cambria Navel trees were equally susceptible to FCM attack ($\chi^2 = 0.02$, $P = 0.900$). Significantly more larvae were retrieved from fruit collected from juvenile Washington Navel trees during 2015, juvenile Nova Mandarin trees and juvenile Midnight Valencia trees than fruit from mature trees (Table 2.7), which correlates with the significantly higher FCM damage levels recorded (Fig. 2.6). No significant differences in larval development rate were measured between fruit from juvenile and mature trees in any of the trials. Although not significant, higher percentages of fifth instar larvae were retrieved from juvenile tree fruit than mature tree fruit harvested from Washington Navel orchards during 2015 and 2017, and from Cambria Navel orchards and Midnight Valencia orchards during 2017 (Fig. 2.7). Percentages of fifth instars collected from Washington Navel oranges during 2015 and 2016 and from Cambria Navel oranges and Midnight Valencia oranges during 2017 were 63.64%, 53.85%, 28.95% and 24.11% in fruit from juvenile trees and 36.36%, 50%, 19.35% and 21.88% in fruit from mature trees respectively. A higher percentage of pupae were retrieved from fruit collected from juvenile Washington Navel trees (14.89% pupae) than juvenile tree fruit (2.7% pupae) during 2017. Although a higher percentage fifth instar larvae were retrieved from fruit harvested from juvenile Cambria Navel trees than fruit from mature trees, a higher percentage of pupae (12.9% pupae) were retrieved from mature tree fruit than juvenile tree fruit (2.63% pupae).

Table 2.7 Survival of FCM on fruit from juvenile and mature trees. Significant P-values are presented in bold.

Cultivar and harvest year	Number of larvae from juvenile tree fruit	Number of larvae from mature tree fruit	Z-value	P-value
Washington 2015	22 a	11 b	-2.11	**0.035**
Washington 2016	58 a	53 a	-1.67	0.094
Washington 2017	44 a	37 a	-1.55	0.122
Nova 2017	161 a	56 b	-4.17	**< 0.001**
Cambria 2017	38 a	31 a	-0.66	0.580
Midnight 2017	118 a	71 b	-3.51	**< 0.001**

Values for total number of larvae are compared for each row respectively. Different letters following values denote significant differences (comparison of mean ranks, $P < 0.05$).

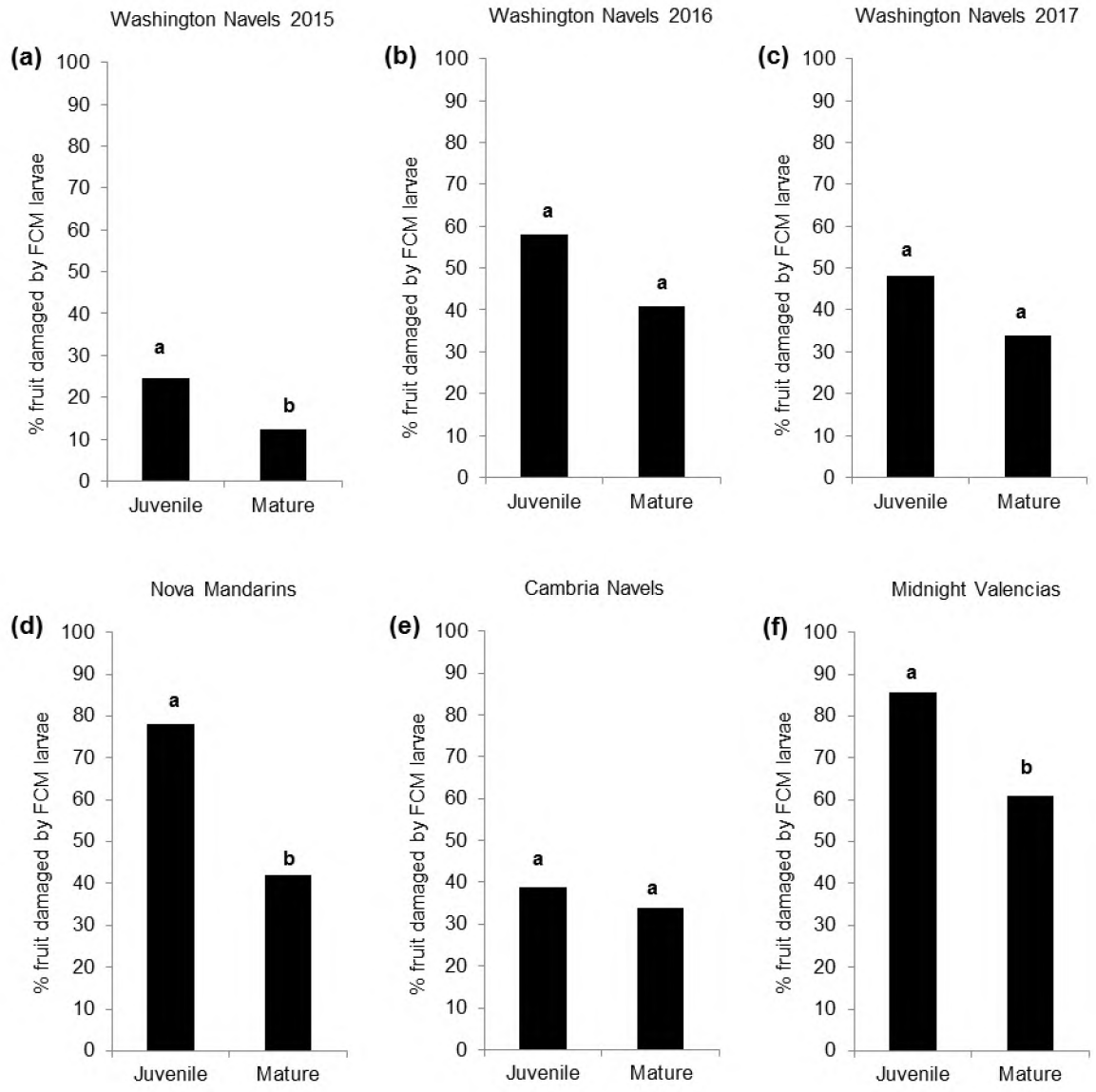

Figure 2.6: Susceptibility of fruit from mature and juvenile (a) Washington Navel oranges harvested during 2015 (n = 90), (b) Washington Navel oranges harvested during 2016 (n = 90), (c) Washington Navel oranges harvested during 2017 (n = 90), (d) Nova Mandarin oranges (n = 90), (e) Cambria Navel oranges (n = 90) and (f) Midnight Valencia oranges (n = 90). Different letters above bars denote significant differences (chi-square tests, $P < 0.05$).

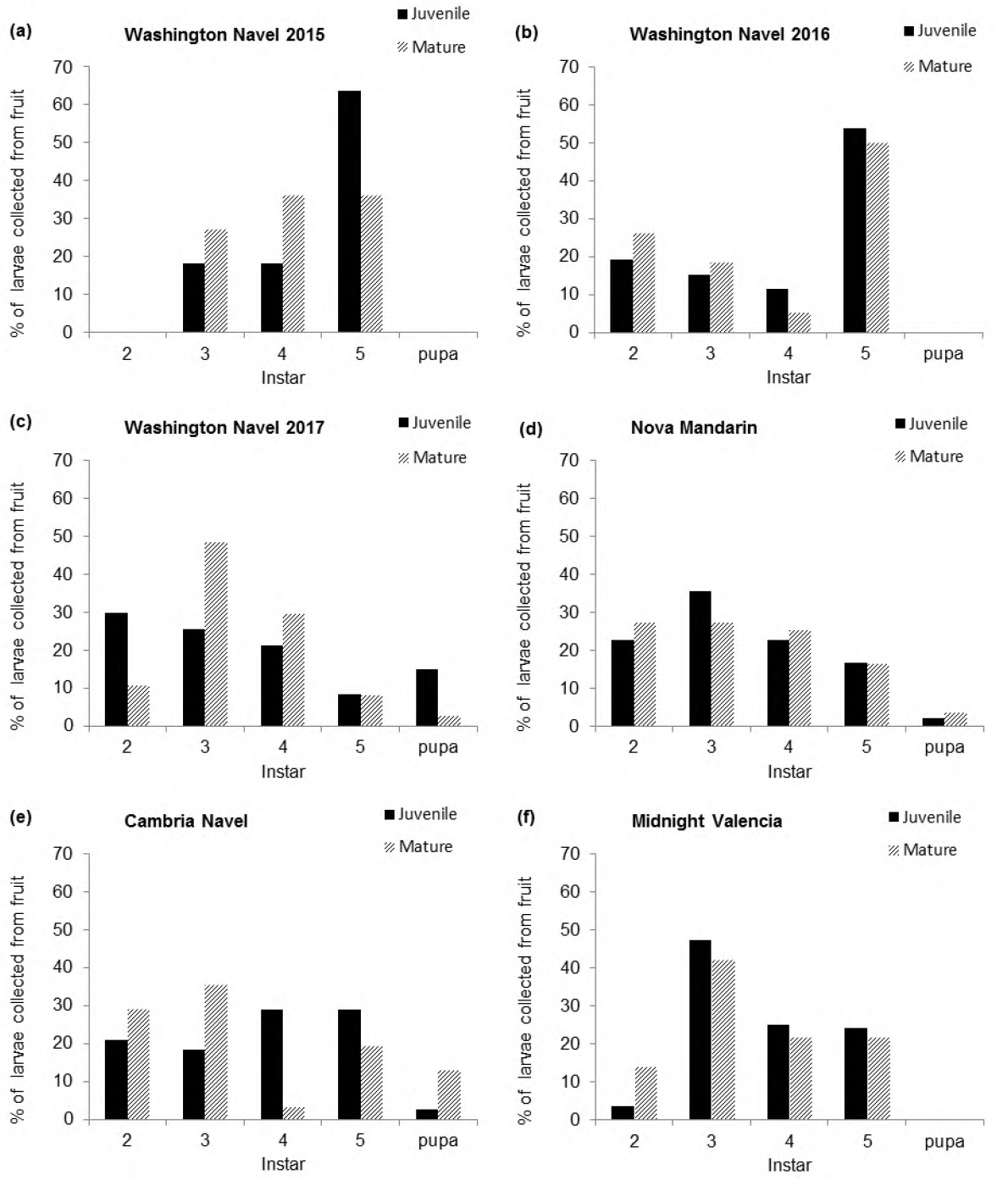

Figure 2.7: Comparison of larval development in fruit from juvenile and mature trees. The distribution of FCM life stages are presented as a percentage of total larvae retrieved from (a) Washington Navel oranges harvested during 2015, (b) Washington Navel oranges harvested during 2016, (c) Washington Navel oranges harvested during 2017, (d) Nova Mandarin oranges, (e) Cambria Navel oranges and (f) Midnight Valencia oranges.

2.4.6 Developmental rate, weight and fecundity of FCM reared on artificial diet

The two-way ANOVA for mean larval weight (Fig. 2.8) showed no interaction between main effects gender (2 levels, male and female) and diet (3 levels, control, juvenile and mature) ($F_{(2,\ 444)}$ = 0.38, P = 0.685). Mean weight of larvae was determined regardless of instar. The mean weight of female larvae (0.0232 ± 0.0015 g) reared on a diet containing 15% fruit powder from mature Washington Navel trees was significantly lower than the mean weight of female larvae reared on both a diet containing 15% dried fruit powder from juvenile Washington Navel trees (mean weight = 0.0265 ± 0.0013 g, P < 0.001) and a control diet (0.0260 ± 0.0013 g, P = 0.004) which contained maize meal only. The mean weight of male larvae (0.0190 ± 0.0016 g) reared on the mature tree diet was also significantly lower than the weight of male larvae (0.0224 ± 0.0015 g) reared on the juvenile tree diet (P = 0.003). No significant differences in mean weight between both male and female pupae were recorded for FCM reared on the three different diets (Fig. 2.8).

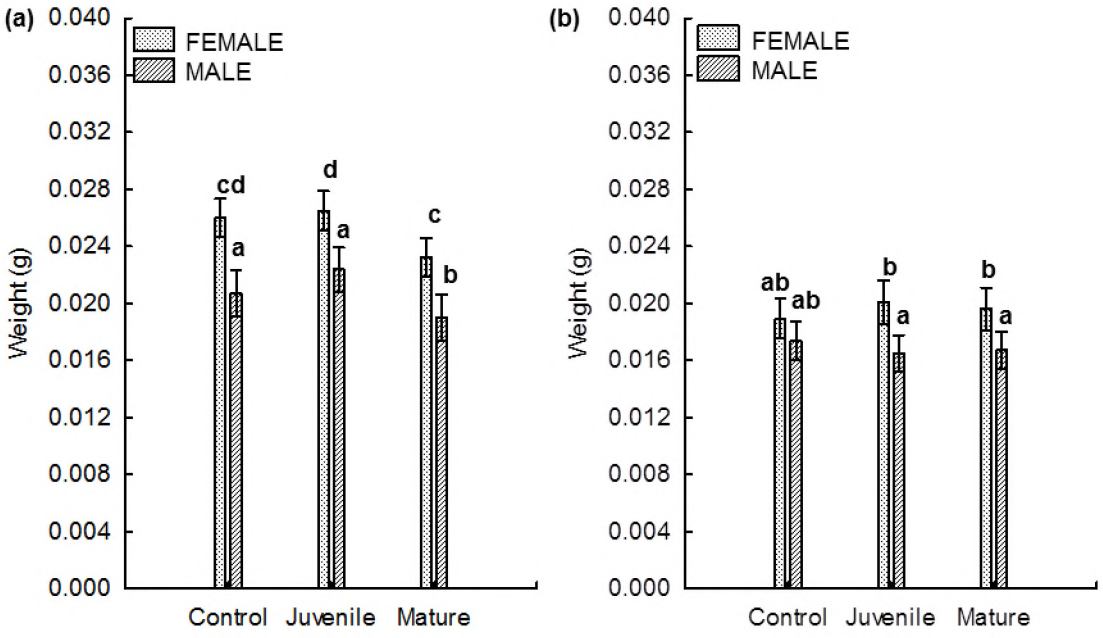

Figure 2.8: Mean weight of (a) larvae (n = 150) and (b) pupae (n = 75) reared on diets containing 15% dried fruit powder from mature and juvenile Washington Navel trees and a control diet containing only maize meal. Different letters above bars denote significant differences (comparison of means, P < 0.05).

The results for larval development of FCM reared on three diets were analysed using a one-way ANOVA and showed significant differences ($F_{(2, 6)}$ = 11.203, P = 0.009) between diets (Fig. 2.9). The mean percentage of pupae retrieved from the control diet (55.89 ± 5.29%) was significantly higher than the mean percentage of pupae retrieved from the juvenile tree diet (32.83 ± 5.17%, P = 0.011) and the mature tree diet (27.84 ± 2.32%, P = 0.004).

The one-way ANOVA for mean eggs oviposited by female FCM reared on different diets showed no significant differences ($F_{(2, 40)}$ = 0.27, P = 0.76) between diets.

The results of a chi-square test showed that there was no significant difference between the female to male ratio of FCM (n = 225) reared on the three different diets (χ^2 = 0.57, P = 0.751). FCM reared on the mature tree diet had the highest female to male ratio of 1.24, followed by the juvenile tree diet with a ratio of 1.14 and then the control diet with the lowest female to male ratio of 1.06.

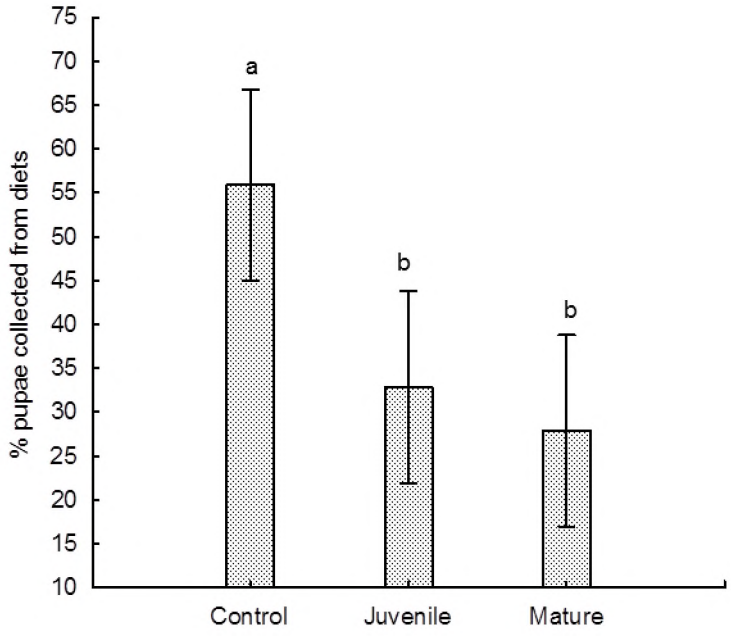

Figure 2.9: Comparison of larval development, expressed as percentage of pupae collected from diets containing 15% dried fruit powder from mature and juvenile Washington Navel trees and a control diet containing only maize meal. Different letters above bars denote significant differences (comparison of means, P < 0.05).

2.4.7 Fruit powder dose bioassay

The two-way ANOVA for mean survival (Fig. 2.10) showed no interaction between main effects, diet (two levels, juvenile and mature) and percentage fruit powder (5, 10, 15 and 30) ($F_{(3, 40)}$ = 0.63, P = 0.601). The result of a one-way ANOVA for pooled mean survival showed significant differences ($F_{(1, 40)}$ = 9.12, P = 0.004) between diets. Mean survival of 58.5 ± 3.74% in juvenile tree diets was significantly higher than mean survival of 49.17 ± 3.91% recorded in mature tree diets.

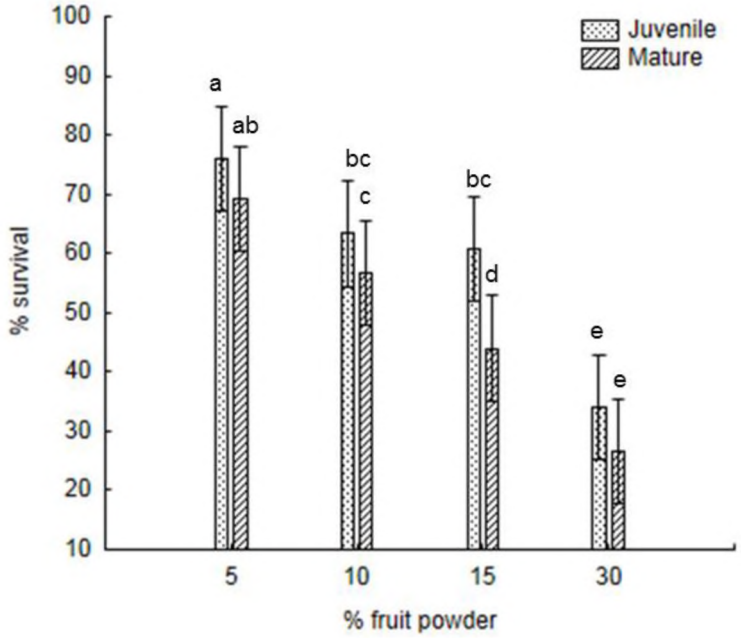

Figure 2.10: Mean survival of FCM larvae (n = 150) placed onto diets containing increasing percentages of dried fruit powder from juvenile and mature trees respectively. Different letters above bars denote significant differences (comparison of means, P < 0.05).

2.5 Discussion

Plant volatiles play a major role in oviposition selection of insect herbivores (Renwick 1989, Bruce *et al.* 2005). Certain volatile compounds produced in citrus leaves and fruit have been shown to have a strong influence on oviposition choice and the number of eggs laid by certain insect species. Newton (1989) determined that FCM prefer to lay eggs on damaged citrus fruit and thus concluded that olfactory stimuli may have more influence on selecting oviposition sites than visual stimuli. A study conducted by Ioannu *et al.* (2012) showed that the oviposition preference of female Mediterranean fruit fly is greatly influenced by the type and quantity of essential oils produced by different citrus types. Methanol extracted from citrus leaves has also been shown to induce oviposition behaviour of the citrus feeding swallowtail butterfly, *Papilio xuthus* Linnaeus (Lepidoptera: Papilionidae) (Ohsugi *et al.* 1985). Although only four citrus volatiles were tested, a study conducted by Soutar *et al.* (2015) showed gravid female FCM to prefer a blend of naphthalene, ocimene and β-caryophyllene for oviposition above each volatile compound on its own in Y-tube experiments. Possible differences in volatile compounds produced by juvenile and mature citrus trees could cause either juvenile or mature trees to be more attractive for oviposition by FCM.

Results of this laboratory study have shown that fruit from juvenile trees were significantly more attractive for oviposition than fruit from mature trees of the same cultivar, with the exception of Midnight Valencia oranges. However, although care was taken not to harvest fruit from damaged branches, this exception may be due to damage caused to trees by frost followed by warm winds (per obs, W. Kirkman, pers comm) which could have changed the volatile composition of the Midnight Valencia fruit used in the experiment. Volatiles from fruit from juvenile Washington Navel orchards peaked at significantly higher levels (pico amp) for a total of 11 volatile compounds (Fig. 2.5) recorded in SPME-GC/MS detection, than volatiles from fruit from mature trees harvested from the same farm. The higher emission of such volatile compounds could explain why fruit from juvenile trees are preferred by FCM for oviposition above fruit from mature trees. However, further studies are required to confirm that this finding is not an anomaly.

Greater oviposition preference of FCM for fruit from juvenile trees was not necessarily linked to higher susceptibility to FCM. Cambria Navel oranges from juvenile trees were equally susceptible to FCM attack as fruit from mature trees, even though fruit from juvenile trees were significantly more preferred for oviposition. In the case of Midnight Valencia oranges the opposite was true. Fruit from juvenile Midnight Valencia trees were significantly more susceptible to FCM attack than fruit from mature trees but were equally preferred for oviposition. Various studies have indicated that higher oviposition of host plants is not necessarily correlated to higher survival of offspring (Zalucki & Kitching 1982, Thompson 1988, Berdegué *et al.* 1998). Once larvae have hatched, the ability of insects to feed and develop on plant hosts will largely depend on physical and internal chemical properties of the plant. A study conducted by Love *et al.* (2014) showed that Cambria Navel oranges are less susceptible to FCM attack than other late maturing Navel oranges. The generally low susceptibility of Cambria Navel oranges could explain why tree age did not have a significant influence on fruit susceptibility to FCM.

Love *et al.* (2014) concluded that Fischer Navels were possibly less susceptible to FCM attack than Fukumoto and Newhall Navel oranges because they were more difficult to penetrate due to higher peel mass. However, results of this study indicate that higher peel masses and rind thickness may possibly increase fruit susceptibility to FCM attack. Of all the fruit quality parameters tested, higher peel masses and rind thickness were the only parameters that could consistently be linked to significantly higher FCM attack. All fruit collected from juvenile trees with the exception of Cambria Navels had significantly thicker rinds than fruit collected from mature trees of the same cultivar. Higher FCM susceptibility in fruit with thicker peels might be due to the higher nutritional value of fruit peels than fruit pulp, which mostly consists of water.

According to Levin (1976), plant defence against herbivores may depend on many different qualities such as plant texture and composition, the presence or absence of essential nutrients, pH or osmotic pressure of plants or the presence or absence of harmful secondary chemical products. Surprisingly, no significant differences in the macro or micro nutrient content were recorded between fruit

harvested during 2017 from juvenile and mature Washington Navel trees. These results are in contrast to those reported by Khalid *et al.* (2012) who recorded higher manganese and iron content in fruit from juvenile Kinnow Mandarin trees than from mature trees. However, results of this study did show substantially higher protein and ash content in fruit from juvenile Washington Navel trees than fruit from mature trees harvested during 2015.

The addition of 15% fruit powder from mature Washington Navel trees did not have a significant influence on the female to male ratio of larvae surviving and developing in the diet compared to a diet containing 15% fruit powder from juvenile trees or a control diet which did not contain any fruit powder. Although the mean weight of larvae reared on the mature tree diet was significantly lower than the weight of larvae reared on the juvenile tree and control diet, the mean weights of pupae were not significantly lower, indicating that larval growth is slightly retarded in the mature tree diet, but insects still reached their full weight by the time they pupated, which explains why no significant differences in fecundity between FCM reared on the three diets were recorded. In contrast to what was expected, FCM development in the control diet, which consisted of maize meal only and had a lower nutrient content than diets containing fruit powder, was significantly faster than larval development in the juvenile and mature tree diets. The reduced growth rate in diets containing fruit powder could be because of harmful secondary metabolites present in citrus fruit which is not present in maize meal. Plants are known to produce various secondary metabolites which aid in defending them against herbivores. These metabolites are diverse and could range from terpenoids to steroids, alkaloids, phenolic, nonprotein amino acids and many more (Mithöfer & Boland 2012). The presence of such harmful secondary plant metabolites could explain the correlation between increased fruit powder content and lower larval survival and development recorded in this study. The significantly lower overall survival and development of larvae reared on mature tree diet than larvae reared on the juvenile tree diet could also indicate that mature citrus trees might produce higher concentrations of plant defence chemicals than juvenile trees.

The results of this laboratory study indicate that juvenile citrus trees are more susceptible to FCM infestation than mature trees, but the degree of vulnerability

varies depending on cultivar. In addition to internal plant defence differences between mature and juvenile trees, differences in the microclimates of juvenile and mature orchards will also have an impact on FCM and their natural enemies. The influence of age on orchard microclimates and FCM ecology will be discussed in Chapters 4 and 5.

CHAPTER 3

The influence of nutritional differences between fruit from juvenile and mature citrus trees on false codling moth susceptibility to entomopathogens

3.1 Introduction

According to Cory & Hoover (2006), differences in plant chemistry can alter the susceptibility of insects to pathogen infection. Poor plant nutritional quality has been shown to not only reduce insect performance, but may also enhance or reduce their susceptibility to diseases. Some plant chemicals have shown the ability to modify the growth and physiology of insects which affect their susceptibility to pathogens either positively or negatively (Ali *et al.* 1998). For example, some phytochemicals such as tannins bind to virus occlusion bodies (OBs) in the insect midgut, thus reducing subsequent virus infectivity (Keating *et al.* 1990, Felton & Duffey 1990), while alkylation has been shown to improve *Bacillus thuringiensis* (Berliner) infectivity by enhancing the solubility of crystal proteins (Ludlum *et al.* 1991). A study conducted by Stevenson *et al.* (2010) showed that isoflavonoids produced by chickpea, *Cicer arietinum* (Linneaus), are able to deactivate OBs of nucleopolyhedroviruses (NPVs) on the surface of chickpea plants, even before being consumed by insect hosts. The degree of pathogen infectivity, especially of viruses and bacteria has been shown to vary greatly depending on the food source of the insect host (Kouassi *et al.* 2001, Ali *et al.* 2004). Similar results have been recorded for EPN (Barbercheck *et al.* 1995, Hazir *et al.* 2016) and EPF (Gatarayiha *et al.* 2010). Although interactions between plants and entomopathogens are known, the exact cause for these variations in pathogen susceptibility is unclear.

Results from Chapter 2 showed Washington Navels harvested in 2015 from juvenile trees to be significantly less susceptible to false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) than fruit from mature trees, even though from the same farm (Douglasdale Farm Table 2.1). The study concluded that the lower susceptibility of fruit from mature trees to FCM damage was possibly because mature trees produce higher concentrations of

phytochemicals, which are harmful to insects. In this study we aimed to determine if differences in plant chemistry between fruit from mature and juvenile citrus orchards had an influence on FCM susceptibility to entomopathogenic fungi (EPF), entomopathogenic nematodes (EPN) and Cryptophlebia leucotreta granulovirus (CrleGV).

3.2 Materials and methods

3.2.1 False codling moth cultures

Egg sheets were obtained from a commercial culture held at River Bioscience, Addo, South Africa. FCM cultures were reared on a diet which was formulated by Moore *et al.* (2014) and consists of maize meal, wheat germ, milk powder, brewer's yeast, nipagin and ascorbic acid.

3.2.2 Preparation of diets for insect rearing and CrleGV bioassays

Three diets containing 15% dried fruit powder from mature and juvenile Washington Navel trees (harvested during 2015) from Douglasdale Farm (Table 2.1) and a control diet which contained maize meal only were prepared as described in section 2.2.8. Diets used for CrleGV bioassays were prepared in the same manner, with the exception that they were not baked in glass jars but in square baking trays. A thin layer of diet (approximately 1 cm thick) was spread onto baking trays. Baking trays were sealed with a layer of silver foil and baked in an oven at 180ºC for 25 minutes.

3.2.3 Entomopathogenic fungi bioassays

FCM larvae reared on three different diets, as described in section 3.2.2, were exposed to increasing concentrations of EPF conidia. *Beauveria bassiana* (Balsamo) Vuillemin (isolate G Ar 17 B3) spores were obtained from Citrus Research International, following mass production by Agrauxine (Loches, France). Spore suspensions were prepared by adding a small quantity of dry spores to 20 ml

distilled water (autoclaved at 120ºC for 20 min), supplemented with 0.01% Tween 20. The suspensions were vortex mixed for 5 min before determining the stock concentration. A Neubauer haemocytometer (Hirschmann®, Germany) was used to determine stock suspension concentrations and to prepare dilutions of $1x10^5$, $1x10^6$ and $1x10^7$ conidia.ml^{-1} required for bioassays. Prior to use, the haemocytometer and cover slips were rinsed with 70 % ethanol and dried with paper towel. Two counts were made for each replicate using a 1/100 dilution. The average count was used in further calculations.

The concentration of the stock suspension was then determined using the following formula: conidia.ml^{-1} = df x d x c.

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

Fifty grams of sieved, autoclaved sand (120ºC for 20 min) mixed with 5 ml of a $1x10^5$, $1x10^6$ or $1x10^7$ conidia.ml^{-1} suspension were added to a Petri dish (90 mm diameter, Fig. 3.1). Five ml of water only were added to soil used for control plates. Ten 5th instar FCM were placed into each dish and incubated for 7 d at 25 °C. Five Petri dishes were used per conidia suspension and insect rearing diet combination. Bioassays were replicated three times on separate occasions. FCM mortality was determined after 7 d. Larvae and pupae that did not move after gentle prodding were regarded as dead. Live larvae (now pupae) were removed from Petri dishes and placed into plastic pharmaceutical vials (40 ml) (Omnisurge, South Africa) containing sterilised, sieved sand (Fig. 3.1). No more than three pupae were placed into each vial. After adding pupae to the vials, they were plugged with sterile cotton wool and incubated as before. Any pupae that did not eclose 10 d after the first moth emerged and that did not move after gentle prodding were regarded as dead. The experiment in its entirety was repeated twice on different dates.

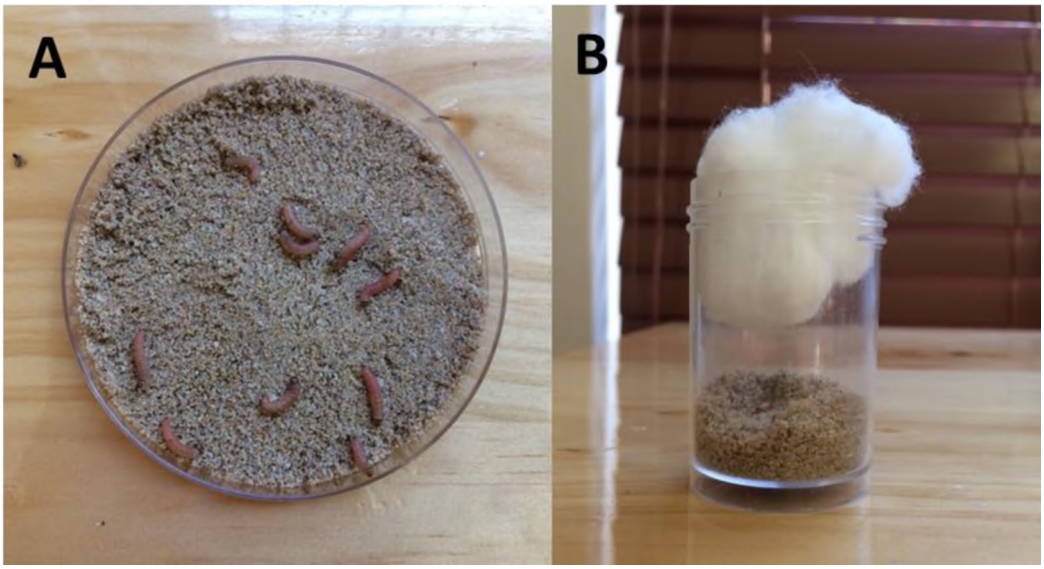

Figure 3.1: (A) Petri dish containing 10 FCM larvae on autoclaved sand mixed with 5 ml conidia suspension. (B) FCM eclosion chamber.

3.2.4 Entomopathogenic nematode bioassays

FCM larvae and pupae were individually exposed to *Heterorhabditis bacteriophora* (Poinar) IJs obtained from River Bioscience, Addo, South Africa in multiwell bioassay plates (24 wells, flat bottom, Nunc™, New York, United States of America). Two bioassay plates, each containing 20 larvae or pupae, were used per nematode concentration and insect rearing diet combination. Each well was lined with a circular paper disc (13 mm diameter) before FCM larvae or pupae were added (Fig. 3.2). FCM were then inoculated individually with the required concentration of nematodes in 50 µl of water (Navon & Ascher 2000). Control plates received 50 µl of distilled water only. Individual larvae were inoculated with 12, 25 and 50 IJs, while pupae, which are less susceptible to EPN (Malan *et al*. 2011), were inoculated with 50, 100 and 200 IJs. To retain larvae in their individual wells, multiple layers of paper towelling were added onto trays before closing them with a lid. Four trays were then grouped together and tightly bound with rubber bands. After inoculation, plates were placed inside plastic containers, lined with moistened paper towels and closed with the lid to maintain high humidity levels of approximately 95% RH. Plastic containers were then incubated in a dark growth chamber at 25 °C for 48 h, after which FCM mortality was determined by means of gentle prodding. The experiment was repeated three times on separate occasions.

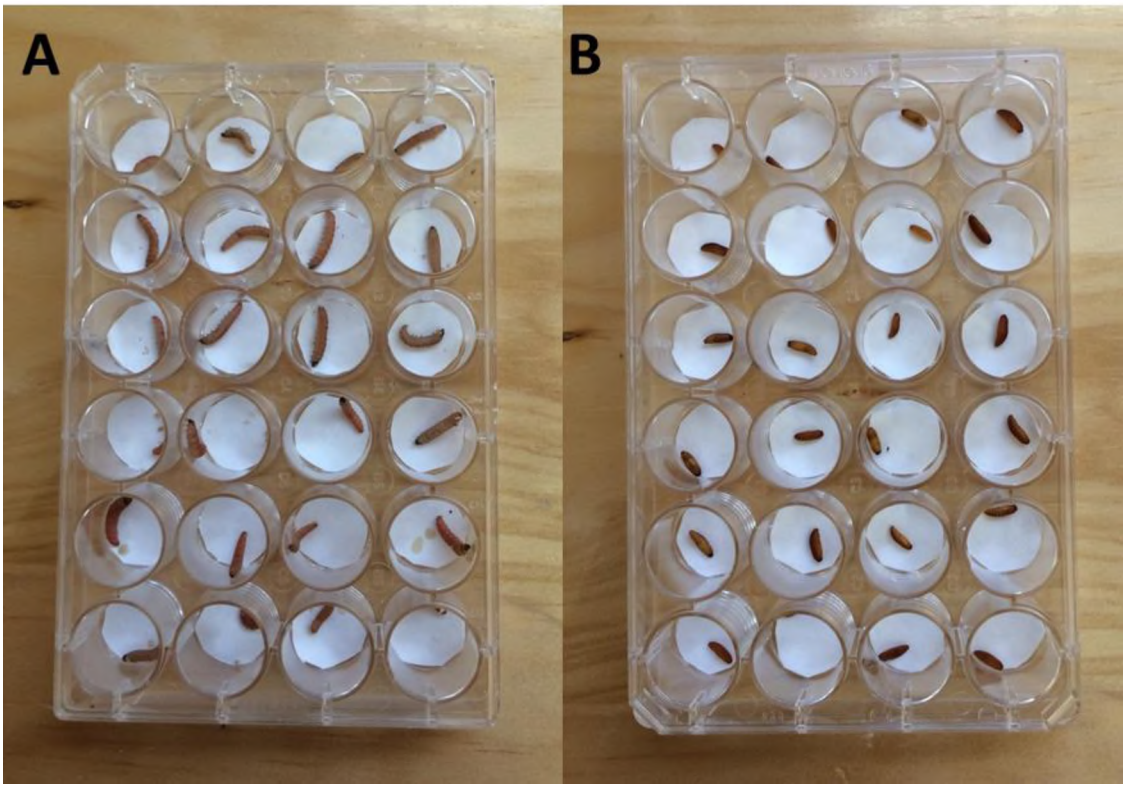

Figure 3.2: Bioassay plates containing (A) larvae and (B) pupae.

3.2.5 CrleGV droplet feeding bioassay

The droplet feeding bioassay technique (Jones *et al.* 1993, Pereira-da-Conceicoa *et al.* 2012) was used to determine the susceptibility of neonate FCM to CrleGV when feeding on diets with different nutrient composition as described in section 3.2.2. Jars containing recently hatched FCM were covered with Parafilm M® (Bemis, United States of America) and secured with a lid. Neonates were then left to crawl onto the Parafilm M® and were removed for droplet feeding after adequate numbers of neonates were present. Larvae were then droplet fed with three different concentrations of virus inoculum. Seven-fold serial dilutions (1:7 dilution factor) were carried out using the virus stock concentrations obtained from River Bioscience, Addo, South Africa. The virus occlusion bodies (OBs) were serially diluted in sterile microfuge tubes and distilled water to obtain final concentrations of 2×10^4, 5×10^5, 7×10^6 OBs/ml. Thereafter, 1% Brilliant blue R dye (USB Corporation, United States of America) was added to each virus suspension. Numerous virus suspension droplets of 2 µl each were placed onto the Parafilm M® to allow neonate FCM to feed

without drowning (Fig. 3.3). Control larvae were fed with sterile distilled water containing 1% blue dye only.

Bioassays were conducted in multiwell bioassay trays. Plugs of each diet as described in section 3.2.3, measuring 1 x 1 cm, were added to each of 20 cells per bioassay tray (Fig. 3.3). After larvae had finished feeding on the various virus-dye suspensions, they were removed and placed individually into bioassay cells containing the respective diets. Three bioassay trays with 20 larvae each were used per virus concentration for the control diet (60 larvae treated per replication), five trays with 20 larvae each per virus suspension were used for diets containing 15% fruit powder from juvenile Washington Navel oranges (100 larvae treated per replication) and eight trays with 20 larvae each per virus suspension were used for diets containing 15% fruit powder from mature Washington Navel oranges (160 larvae treated per replication). The number of larvae used per treatment was adapted due to the higher control mortality recorded in the control treatments of diets containing 15% fruit powder. The experiment was replicated three times on separate occasions.

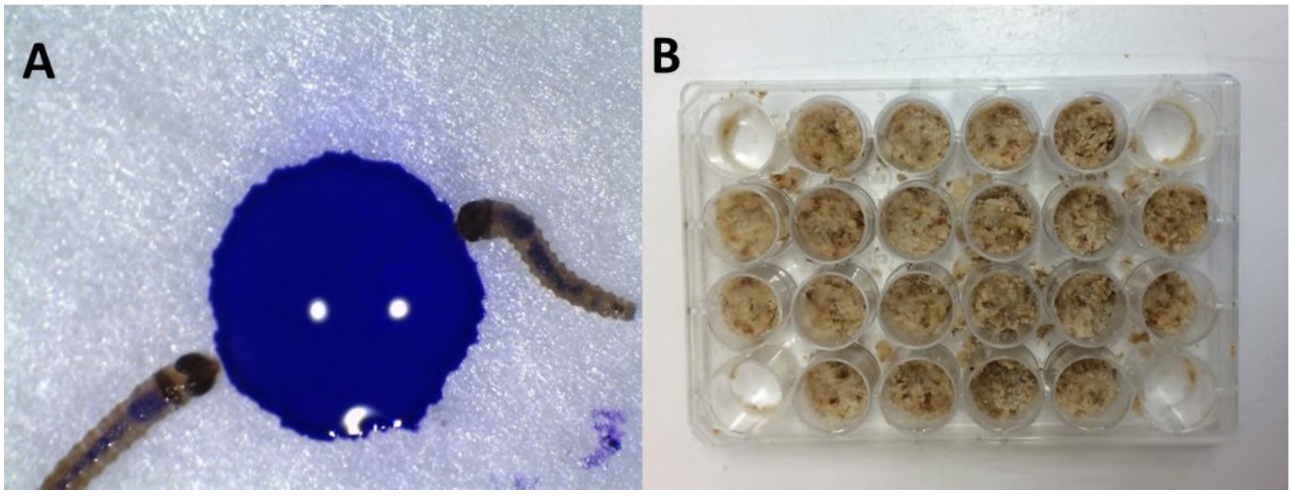

Figure 3.3: (A) Blue gut of neonate FCM larva ingesting a virus-dye suspension (Photo credit: J.K. Opoku-Debrah). (B) A 24-well bioassay tray with artificial diet containing 15% fruit powder.

3.3 Statistical analyses

All statistical analyses were performed using Statistica version 13.2, 2017 (Statsoft S. Africa Research (Pty) Ltd, Johannesburg, South Africa). Data sets were analysed using ANOVA. Post-hoc comparisons of means were compared using Fisher's LSD test. Significant differences were determined on a 95% probability level. Unless specifically stated that it was not done, data of all trials were corrected using Abbott's formula (Abbott, 1925), to compensate for larvae or pupae that died of natural causes other than the entomopathogen (EPF, EPN or CrleGV) tested.

3.4 Results

3.4.1 EPF bioassay

The two-way ANOVA for mean mortality (Fig. 3.4) showed no interaction between main effects, diet (three levels, control, juvenile and mature) and log dose (3, 6 and 7) ($F_{(4, 126)}$ = 0.04, P = 0.997). No significant differences were measured between treatments for any of the three conidia concentrations tested. The results of insect mortality, as influenced by diet were pooled together and analysed using a one-way ANOVA. There was no significant difference ($F_{(2, 126)}$ = 0.68; P = 0.51) between diets. Mean control mortality recorded was 9.6 ± 0.16%, 9.07 ± 0.25%, and 9.4 ± 0.25% for larvae reared on the control diet, juvenile tree diet and mature tree diet respectively.

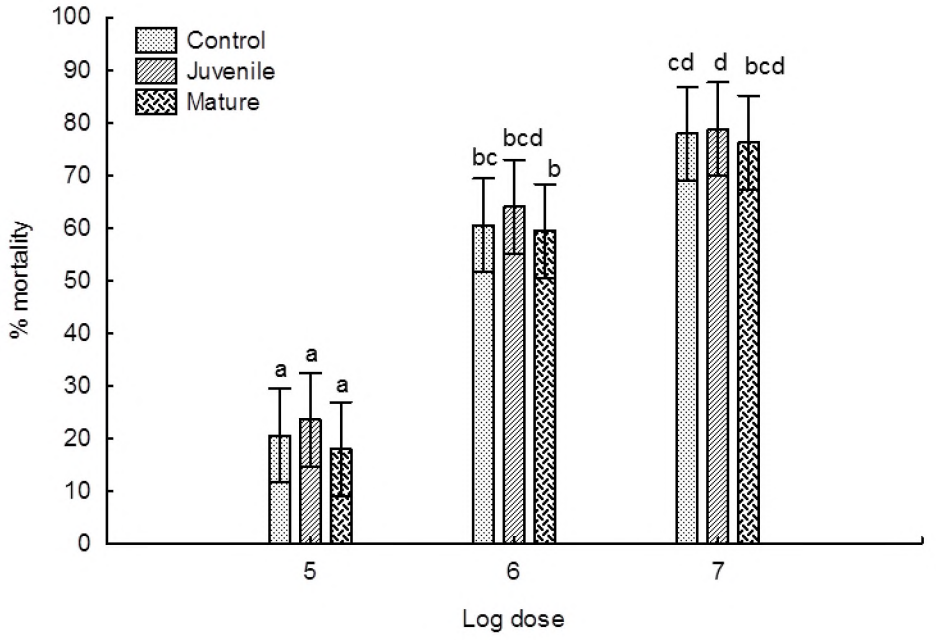

Figure 3.4: Mean mortality of FCM larvae (n = 150) reared on three different diets exposed to increasing concentrations of fungal conidia. Different letters above bars denote significant differences (comparison of means, $P < 0.05$).

3.4.2 Entomopathogenic nematode bioassay

The two-way ANOVA for mean mortality (Fig. 3.5) showed no interaction between main effects, diet (three levels, control, juvenile and mature) and dose (12, 25 and 50) ($F_{(4, 45)} = 0.06$, $P = 0.992$). Although mean mortality in the mature tree diet was consistently higher than in the juvenile tree and control diet, no significant differences were recorded between diets for any of the three nematode concentrations tested. The results of insect mortality, as influenced by nematode concentration were pooled together by diet and analysed using a one-way ANOVA. Results showed significant differences ($F_{(2, 45)} = 6.0$, $P = 0.005$) between diets. Mean mortality of larvae reared on the control diet (81.47 ± 3.31% mortality) was significantly lower ($P = 0.023$) than mean larval mortality recorded in the mature tree diet (84.88 ± 3.39% mortality). Mean control mortality recorded in was 0.28± 0.11%.

Mean mortality of pupae were analysed using a two-way ANOVA (Fig. 3.5) and showed no interaction between main effects, diet (three levels, control, juvenile and mature) and dose (50, 100 and 200) ($F_{(4, 45)} = 0.32$, $P = 0.861$). There were no

significant differences between diets for any of the three nematode concentrations tested. The results of insect mortality, as influenced by nematode concentration were pooled together by diet and analysed using a one-way ANOVA. Results showed no significant differences ($F_{(2, 45)}$ = 0.077, P = 0.926) between diets. Mean control mortality recorded in was 0.11± 0.08%.

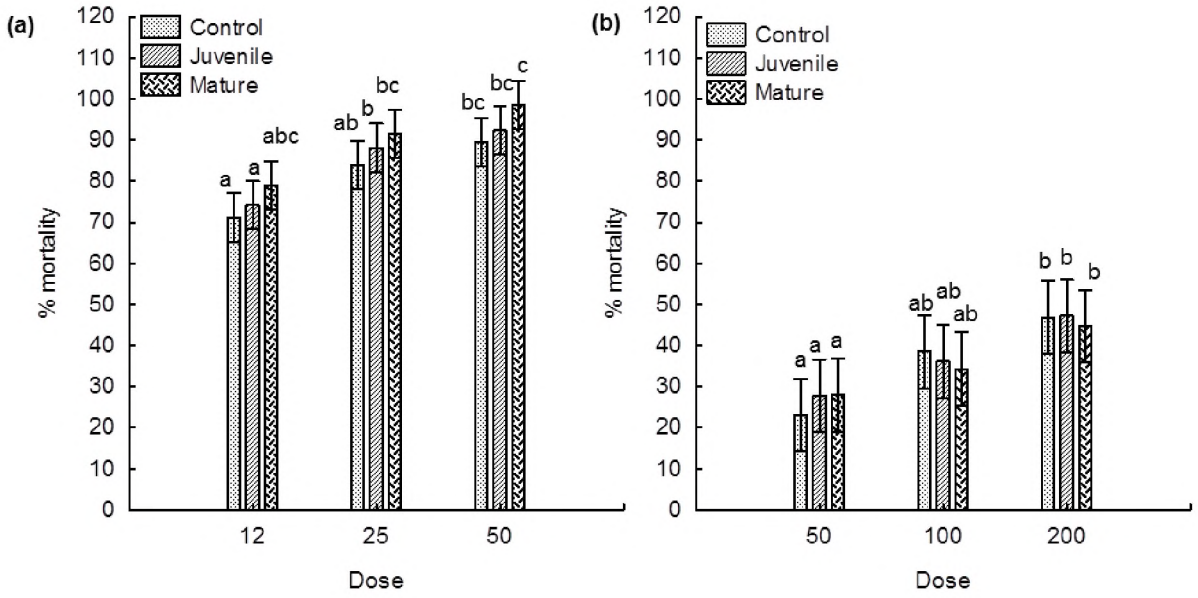

Figure 3.5: Mean mortality of FCM (a) larvae (n = 144) and (b) pupae (n = 144) reared on three different diets exposed to increasing concentrations of nematodes. Different letters above bars denote significant differences (comparison of means, P < 0.05).

3.4.3 CrleGV droplet feeding bioassay

Results of the one-way ANOVA for mean mortality (not corrected by Abbotts formula) in the controls of the three different diets showed significant differences ($F_{(2, 24)}$ = 55.32 P < 0.001) between diets. Mean mortality in diets containing 15% fruit powder from mature trees (66.11 ± 2.86% mortality) were significantly higher than in diets containing 15% fruit powder from juvenile trees (49.44 ± 3.27% mortality, P = 0.002) and control diets which contained no fruit powder (22.22 ± 2.78% mortality, P < 0.001). Mortality in the juvenile tree diet was also significantly higher (P < 0.001) than in the control diet.

The two-way ANOVA for mean mortality (Fig. 3.6) showed no interaction between main effects, diet (three levels, control, juvenile and mature) log dose (4.3, 5.7 and 6.9) ($F_{(4, 135)}$ = 0.42, P = 0.795). The results of insect mortality, as influenced by diet were pooled together and analysed using a one-way ANOVA. Results showed that there were significant differences ($F_{(2, 135)}$ = 3.32, P = 0.039) in mean mortality between diets. Mean mortality of larvae reared on the mature tree diet (60.68 ± 5.37% mortality) was significantly lower (P = 0.033) than mean larval mortality recorded in the juvenile tree diet (71.54 ± 6.83%).

Although mean mortality in mature tree diets were significantly higher than in juvenile tree diets at the lowest virus dose of 2 $x10^4$ OBs/ml, the one-way ANOVA for mean mortality (not corrected by Abbotts formula) showed no significant difference (P = 0.95) between the two diets. Mean mortality in juvenile tree diets (80.67 ± 4.44%) and mature tree diets (81.67 ± 3.44%) were almost exactly the same. Mortality in the control diet (56.11± 5.46%) was significantly lower than in both the juvenile (P < 0.001) and mature tree diets (P < 0.001).

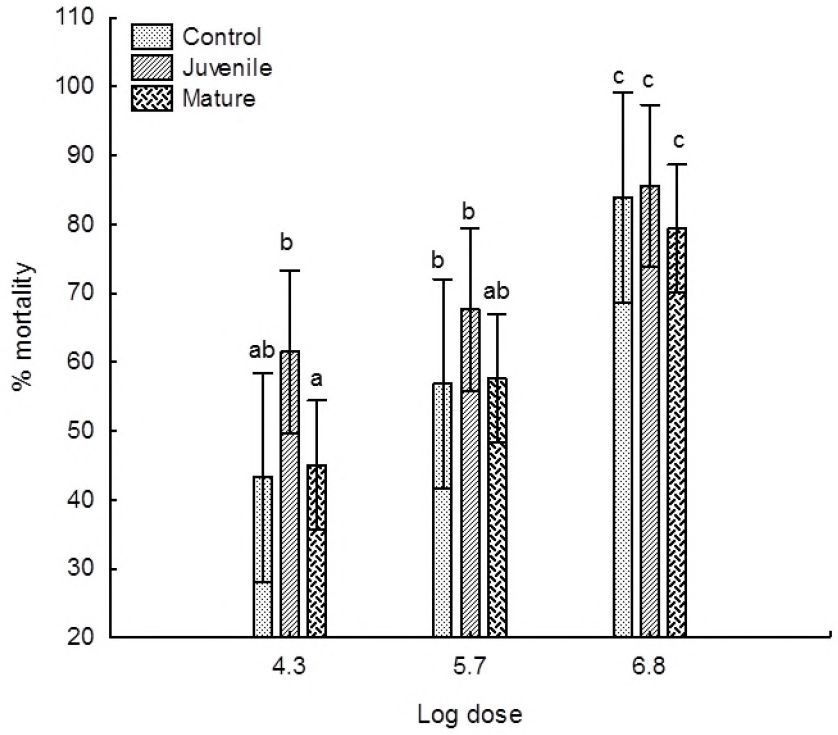

Figure 3.6: Mean mortality of FCM larvae reared on three different diets exposed to increasing concentrations of CrleGV OBs. Different letters above bars denote significant differences (comparison of means, $P < 0.05$).

3.5 Discussion

Plants have developed a multitude of diverse chemicals which aid in defending them against insect herbivores (Mithöfer & Boland 2012). Many of these defence chemicals are toxic, repellent or anti-nutritive for herbivores (Mithöfer & Boland 2012). Some plant chemicals have also been shown to alter the ability of entomopathogens to infect their hosts (Cory & Hoover 2006). Results of this study showed no significant differences between the susceptibility of FCM reared on diets containing 15% fruit powder from mature Washington Navel trees, diets containing 15% fruit powder from juvenile trees or a control diet which contained no fruit powder, to either EPF or EPN.

The significantly lower larval mortality recorded in mature tree diets at the lowest virus dose of 2 x10^4 OBs/ml compared to the juvenile tree diets could be due to higher concentrations of secondary metabolites such as tannins which have been shown to reduce host susceptibility to viruses by binding virus OBs in the midgut of

insects (Keating *et al.* 1990, Felton & Duffey 1990). The inability of virus binding metabolites to significantly reduce mortality at higher virus doses could possibly be because only a limited amount of chemicals are obtained during feeding which can only bind a limited number of OBs. The slightly lower mortality recorded in the control diet than the juvenile tree diet at the two lowest virus doses of 2×10^4 and 5×10^5 OBs/ml, could be because of higher larval performance recorded in this diet in Chapter 2 than both the juvenile tree and mature tree diets.

Although this study has shown that FCM susceptibility to CrleGV is significantly lower in larvae reared on diets containing 15% fruit powder from mature Washington trees than larvae reared on diets containing 15% fruit powder from juvenile trees, at low virus doses, higher natural mortality was recorded in mature tree diets than in juvenile tree diets. Thus the combined mortality caused by virus and diet were equal for both diets. Furthermore, the tree canopies of juvenile citrus trees are less dense than the tree canopies of mature trees. Thus, virus particles will be more exposed to ultraviolet (UV) radiation in juvenile orchards than in mature orchards. Baculoviruses have been shown to be adversely affected by exposure to UV radiation which reduces their efficacy as biopesticides (Moore 2002, Mwanza *et al.* 2016). Therefore, the efficacy CrleGV will be higher in mature orchards than in juvenile orchards.

CHAPTER 4

The influence of orchard age on FCM ecology

4.1 Introduction

Structural complexity increases as plants mature and has been shown to increase population numbers of invertebrate natural enemies (Boege 2005). Ontogenetic changes in plant structure, as they mature, include reduced or increased thorniness, changes in leaf shape and the development of lateral branches, adventitious roots and reproductive structures (Poethig 1990).

Langellotto & Denno (2004) conducted a meta-analysis of 43 studies which covered 62 independent taxa. Results of their meta-analysis showed that seven out of the nine natural enemy guilds studied, responded positively to increased habitat complexity. A strong negative response to simplified habitat structure was recorded in spiders, followed by hemipterans, mites and parasitoids. The results of the meta-analysis corresponds with results reported by Pekár (2003), who recorded higher abundance and species diversity of spiders in mature apple orchards, which had larger more complex tree structures juvenile orchards. According to Langellotto & Denno (2004), the reason for higher abundance of natural enemies in complex-structured habitats is unclear, but could be due to more effective prey capture or increases in alternative food sources and refuge from intra-guild predation.

In contrast to results of the meta-analysis conducted by Langellotto & Denno (2004), a review on the influence of plant architecture on insect population numbers, conducted by Simon *et al.* (2007), argues that complex tree architecture impairs the foraging ability of most natural enemies as their prey have more places to hide. However, structural simplicity in juvenile trees will also have a negative impact on the foraging ability of natural enemies. According to Van Driesche & Bellows (1996), parasitoids are more exposed to wind and dust in younger, less structurally complex orchards, which can increase grooming and reduce foraging, oviposition and lengths of visits on dusty foliage. Reduced complexity in tree structure may also increase parasitoid exposure to chemicals. Natural enemies, such as parasitoids are known to be more sensitive to pesticide applications than some pest species, as they are less

cryptic and more mobile (Samways 2005). Differences in plant structure will also influence the production and environmental persistence of insect pathogens (Cory & Hoover 2006). For example, results of a study conducted by Killick & Warden (1991), showed significantly lower ultra violet (UV) radiation in the lower branches of pine trees and consequently, higher mortality of pine beauty moth, *Panolis flammea* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), due to fewer virus infections being recorded in the lower branches than branches higher up in the tree canopy.

Although increased complexity in plant structure has been shown to increase natural enemy abundance, some insect herbivores have also been shown to be positively affected by increased complexity in tree structure (Simon *et al.* 2007). A study conducted by Simon *et al.* (2006) determined the influence of tree architecture on apple pests. The development of aphids and mites were compared in two training systems (used to manipulate tree architecture): the more open and aerated centrifugal training (CT) system and the Original Solaxe (OS) system, which has a denser tree canopy. Infestations of both aphids and mites were higher in the OS system than the CT system, possibly because the higher shoot density of the OS system allowed easier access to growing shoots. Codling moth population numbers have also been shown to be higher in mature orchards due to higher abundance in protective pupation sites (Wearing & Skilling 1975).

The influence of differences in plant physiology between juvenile and mature citrus trees on FCM survival and host susceptibility was discussed in Chapters 2 and 3. In this chapter the influence of differences in tree architecture between juvenile and mature citrus trees on the population dynamics of false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) and its arboreal natural enemies, which include parasitoids and viruses, was investigated.

4.2 Materials and Methods

4.2.1 Moth catches

One Chempac® (Suider-Paarl, South Africa) yellow delta trap was hung in the South Western side of each orchard (see section 4.2.4 for description of orchards used) on the 5th tree in the 5th row at a height of approximately 1.5 m to record FCM moth catches (Moore 2017). Traps were also hung at a height of approximately 1.5 m in unplanted areas adjacent to orchards in an opening in the thicket. Each yellow delta trap contained a sticky pad and a female FCM pheromone lure obtained from Chempac. Pheromone lures were replaced every 12 weeks, as per registered recommendations, and sticky pads were replaced every four to six weeks. FCM trap catches were recorded weekly, differentiating between wild and sterile moths. Sterile insect technique was applied on an area wide basis for the duration of this study. Sterile moths released in the Sundays River Valley, Eastern Cape, South Africa, were reared in a laboratory at XSIT, Citrusdal, South Africa on a diet which contains pink dye. When sterile male moths are crushed, a pink discharge is visible that is not visible when wild moths are crushed.

4.2.2 Egg counts and parasitism

Ten fruit on each of 10 trees were inspected fortnightly in each juvenile and mature orchard monitored to detect FCM eggs and to determine if the eggs were parasitized or not. Parasitised eggs are black, whereas non-parasitised eggs are cream, pink or dark brown (just before hatching) (Georgala 1969, Daiber 1979a, Newton 1998) (Fig. 4.1). Sample trees were located diagonally across orchards, from the South western corner to the North eastern corner of each orchard (Fig. 4.2). The first sample tree of each orchard was the same tree in which the yellow delta trap was hung. Successive sample trees were located three to four trees apart in the adjacent rows.

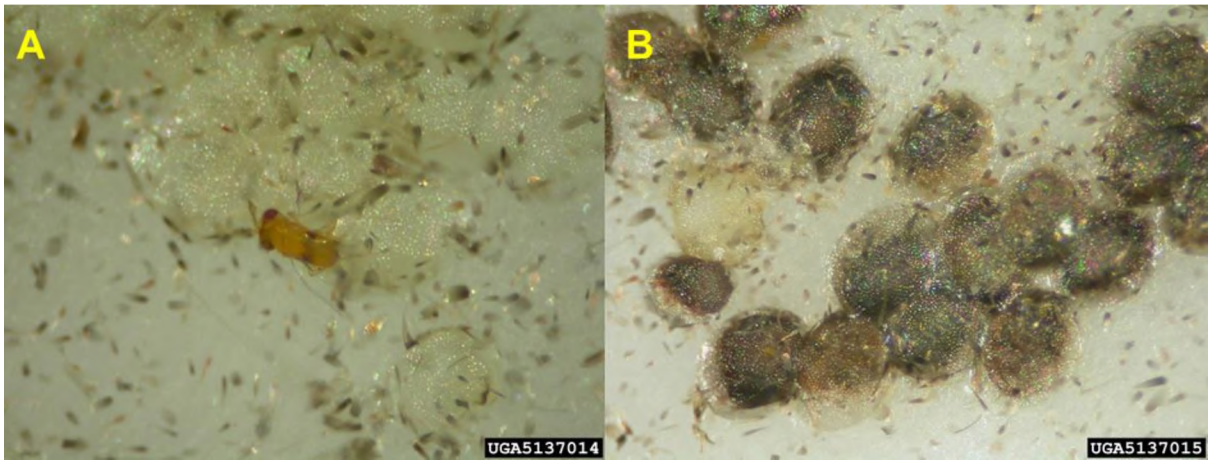

Figure 4.1: (A) Egg parasitoid and FCM eggs before parasitism (Photo credit: J.H. Hofmeyr, https://www.invasive.org/browse/detail.cfm?imgnum=5137013). (B) Parasitised FCM eggs (Photo credit: J.H. Hofmeyr, https://www.invasive.org/browse/detail.cfm?imgnum=5137015).

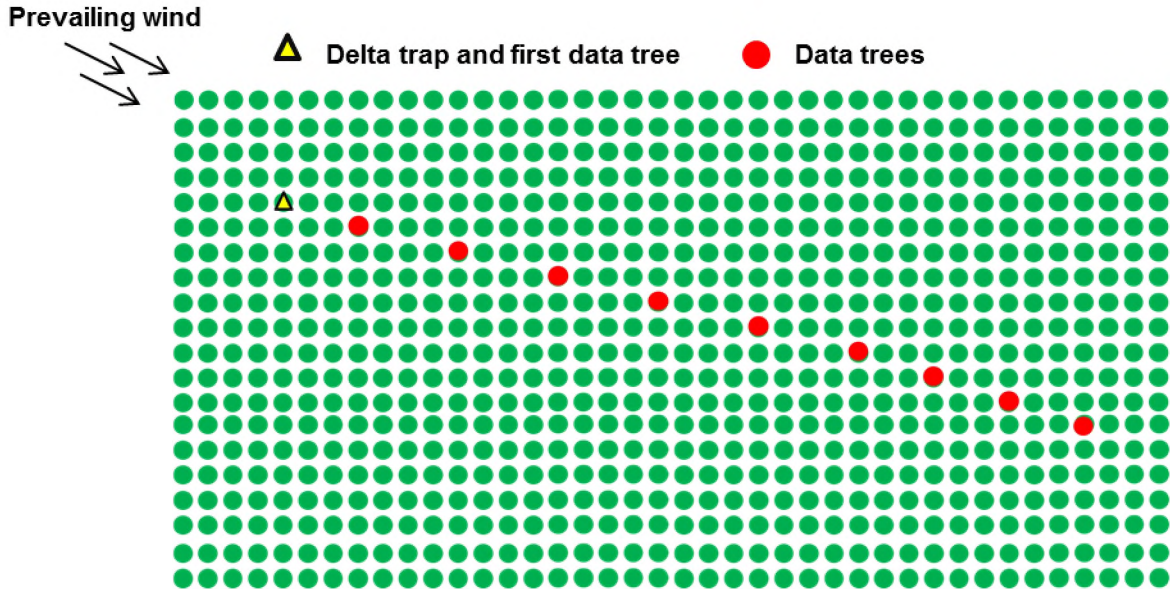

Figure 4.2: Schematic diagram of the position the yellow delta trap and data trees.

4.2.3 False codling moth fruit infestation, larval parasitism and virus infection

When present, dropped fruit were collected weekly from the orchard floor underneath the same 10 trees per orchard that were marked for egg parasitism inspections. The fruit collected were brought back to the laboratory and dissected to search for larvae or signs of larval damage. Any live larvae found were placed individually into 30 ml glass sample vials (Dalgen, South Africa) with artificial diet (Moore *et al*. 2014), obtained from River Bioscience (Addo, South Africa) and closed

with a sterile cotton wool plug, which also served as a pupation substrate for larvae. Glass tubes were checked every second day for signs of virus infection and for the emergence of FCM and parasitoids. Any dead larvae that showed signs of virus infection were also recorded. Virus infected larvae look swollen and display colour changes which include black speckling, milky white, brown or grey colouring, or white to yellow patches below the cuticle which turn into brown lesions (Moore 2002).

4.2.4 Study sites

Details of study sites are displayed in Table 4.1. Refugia adjacent to six citrus orchards (two at Riverbend Farm, two at Miskruier Farm, one at Buffelsbos Farm and one at Woodridge Farm) were monitored from February 2015 to May 2015. Six non-bearing orchards (one to three years old), six juvenile orchards (four to eight years old) and six mature orchards (nine years and older) were monitored from February 2015 until July 2017. Although referred to as non-bearing orchards throughout this study, orchards placed in the non-bearing maturity group were non-bearing during 2015 and 2016, but were bearing fruit for the first time during 2017. Two Midnight Valencia orchards, two Mandarin orange orchards and two Navel orange orchards were monitored per maturity group. Although the Mandarin orange orchards chosen for the mature grouping were only five years old at the beginning of the study, the trees were already large, at a height of approximately 2.5 m, with a very dense canopy.

4.2.5 Temperature and humidity

To compare differences in temperature and humidity between juvenile and mature orchards, one DS1923 Maxim iButton (Maxim Integrated, United States of America) was placed in the South Western side of one mature and one juvenile orchard in the 5th tree of the 5th row at Kleinplaas Farm, Riverbend Farm and Woodridge Farm. At each farm one mature and juvenile orchard were chosen, with the closest possible proximity to each other. Temperature and humidity readings were taken from 22 August until 16 October 2017. Since the purpose of this trial was only to determine if there are differences in temperature and humidity readings

between juvenile and mature orchards, temperature and humidity readings were only recorded for a brief period.

Table 4.1. Details of orchard monitored.

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted	Year monitored
Non-bearing	Riverbend S 33°25.107 E 25°42.677	Witkrans Navel	515	2014	2015, 2016, 2017
		M7 Navel	603	2015	2015
		Orr Mandarin	606	2015	2015, 2016, 2017
		Afourer Mandarin	611	2014	2015
		Midnight Valencia	506	2014	2015, 2016, 2017
		Midnight Valencia	502	2014	2015
	Miskruier S 33°27.427 E 25°41.431	Cambria Navel	11	2013	2016, 2017
		Midnight Valencia	71	2013	2016, 2017
	Halaron S 33°30.591 E 25°39.704	Afourer Mandarin	75	2015	2016, 2017
Juvenile	Woodridge S 33°28.787 E 25°41.665	Cambria Navel	300	2011	2015
		Cambria Navel	303	2012	2015, 2016
		Midnight Valencia	301, 302	2011	2015, 2016

Pesticides applied (active ingredients)

2015, 2016 and 2017: spirotetramat, methomyl, abamectin
2015 and 2016: fenpropathrin, chlorfenapyr
2017: cypermethrin, chlorpyrifos, mancozeb, carbendazim, pyraclostrobin, *B. bassiana,* abamectin, copper hydroxide

2016: None
2017: abamectin, azoxystrobin, CrleGV, benomyl, copper hydroxide, mancozeb, pyriproxyfen, spinetoram, methidathion
2016: None
2017: abamectin, azoxystrobin, CrleGV, benomyl, copper hydroxide, mancozeb, pyriproxyfen, spinetoram, methidathion
2016 and 2017: None

imidacloprid, carbendazim, fenpropathrin, mancozeb, chlorpyrifos, tebucanazole, methomyl, chlorfenapyr, abamectin, pyraclostrobin, spirotetramat, dichlorpop-p, spinetoram, buprofenzin, trifloxystrobin, CrleGV

2015 and 2016: imidacloprid, carbendazim, fenpropathrin, mancozeb, chlorpyrifos, tebucanazole, methomyl, chlorfenapyr, abamectin, pyraclostrobin, spirotetramat, dichlorpop-p, spinetoram, buprofenzin
2015: trifloxystrobin, CrleGV
2016: HearNPV, *B. bassiana*

2015 and 2016: imidacloprid, carbendazim, fenpropathrin, HearNPV, mancozeb, chlorpyrifos, methomyl, chlorfenapyr, abamectin, pyraclostrobin,

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted	Year monitored
	Buffelsbos S 33°27.294 E 25°42.071	Valley Gold	650, 653	2012	2015, 2016, 2017
	Halaron S 33°30.591 E 25°39.704	M7 Navel	71	2012	2016, 2017
	Douglasdale	Washington Navel	55	2011	2017
	Kleinplaas S 33°29.326 E 25°41.926	Midnight Valencia	20, 23	2012	2017
Mature	Miskruier S 33°27.427 E 25°41.431	Washington Navel	50	2005	2015, 2016
		Newhall Navel	49	2005	2015
		Midnight Valencia	51, 52	2005	2015, 2016
	Woodridge S 33°28.787 E 25°41.665	Afourer	341, 342	2010	2015, 2016, 2017

Pesticides applied (active ingredients)

spirotetramat, dichlorpop-p, spinetoram, buprofenzin
2015: trifloxystrobin, CrleGV
2016: tebucanazole, *B. bassiana,* copper hydroxide

2015, 2016 and 2017: fenpropathrin, copper hydroxide
2015: chlorpyrifos, cypermethrin
2016 and 2017: abamectin, *B. bassiana,* chlorfenapyr, pyraclostrobin

2016 and 2017: imidacloprid, abamectin, benomyl, mancozeb
2017: imidacloprid, profenofos, spinetoram, tebucanazole, abamectin, benomyl, mancozeb

carbendazim, abamectin, cyanamide, mancozeb, pyriproxyfen, profenofos, tebucanazole, buprofenzin, CrleGV, axoxystrobin, copper hydroxide, spinetoram, tau-fluvalinate

benomyl, abamectin, mancozeb, pyriproxyfen, CrleGV, azoxystrobin, copper hydroxide, methidathion

2015 and 2016: abamectin, cyanamide, CrleGV, azoxystrobin, benomyl, mancozeb

2015: tau-fluvalinate, spinetoram, pyriproxyfen, buprofenzin

tau-fluvalinate , benomyl, abamectin, cyanamide, *B. bassiana*, mancozeb, CrleGV, azoxystrobin, pyriproxyfen, buprofenzin

2015 and 2016: benomyl, abamectin, cyanamide, azoxystrobin, mancozeb copper hydroxide

2015:spinetoram, CrleGV, pyriproxyfen, buprofenzin

2015, 2016 and 2017: mancozeb, chlorpyrifos, methomyl, abamectin, pyraclostrobin, spirotetramat, spinetoram
2015: imidacloprid, fenpropathrin, tebucanazole, trifloxystrobin,

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted	Year monitored	Pesticides applied (active ingredients)
						2016: imidacloprid, fenpropathrin, HearNPV, tebucanazole, dichlorpop-P, buprofenzin, copper hydroxide, *B. bassiana* **2017:** tau-fluvalinate, carbendazim, dichlorpop-P, copper hydroxide, buprofenzin, *B. bassiana*
	Halaron S 33°30.591 E 25°39.704	Bahianinha Navel	53	2007	2016, 2017	**2016 and 2017:** imidacloprid, profenofos, tebucanazole, abamectin, Benomyl, mancozeb **2016:** *B. bassiana* **2017:** spinetoram
	Douglasdale	Washington Navel	83	2003	2017	carbendazim, abamectin, cyanamide, mancozeb, pyriproxyfen, profenofos, tebucanazole, buprofenzin, CrleGV, axoxystrobin
	Kleinplaas S 33°29.326 E 25°41.926	Midnight Valencia	17	2007	2017	Benomyl, abamectin, mancozeb, pyriproxyfen, CrleGV, azoxystrobin, copper hydroxide, methidathion

4.3 Statistics

All statistical analyses were performed using Statistica version 13.2, 2017 (Statsoft South Africa Research (Pty) Ltd, Johannesburg, South Africa). The data for FCM trap catches, egg counts and FCM damage were found to not be normal. Therefore, a nonparametric Mann-Whitney U test was used to determine significance between juvenile and mature orchards. A chi-square test, which compared the absence and presence of parasitism in eggs and virus infection in larvae (observed vs. expected) was used to compare egg parasitism and virus infected larvae recorded in juvenile and mature orchards. All other data were analysed by a Kruskal-Wallis test to determine significance and a multiple comparisons of mean ranks post hoc test was used to determine where the significant differences were (Fowler *et al.* 2005). Significant differences were determined on a 95% probability level.

4.4 Results

4.4.1 Navel orchards

During 2015, mean trap catches of 0.5 ± 0.10 moths per trap per week in juvenile (Cambria Navel) orchards were significantly lower ($Z = -3.85$, $P < 0.001$) than in mature orchards (Washington Navel and Newhall Navel) with 1.49 ± 0.11 moths per trap per week (Table 4.2). Significantly lower mean egg counts ($Z = -7.30$, $P < 0.001$) of 0.09 ± 0.02 eggs per tree per week were recorded in juvenile orchards than mature orchards, with a mean of 0.72 ± 0.07 eggs per tree per week. Egg parasitism was significantly higher in mature orchards ($\chi 2 = 4.92$, $P = 0.026$) as 33.63% (n = 116) of eggs recorded were parasitised compared to 9.52% (n = 21) egg parasitism in juvenile orchards (Fig. 4.3). The mean number of non-parasitised eggs (0.08 ± 0.0.02 viable eggs per tree per week) recorded in juvenile orchards was not significantly lower ($Z = 0.16$, $P = 0.876$) than the mean of all eggs recorded (0.09 ± 0.02 eggs per tree per week). Thus, egg parasitism did not significantly reduce egg viability in juvenile orchards. In contrast, egg parasitism significantly reduced the mean number of viable eggs recorded in mature orchards to 0.48 ± 0.06 eggs per

tree per week (Z = 2.34, P =0.019). Although egg parasitism was significantly higher in mature orchards, the mean number of viable eggs recorded was still significantly higher than the mean number of viable eggs recorded in juvenile orchards (Z = -7.30, P < 0.001). Only one FCM larva was collected from one of the two juvenile orchards monitored during 2015 while 85 larvae were collected from mature orchards.

Due to low FCM infestation recorded in juvenile Cambria Navel orchards during 2015, one Cambria Navel orchard was replaced with an M7 Navel orchard at Halaron Farm during 2016 (Table 4.1). The Newhall orchard at Miskruier Farm was also replaced with a Bahianinha Navel orchard at Halaron Farm, to reduce the effect that any difference in farming practices may have on FCM infestation levels. No FCM larvae were recorded in the remaining Cambria Navel orchard during 2016 and therefore the data collected from this orchard were excluded from analyses. Mean moth counts of 4.53 ± 0.24 moths per trap per week recorded in the juvenile M7 orchard was significantly higher (Z = -4.29, P < 0.001) than in mature orchards (Washington Navel and Bahianinha Navel) with a mean of 1.78 ± 0.80 moths recorded per trap per week. No significant difference in mean egg counts (Z = 0.14, P = 0.892) was recorded between juvenile orchards (0.42 ± 0.08 eggs per tree per week) and mature orchards (0.45 ± 0.05 eggs per tree per week). Egg parasitism of 56.38% (n = 94) in mature orchards was significantly higher (χ^2 = 3.06, P < 0.001) than measured in the juvenile orchards, with only 2.63% (n = 38) egg parasitism recorded (Fig. 4.3). Since only one parasitised egg was recorded in the juvenile orchard, egg parasitism did not have a significant effect on egg viability. Egg parasitism significantly reduced (Z = 2.38, P = 0.001) the mean number of viable eggs recorded in mature orchards from 0.45 ± 0.05 eggs per tree per week to 0.20 ± 0.04 eggs per trap per week. The mean number of viable eggs recorded in mature orchards was significantly lower (Z = -2.66, P = 0.008) than a mean 0f 0.41 ± 0.19 viable eggs per tree per week recorded in juvenile orchards. Mean FCM infestation of 0.20 ± 0.02 larvae per tree per week per week recorded in the juvenile orchard was higher than a mean 0.14 ± 0.04 larvae recorded in the mature orchards, however not significantly so (Z = -1.03, P = 0.301).

Since FCM infestation was still exceptionally low in the juvenile Cambria Navel orchard during 2016, the juvenile Cambria Navel orange orchard at Woodridge

and mature Washington Navel orchards at Miskruier were replaced by a juvenile and mature Washington Navel orchard on Douglasdale Farm (Table 4.1) during 2017. The data collected from the Washington Navel orchards were separated for analyses from the other juvenile and mature Navel orchards, as differences in FCM infestation levels could be caused by differences in both farm management practices and cultivar differences, and these could thus be excluded. Significantly higher mean moth counts ($Z = 2.03$, $P = 0.042$) of 4.93 ± 0.80 moths per trap per week were recorded in the juvenile Washington Navel orchard than the mature orchard, with a mean of 3.18 ± 0.80 moths (Table 4.2). Mean egg counts were also higher in the juvenile Washington Navel orchard (1.56 ± 0.22 eggs per tree per week) than the mature orchard (1.11 ± 0.17 eggs per tree per week) but also not significantly so ($Z = -1.53$, $P = 0.127$). Egg parasitism was significantly higher ($\chi^2 = 10.40$, $P < 0.001$) in the mature orchard (12.35% parasitism, n = 89) than the juvenile orchard (1.6%, n = 125) (Fig. 4.3). However, the mean number of 0.98 ± 0.16 viable eggs per tree per week recorded in mature orchards was not significantly lower ($Z = 0.58$, $P = 0.563$) than the mean of all eggs (1.11 ± 0.17 eggs per tree per week) recorded. The mean number of 1.53 ± 0.22 viable eggs per tree per week recorded in the juvenile orchards was higher than a mean of 0.98 ± 0.16 viable eggs per tree per week recorded in mature orchards, however it was only significantly higher at a 90% confidence level ($Z = -1.93$, $P = 0.053$). No significant difference ($Z = -0.07$, $P = 0.946$) was recorded in mean FCM infestation between the juvenile orchard and the mature orchard, with a mean of 2. 45 ± 0.21 and 2.78 ± 0.28 larvae recorded per tree per week respectively.

During 2017, higher mean moth counts of 7.21 ± 1.73 moths per week were recorded in the juvenile M7 orchard than the mature Bahianinha Navel orchard, with a mean moth count of 5.04 ± 0.95 moths per trap per week, however not significantly so ($Z = 0.24$, $P = 0.811$) (Table 4.2). Mean egg count of 0.97 ± 0.12 eggs per tree per week recorded in the juvenile orchard was significantly higher ($Z = -4.50$, $P < 0.001$) than mean egg count of 0.34 ± 0.07 eggs per tree per week recorded in the mature orchard. Egg parasitism was significantly higher ($\chi^2 = 12.00$, $P < 0.001$) in the mature orchard (25.81% parasitism, n = 38) than in the juvenile orchard (2.94% parasitism, n = 98) (Fig. 4.3). However, the mean number of 0.26 ± 0.06 viable eggs per tree per week recorded in mature orchards was not significantly lower ($Z = 0.60$, $P = 0.549$)

than the mean of all eggs (0.34 ± 0.07 eggs per tree per week) recorded. The mean number of 0.93 ± 0.11 viable eggs per tree per week recorded in the juvenile orchards was significantly higher (Z = -5.06, P < 0.001) than the mean number of viable eggs recorded in mature orchards. Mean FCM infestation of 0.76 ± 0.20 larvae per tree per week was significantly higher (Z = -5.53, P < 0.001) in the juvenile orchard than the mature orchard, with 0.57 ± 0.10 larvae per tree per week recorded.

Table 4.2 Mean numbers of FCM moth catches, eggs counts (all eggs) and viable egg counts (non-parasitised eggs) recorded in Navel orange orchards. Means (± standard errors) are presented. Significant P-values are presented in bold.

	Navel oranges 2015			
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	0.50 (1.00) a	1.49 (0.11) b	-3.85	**< 0.001**
Eggs	0.09 (0.02) a	0.72 (0.07) b	-9.73	**< 0.001**
Viable eggs	0.08 (0.02) c	0.48 (0.06) c	-7.30	**< 0.001**
Larvae	0.003(0.003) a	0.26 (0.01) b	7.79	**< 0.001**
	Navel oranges 2016			
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	4.53 (0.24) a	1.78 (0.80) b	-4.29	**< 0.001**
Eggs	0.42 (0.08) a	0.45 (0.05) a	0.14	0.892
Viable eggs	0.41 (0.19) a	0.20 (0.04) b	-2.66	**0.008**
Larvae	0.20 (0.02) a	0.14 (0.04) a	-1.03	0.301
	Washington Navel oranges 2017			
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	4.93 (0.80) a	3.18 (0.80) b	2.03	**0.042**
Eggs	1.56 (0.22) a	1.11 (0.17) a	-1.53	0.127
Viable eggs	1.53 (0.22) a	0.98 (0.16) a	-1.93	0.053
Larvae	2.45 (0.21) a	2.78 (0.28) a	-0.07	0.946
	Navel oranges 2017			
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	7.21 (1.73) a	5.04 (0.95) a	0.24	0.811
Eggs	0.97 (0.12) a	0.34 (0.07) b	-4.50	**< 0.001**
Viable eggs	0.93 (0.11) a	0.26 (0.06) b	-5.06	**< 0.001**
Larvae	0.76 (0.20) a	0.57 (0.10) b	-5.53	**< 0.001**

Values are compared for each row only. Different letters following values denote significant differences (comparison of mean ranks, P < 0.05).

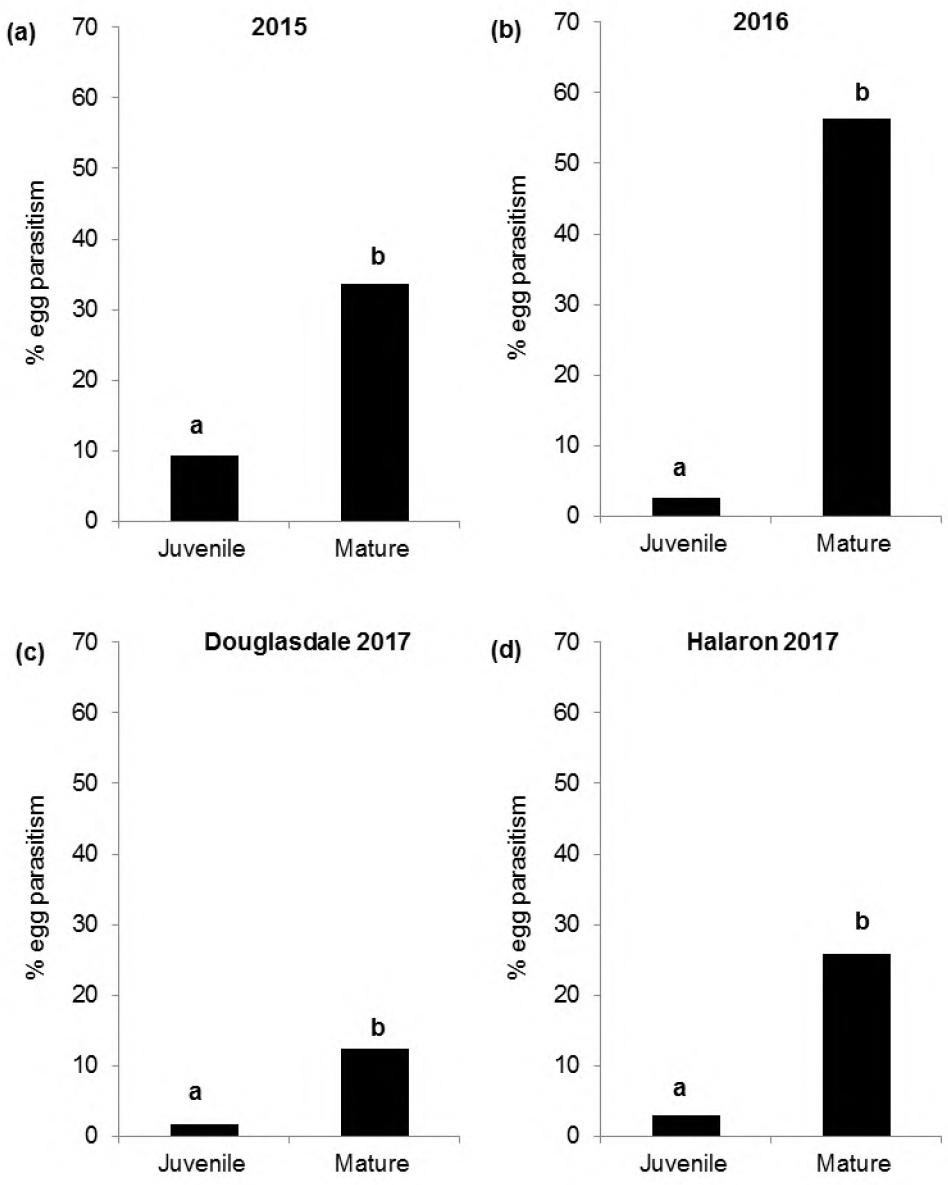

Figure 4.3: Egg parasitism (percentage of all eggs) recorded in juvenile and mature Navel orange orchards during (a) 2015, (b) 2016 and 2017 at (c) Douglasdale Farm and (d) Halaron Farm. Different letters above bars denote significant differences (chi-square tests, $P < 0.05$).

4.4.2 Mandarin orchards

During 2015, no significant difference ($Z = -1.61$, $P = 0.108$) in mean moth counts were recorded between juvenile and mature orchards, with a mean of 0.43 ± 0.08 and 0.76 ± 0.13 moths recorded per trap per week respectively (Table 4.3). Slightly higher mean egg counts of 0.23 ± 0.04 eggs per tree per week were recorded in mature orchards than juvenile orchards, with a mean of 0.19 ± 0.03 eggs per tree per week. However, this was not significant so ($Z = -0.78$, $P = 0.438$). Egg

parasitism of 10.53% (n = 38) recorded in juvenile orchards was higher than egg parasitism of 5.88% (n = 51) recorded in juvenile orchards, however, the difference was not significant (χ^2 = 0.196, P = 0.658) (Fig. 4.4). Although egg parasitism was higher in juvenile orchards, the mean number of 0.17 ± 0.03 viable eggs per tree per week was not significantly lower than the mean of all eggs (0.19 ± 0.03 eggs per tree per week) recorded (Z = 0.27, P = 0.79). The mean number of viable eggs recorded in mature orchards of 0.22 ± 0.04 viable eggs per tree per week was slightly higher than mean viable egg counts of 0.17 ± 0.03 viable eggs per tree per week recorded in juvenile orchards, but not significantly so (Z =0.95, P = 0.341). Low FCM infestation levels were measured in both juvenile and mature orchards, with only one and three larvae recorded respectively.

Similar mean moth catches were recorded for juvenile and mature orchard during 2016, with a mean of 1.13 ± 0.6 and 1.00 ± 0.13 moths per trap per week recorded respectively (Table 4.3). Although higher mean egg counts of 0.22 ± 0.04 eggs per tree per week were recorded in juvenile orchards than mature orchards, with a mean of 0.15 ± 0.03 eggs per tree per week, the difference was not significant (Z = 1.26, P = 0.208). Egg parasitism of 32.14% (n = 38) recorded was significantly higher (χ^2 = 27.00, P < 0.001) than 1.92% (n = 52) parasitism recorded in juvenile orchards (Fig. 4.4). Although egg parasitism was significantly higher in mature orchards, the mean number of viable eggs recorded (0.12 ± 0.03 eggs per tree per week) was not significantly higher (Z = 0.62, P = 0.536) than the mean of all eggs recorded (15 ± 0.03 eggs per tree per week). The mean number of viable eggs recorded in mature orchards (0.12 ± 0.03 viable eggs per trap per week) was significantly lower (Z = 2.34, P = 0.019) than the mean number of viable eggs recorded in juvenile orchards (0.21 ± 0.03 viable eggs per tree per week). Although higher mean FCM infestation of 0.04 ± 0.002 larvae per tree per week was recorded in juvenile orchards compared 0.02 ± 0.006 larvae per tree per week recorded in mature orchards, it was only significantly higher at a 90% confidence level (Z = -1.82, P = 0.068).

Higher mean moth catches of 0.85 ± 0.22 moths per trap per week were recorded in juvenile orchards than mature orchards, with a mean of 0.29 ± 0.8 moths per trap per week recorded during 2017, however it was only significantly higher at a

90% confidence level (Z = 1.71, P = 0.087) (Table 4.3). No eggs were recorded in mature orchards. Mean egg counts of 0.06 ± 0.02 eggs per tree per week were recorded in juvenile orchards, of which none were parasitised. FCM infestation in juvenile orchards of 0.034 ± 0.01 larvae per tree per week was significantly higher (Z = -2.86, P = 0.0.004) than 0.004 ± 0.002 larvae per tree per week recorded in mature orchards.

Table 4.3 Mean numbers of FCM moth catches, eggs counts (all eggs) and viable egg counts (non-parasitised eggs) recorded in Mandarin orchards. Means (± standard errors) are presented. Significant P-values are presented in bold.

Mandarin orchards 2015				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	0.43 (0.08) a	0.76 (0.13) a	-1.61	0.108
Eggs	0.19 (0.03) a	0.23 (0.04) a	-0.78	0.438
Viable eggs	0.17 (0.03) a	0.22 (0.04) a	1.12	0.262
Larvae	0.003 (0.004) a	0.007 (0.03) a	0.95	0.341
Mandarin orchards 2016				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	1.13 (0.36) a	1.00 (0.13) a	-0.23	0.82
Eggs	0.22 (0.04) a	0.15 (0.03) a	1.26	0.208
Viable eggs	0.21 (0.03) a	0.12 (0.03) b	2.34	**0.019**
Larvae	0.04 (0.002) a	0.02 (0.006) a	-1.82	0.068
Mandarin 2017				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	0.85 (0.22) a	0.29 (0.08) a	1.71	0.087
Eggs	0.06 (0.02) a	0.00 (0.00) b	3.41	**< 0.001**
Viable eggs	0.06 (0.02) a	0.00 (0.00) b	3.41	**< 0.001**
Larvae	0.034 (0.01) a	0.004 (0.002) b	-2.86	**0.004**

Values are compared for each row only. Different letters following values denote significant differences (comparison of mean ranks, P < 0.05).

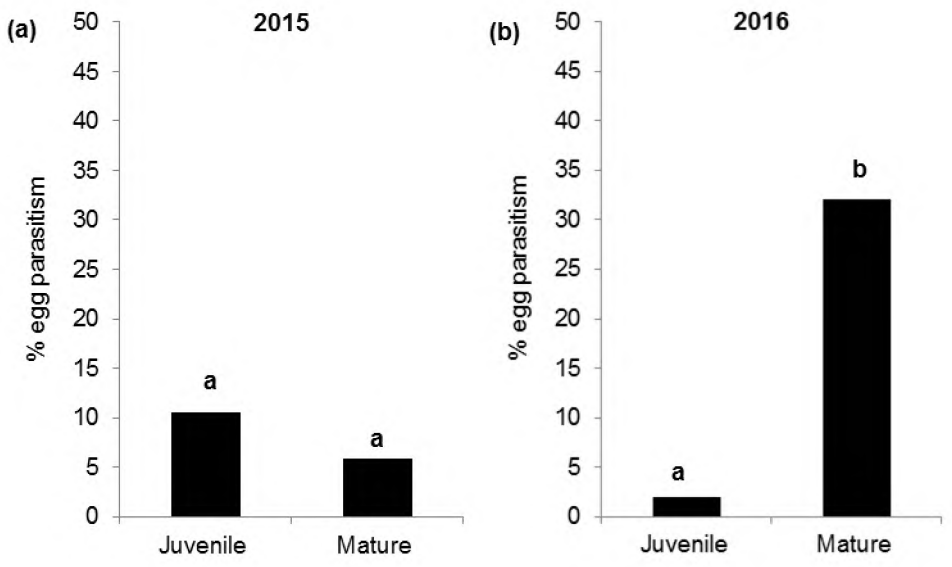

Figure 4.4: Egg parasitism (percentage of all eggs) recorded in Mandarin orchards during (a) 2015 and (b) 2016. Different letters above bars denote significant differences (chi-square tests, $P < 0.05$).

4.4.3 Midnight Valencia orchards

Significantly higher mean moth counts ($Z = 5.00$, $P < 0.001$) of 1.28 ± 0.17 moths per trap per week were recorded in juvenile orchards than in mature orchards, with a mean of 0.27 ± 0.07 moths per trap per week recorded during 2015 (Table 4.4). Although trap catches were higher in juvenile orchards, higher mean egg counts of 0.32 ± 0.04 eggs per tree per week were recorded in mature orchards than in juvenile orchards, with a mean of 0.28 ± 0.04 eggs per tree per week, however this was not significant ($Z = 0.62$, $P = 0.624$). Egg parasitism of 38.67% ($n = 83$) recorded in mature orchards was significantly higher ($\chi^2 = 9.09$, $P = 0.003$) than the 16.67% ($n = 72$) parasitism recorded in juvenile orchards (Fig. 4.5). Egg parasitism significantly reduced ($Z = 2.18$, $P = 0.029$) the mean number of viable eggs recorded in mature orchards from 0.32 ± 0.04 eggs per tree per week to 0.18 ± 0.03 eggs per tree per week. Egg parasitism did not significantly reduce ($Z = 0.76$, $P = 0.446$) the mean number of viable eggs recorded in juvenile orchards (0.23 ± 0.03 eggs per tree per week) compared to the mean of all eggs recorded (0.28 ± 0.04 eggs per tree per week). Although egg parasitism was significantly higher in mature orchards than juvenile orchards, the mean number of viable eggs (0.18 ± 0.03 eggs per tree per week) recorded in mature orchards was not significantly lower ($Z = -4.09$, $P = 0.624$) than the mean number of viable eggs recorded in juvenile orchards (0.23 ± 0.03

eggs per trap per week). FCM infestation was very low in both mature and juvenile orchards, with only one and two larvae recorded in mature and juvenile orchards respectively.

No significant difference ($Z = -0.44$, $P = 0.624$) in mean moth counts was recorded between juvenile and mature orchards during 2016, with a mean of 0.31 ± 0.07 and 0.34 ± 0.04 moths per trap per week recorded respectively (Table 4.4). Mean egg counts were significantly higher ($Z = -4.40$, $P < 0.001$) in mature orchards with a mean of 4.00 ± 0.05 eggs recorded per tree per week compared to 0.13 ± 0.02 eggs per tree per week recorded in juvenile orchards. Egg parasitism of 45.54% (n = 112) recorded in mature orchards was significantly higher ($\chi^2 = 14.40$, $P <0.001$) than the 10.81% (n = 37) egg parasitism recorded in juvenile orchards (Fig. 4.5). The mean number of viable eggs recorded in mature orchards (0.22 ± 0.03 eggs per tree per week) was significantly lower ($Z = 2.6$, $P = 0.09$) than the mean of all eggs recorded (4.00 ± 0.05 eggs per tree per week). The mean number of viable eggs recorded in juvenile orchards (0.12 ± 0.02 eggs per tree per week) was not significantly lower ($Z = 0.16$, $P = 0.874$) than the mean of all eggs (0.13 ± 0.02 eggs per tree per week) recorded. Although egg parasitism was significantly higher in mature orchards than juvenile orchards, the mean number of viable eggs recorded in juvenile orchards was still significantly lower ($Z = -2.20$, $P = 0.028$) than the mean number of viable eggs recorded in mature orchards. Similar to 2015, FCM infestation was very low in both mature and juvenile orchards, with only two larvae recorded in mature orchards and two larvae recorded in juvenile orchards.

Due to the low FCM infestation levels recorded during 2015 and 2016, all orchards were replaced with juvenile and mature Midnight Valencia orchards at Kleinplaas Farm (Table 4.1). Mean moth counts of 5.75 ± 0.52 recorded in juvenile orchards was significantly higher ($Z = 2.11$, $P = 0.034$) than mean moth counts of 5.59 ± 0.97 recorded in mature orchards (Table 4.4). Mean egg counts were significantly higher ($Z = 5.02$, $P < 0.001$) in juvenile orchards, with a mean of 0.46 ± 0.06 eggs recorded per tree per week, than in mature orchards, with 0.16 ± 0.03 eggs per tree per week recorded. Egg parasitism of 29.00% (n = 41) recorded in mature orchards was significantly higher ($\chi^2 = 6.04$, $P = 0.014$) than the 12.6% (n = 119) egg parasitism recorded in juvenile orchards (Fig. 4.5). Although egg parasitism

was higher in mature orchards, the mean number of viable eggs (0.11 ± 0.03 eggs per tree per week) was not significantly lower (Z = 0.62, P = 0.537) than the mean of all eggs (0.16 ± 0.03 eggs per tree per week) recorded. The mean number of viable eggs recorded in juvenile orchards (0.40 ± 0.05 eggs per tree per week) was significantly higher (Z = 5.49, P < 0.001) than the mean number of viable eggs (0.11 ± 0.03 eggs per tree per week) recorded in mature orchards. FCM infestation of 0.15 ± 0.02 larvae per tree per week was significantly higher (Z = 3.38, P < 0.001) in juvenile orchards than the 0.06 ± 0.01 larvae per tree per week recorded in mature orchards.

Table 4.4 Mean numbers of FCM moth catches, eggs counts (all eggs) and viable egg counts (non-parasitised eggs) recorded in Midnight Valencia orchards. Means (± standard errors) are presented. Significant P-values are presented in bold.

Midnight Valencia orchards 2015				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	1.28 (0.17) a	0.27 (0.07) b	5.00	**< 0.001**
Eggs	0.28 (0.04) a	0.32 (0.04) a	0.62	0.532
Viable eggs	0.23 (0.03) a	0.18 (0.03) a	-4.09	0.624
Larvae	0.004 (0.004) a	0.002 (0.002) a	0.03	0.977
Midnight Valencia orchards 2016				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	0.31 (0.07) a	0.34 (0.01) a	-0.44	0.66
Eggs	0.13 (0.02) a	0.40 (0.05) b	-4.40	**< 0.001**
Viable eggs	0.12 (0.02) a	0.22 (0.03) b	-2.20	**0.028**
Larvae	0.003 (0.002) a	0.01 (0.005) a	1.42	0.156
Midnight Valencia orchards 2017				
	Mean juvenile	**Mean mature**	**Z-value**	**P-value**
Moth catches	5.75 (0.52) a	5.59 (0.97) b	2.11	**0.034**
Eggs	0.46 (0.06) a	0.16 (0.03) b	5.02	**< 0.001**
Viable eggs	0.40 (0.05) a	0.11 (0.03) b	5.49	**< 0.001**
Larvae	0.15 (0.02) a	0.06 (0.01) b	3.38	**< 0.001**

Values are compared for each row only. Different letters following values denote significant differences (comparison of mean ranks, P < 0.05).

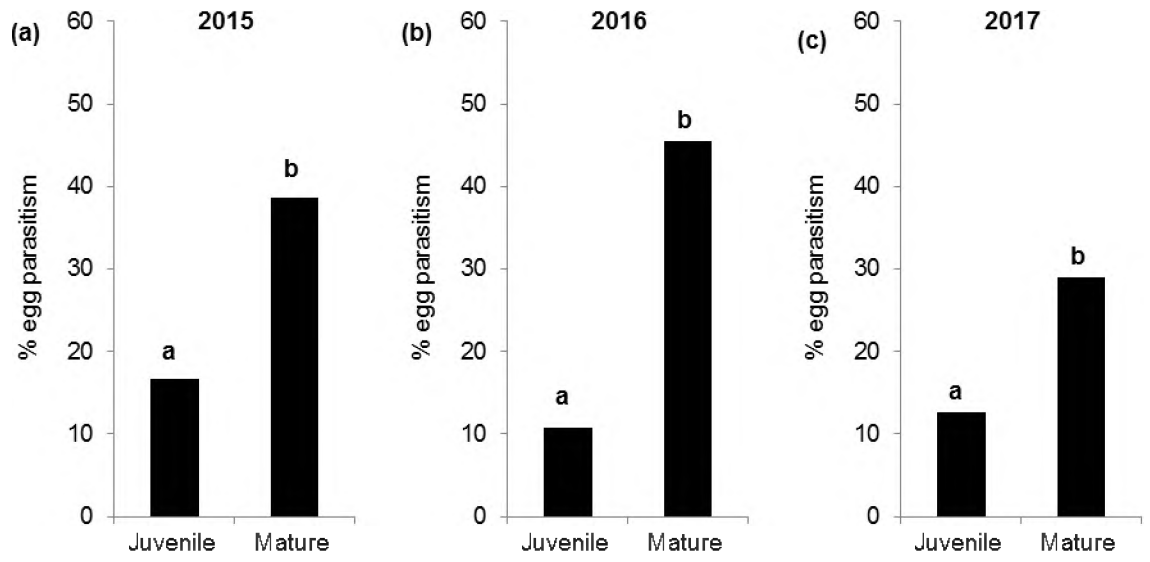

Figure 4.5: Egg parasitism (percentage of all eggs) recorded in Midnight Valencia orange orchards during (a) 2015, (b) 2016 and (c) 2017. Different letters above bars denote significant differences (chi-square tests, $P < 0.05$).

4.4.4 Refugia and non-bearing orchards

Significant differences in mean ranks of moth counts were recorded between refugia, non-bearing orchards, juvenile orchards and mature orchards during February 2015 to May 2015 ($H = 90.55$, $P < 0.001$). Mean ranks of moth counts recorded in refugia and non-bearing orchards were significantly lower than mean ranks of moth catches recorded in juvenile and mature orchards, as only one and two moths were recorded in refugia and non-bearing orchards respectively. No significant difference in mean ranks of moth catches were recorded between juvenile and mature orchards ($P = 0.72$).

Significant differences in mean ranks of moth catches were recorded between sample years ($H = 57.33$, $P < 0.001$). Mean ranks of moth counts recorded during 2017 were significantly higher than mean ranks of moth counts recorded during 2015 ($P < 0.001$) and 2016 ($P < 0.001$). No significant difference in mean ranks of moth catches were recorded between 2015 and 2016 ($P = 0.644$). Significant differences in mean ranks of moth catches were recorded between orchard maturity during 2015 ($H = 40.56$, $P < 0.001$), 2016 ($H = 64.98$, $P < 0.001$) and 2017 ($H = 123.69$, $P > 0.001$). Non-bearing orchards were significantly lower than mean ranks of moth

catches recorded in both juvenile and mature orchards during 2015, 2016 and 2017. No significant differences in mean ranks of moth catches were recorded in juvenile and mature orchards during 2015, 2016 or 2017.

4.4.5 Larval parasitism and virus infections

No larval parasitism was recorded in any larvae collected from orchards monitored for the duration of this study. A higher percentage virus infection was recorded in larvae collected from mature orchards (7.23% infection, n = 308) than in larvae collected in juvenile orchards (4.45% infection, n = 494), however it was only statistically significant at a 90% confidence level ($\chi^2 = 2.91$, P = 0.088).

4.4.6 Temperature and humidity

Mean temperatures recorded in juvenile orchards were between 0.22 °C and 0.52 °C higher than mean temperatures recorded in mature orchards (Table 4.5). Maximum temperatures recorded in juvenile orchards were between 1.52 °C and 4.55 °C higher than maximum temperatures recorded in mature orchards. Although maximum temperatures were higher in juvenile orchards than mature orchards, minimum temperatures were between 0.46 °C and 0.94 °C lower than minimum temperatures recorded in mature orchards. Mean humidity was between 1.96% and 2.93% lower than mean humidity recorded in mature orchards. Minimum humidity levels were between 0.06% and 2.45% lower than minimum humidity recorded in mature orchards

Table 4.5 Minimum, maximum and overall average humidity levels and temperatures recorded.

	Temperature °C			Humidity (%)		
	Average	**Min**	**Max**	**Average**	**Min**	**Max**
Riverbend juvenile	15.95	0.08	40.62	74.76	8.35	100
Riverbend mature	15.66	0.58	38.63	77.47	10.06	100
Woodridge Juvenile	16.50	0.42	42.12	74.09	7.06	100
Woodridge mature	15.98	0.54	37.58	76.05	9.51	100
Kleinplaas juvenile	16.66	1.1	39.62	73.47	9.53	100
Kleinplaas mature	16.44	1.56	38.11	76.40	9.59	100

4.5 Discussion

Higher mean ranks moth counts were recorded in juvenile and mature orchards during all three sample years compared to non-bearing orchards and refugia. Moth catches were still significantly lower in the non-bearing orchard group during 2017 (trees now juvenile) even though it was their first fruit bearing year. Low moth catches recorded during the first fruit-bearing year of citrus orchards monitored in the non-bearing orchard group is probably due to the poor dispersal ability of FCM (Newton 1998, Moore *et al.* 2004, Timm *et al.* 2010, Stotter *et al.* 2014). However, FCM population numbers are expected to increase significantly in successive years as they disperse from nearby refugia to exploit the higher niche area available to FCM, due to the expanse and homogeneity of susceptible vegetation in citrus orchards (Strong *et al.* 1984, Dent 2000). No significant differences in mean ranks of moth catches were measured between juvenile and mature orchards during any of the three sampling years. Significantly higher trap counts recorded in either juvenile or mature trees were not necessarily linked to higher egg counts or higher FCM damage. Although fruit from juvenile trees have been shown to be preferred above fruit from mature trees for oviposition (Chapter 2), this was not always so in the field. However, mean egg counts in juvenile orchards were higher in Washington Navels and significantly higher in Midnight Valencias during 2017, than in mature orchards of the same cultivars and from the same farm.

Egg parasitism was consistently higher (between 10.75% and 53.75% higher) in mature orchards than juvenile orchards and significantly so in most trials with the exception of Mandarins during 2015, where egg parasitism was slightly higher in juvenile orchards than mature orchards, but not significantly so. Results of this study showed egg parasitism of 34% or higher to significantly reduce mean numbers of viable eggs compared the mean of total egg counts recorded. Higher egg parasitism supports results reported by Langellotto & Denno (2004), who reported a strong negative response to simplified habitat structure in spiders, hemipterans, mites and parasitoids. Lower egg parasitism in juvenile orchards compared to mature citrus orchards may also be because parasitoids are more exposed to wind and dust. According to Van Driesche & Bellows (1996), higher exposure to dust can increase grooming and reduce foraging, oviposition and lengths of visits on dusty foliage.

Furthermore, the more open tree canopy and smaller size of juvenile citrus trees compared to mature trees will also allow for improved spray coverage of pesticides (Simon *et al.* 2007). Various studies have shown that egg parasitoids are highly sensitive to chemical pesticides (Cônsoli *et al.* 1998, Hassan *et al.* 1998, Brunner *et al.* 2001, Grützmacher *et al.* 2004, Takada *et al.* 2001). For example, a study conducted by Hassan *et al.* (1998) reported between 90 to 100% reduction in egg parasitism of the Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) by the egg parasitoid *Trichogramma cacoeciae* (Marchal) (Hymenoptera: Trichogrammatidae), after exposure to mancozeb, a fungicide frequently used to control citrus black spot in South African citrus orchards (Table 4.1, Schutte 2009, Kotze *et al.* 2017). Results reported by Brunner *et al.* (2001) showed imidacloprid and abamectin to be highly toxic to the egg parasitoid *T. platneri* (Nagarkatti). In addition, egg parasitoids are more sensitive to pesticide applications than most insect pest species since they are more mobile and less cryptic (Samways 2005). In the case of FCM, larvae are only exposed to pesticides from oviposition until they burrow into fruit shortly after hatching.

In contrast to what was expected, significantly higher mean counts of viable eggs in juvenile orchards compared to mature orchards were not necessarily linked to significantly higher FCM damage and vice versa. During 2016, similar FCM damage levels were recorded in juvenile and mature Midnight Valencia orange orchards, even though the mean numbers of viable eggs were significantly higher in mature orchards. Results recorded in Chapter 2 showed Midnight Valencia oranges from mature trees to be significantly less susceptible (27% less susceptible) to FCM damage than fruit from juvenile trees, which could explain why FCM damage recorded in mature orchards was lower than expected. During 2016, significantly higher mean counts of 0.41 viable eggs per tree per week were recorded in juvenile Navel orange orchards, compared to mean egg counts of 0.21 viable eggs recorded in mature orchards. However, FCM damage recorded in juvenile Navel orange orchards was higher than damage recorded in mature orchards, but not significantly so. Similar results were recorded in Washington Navel orchards during 2017. Although only statistically significant at a 90% confidence level, higher mean counts of viable eggs (1.53 viable eggs per tree per week were) were recorded in juvenile Washington Navel orchards than in mature orchards (0.98 viable eggs per tree per

week). However, the higher mean egg counts recorded in juvenile orchards did not cause significantly higher FCM damage (2.78 larvae per tree per week) in juvenile orchards than in mature orchards (2.45 larvae per tree per week).

When placed under similar or higher pest pressure, fruit damage caused by FCM is expected to be higher in juvenile orchards than in mature orchards for two reasons. Firstly, results recorded in Chapter 2 showed fruit from juvenile trees to be more susceptible to FCM damage than fruit from mature trees of the same cultivar and secondly, results of this study showed that mean temperatures recorded in juvenile orchards were between 0.22 °C and 0.52 °C higher than mean temperatures recorded in mature orchards, which will expedite FCM development (Daiber 1979a, b, c, Stibick 2010). FCM damage in the above mentioned orchards was possibly lower than expected, because similar to parasitoids, although possibly not to the same extent (Samways 2005), neonate FCM are also more exposed to wind, dust and pesticides in juvenile orchards than in mature orchards. In addition, results of this study have also shown that although mean temperatures were higher in juvenile orchards than mature orchards, juvenile trees are more exposed to temperature extremes, which could increase FCM mortality. Maximum temperature were between 1.52 °C and 4.55 °C higher and minimum temperatures between 0.46 °C and 0.94 °C lower in juvenile orchards compared to mature orchards. Mean humidity recorded in juvenile orchards was also between 1.96% and 2.93% lower than mean humidity recorded in mature orchards. FCM eggs have been shown to be negatively affected by low humidity. Daiber (1979a) reported significantly lower survival of FCM eggs at 30% RH compared to 60 and 90% RH.

Persistence of entomopathogenic viruses has been shown to be reduced by exposure to UV radiation (Moore 2002, Cory & Hoover 2006, Mwanza *et al.* 2016). Therefore, virus persistence is expected to be higher in mature orchards because canopies of mature trees are denser than canopies of juvenile trees, which will reduce exposure to UV radiation. Although the effect of viruses on neonate larvae could not be compared, a higher proportion of larvae retrieved from mature orchards were infected by virus than were larvae retrieved from juvenile orchards, which indicate higher persistence of virus in mature orchards. Similar to this study, Killick & Warden (1991) reported higher mortality of pine beauty moth due to virus infections

in the lower branches of pine trees, which were less exposed to UV radiation compared to branches higher up in the tree canopy.

The results of this field study indicate that differences in tree architecture between juvenile and mature citrus trees have a significant effect on FCM population numbers and egg parasitism. Juvenile orchards have been shown to be more vulnerable to FCM infestations due to lower egg parasitism recorded than in mature orchards. Even though higher mean temperatures were recorded in juvenile orchards than in mature orchards (which would expedite larval development), and fruit from juvenile trees have been shown to be more susceptible to FCM damage than fruit from mature trees of the same cultivar (Chapter 2), FCM infestation in juvenile and mature orchards may still be similar when subjected to equal or higher pest pressure. Compared to mature orchards, FCM damage in juvenile orchards may be lower than expected due to higher mortality of eggs and neonate larvae caused by better spray coverage of pesticides, greater exposure to temperature extremes and lower humidity.

CHAPTER 5

The influence of orchard age on the ecology of entomopathogenic fungi, entomopathogenic nematodes and ants

5.1 Introduction

Recent studies have shown that entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) have great potential as biological control agents for the soil inhabiting stages of false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) which include late 5th instar in search of pupation sites, prepupae, pupae and emerging adults. Results of a study conducted by Manrakhan *et al*. (2014) showed that naturally occurring nematode populations can have a significant impact on FCM population numbers, where, FCM infestation was 59% higher in an orchard where the nematicide, cadusafos, was applied compared to a nearby orchard where no nematicides were applied. In another study, EPN achieved up to 80% control of FCM, in citrus orchards where inundative releases of *Heterorhabditis bacteriophora* (Poinar) and *H. zealandica* (Poinar) were applied (Malan & Moore 2016). Inundative applications of EPF have also been shown to significantly decrease FCM infestation in citrus orchards. Results of a field study conducted by Coombes (2015) reported between 34% and 82% reduction of FCM infestation where inundative applications of *Beauveria bassiana* (Balsamo) Vuillemin were made and between 28% and 63% reduction in FCM infestation where *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin was applied.

Although EPF and EPN show great potential for FCM control, the efficacy and persistence of these biological control agents will greatly depend on the environmental condition of the orchard they are applied in, or where they occur naturally. Both EPN and EPF are adversely affected by low humidity and ultraviolet (UV) radiation (Gaugler *et al.* 1992, Jaronski *et. al.* 2010). Soil moisture is considered the most limiting factor for EPF and EPN efficiency. High relative humidity is essential to prevent nematodes from desiccating and to maintain mobility

as nematodes require a water film in which to move (Wright *et al.* 2005). Low relative humidity also has adverse effects on the infectivity of EPF, as soil moisture levels lower than 90% can prevent spore germination, host infection and sporulation of EPF (Hesketh *et al*. 2010).

Other subterranean natural enemies of FCM include mites, *Pediculoides* sp., predatory bugs, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and *Rhynocoris albopunctatus* (Nyiira) (Hemiptera: Reduviidae), and ants (Bownes *et al.* 2014). Although the presence of ants are not always desirable in citrus orchards, as their mutualistic relationship with sap-feeding insects is known to cause outbreaks of scale insects, psyllids and aphids, they have been shown to be beneficial for FCM control. A study conducted by Bownes *et al.* (2014) showed the brown house ant, *Pheidole megacephala* (Fabricius) (Hymenoptera: Formicidae), and the pugnacious ant, *Anoplolepis custodiens* (Smith) (Hymenoptera: Formicidae), to prey on FCM pupae. Higher numbers of surviving pupae were recorded in citrus orchards in which no chemicals were applied for ant control compared to treated orchards.

Ants have been shown to respond negatively to soil disturbances such as clearing natural vegetation (King *et al.* 1998, Gascon *et al.* 1999) and agricultural activities (Perfecto & Snelling 1995, Philpott *et al.* 2006). King *et al.* (1998), recorded higher species richness of ants in forest compared to areas low in vegetation and determined that ant species richness is negatively affected by high soil temperatures. Perfecto & Snelling (1995) also recorded a negative response in ant diversity to reduced diversity in vegetation. Similar results were reported by Samways (1983), who recorded increasing equitability of ant abundance with an increase in the age of citrus orchards. In young citrus orchards, the dominant ant species accounted 96% of ants recorded, compared to 60% of ants recorded in old orchards and only 17.6% of ants recorded in nearby grasslands.

In Chapter 4 the influence of orchard age on the arboreal natural enemies of FCM (parasitoids and virus) was determined. In this chapter the influence of differences in orchard microclimate between non-bearing (one to three years old), juvenile (four to eight years old) and mature orchards (9 years and older) on the occurrence of the subterranean natural enemies of FCM, which include EPF, EPN and ants activity, was investigated. Since the shaded area under citrus trees

increases with age, the study hypothesized that EPN and EPF occurrence will be lowest in non-bearing orchards, followed by juvenile orchards, with the highest occurrence in mature orchards. The study further hypothesized that ant population numbers will be higher in mature orchards than juvenile orchards, as they have had more time to establish after initial soil disturbances during planting.

5.2 Materials and methods

5.2.1 Bait insect cultures

FCM larvae were obtained from a commercial culture held at River Bioscience, Addo, South Africa. FCM cultures were reared on a diet which was formulated by Moore *et al.* (2014) and consists of maize meal, wheat germ, milk powder, brewers' yeast, nipagin and ascorbic acid. Since FCM has shown low susceptibility to EPF (Goble *et al.* 2010), Mealworm, *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) were also used as bait insect. Mealworms are popular bait insects (Meyling 2007, Malan *et al.* 2011) and are easy to rear. Larvae were reared at room temperature in plastic containers on fine wheat bran. To improve humidity, potato peels or apple slices were laid over the surface of the colony.

5.2.2 Soil sampling

Soil samples were collected every second month from April 2015 until June 2017 from six non-bearing, six juvenile and six mature orchards in the Eastern Cape Province, South Africa (Table 5.1). Two Navel orchards, two Mandarin orchards and two Midnight Valencia orchards were sampled per maturity group. Although the Mandarin orchards chosen for the mature grouping were only five years old at the beginning of the study, the trees had already grown to a height of approximately 2.5 m, with a very dense canopy. Eight soil samples, which consisted of seven subsamples each, were collected from eight evenly distributed rows in each orchard (Fig. 5.1). Seven subsamples (approximately 140 g each) were collected with a hand spade from underneath the canopy of seven trees per row at a depth of approximately 15 cm. To reduce edge effect, the first sample was collected from the

third to fifth row and the third to fifth tree of each orchard, depending on the size of the orchard. The following samples were then collected in a zigzag pattern from seven evenly distributed trees per row. Soil samples for each orchard tested were sent to Bemlab, Strand, Western Cape, South Africa to determine the soil texture and pH (Table 5.1).

Eight soil samples, which consisted of seven subsamples, were collected from refugia adjacent to six citrus orchards (two at Riverbend Farm, two at Miskruier Farm, one at Buffelsbos Farm and one at Woodridge Farm) during April 2015. Eight soil sampling points were selected randomly, 6-7 m apart (Fig. 5.2). Seven subsamples were collected at each sampling point, 1-1.5 m apart. Soil samples were labelled and kept in clear plastic bags (32 cm x 21 cm) at 16 °C and baited with insects within two weeks after being collected.

Soil samples were only collected once from refugia, in order to compare EPF and EPN occurrence in non-bearing, juvenile and mature orchards. Orchards were then monitored continuously every second month to determine changes in EPF or EPN occurrence as orchards aged. Although referred to as non-bearing orchards throughout this study, orchards placed in this maturity grouping were non-bearing during 2015 and 2016, but were bearing fruit for the first time during 2017.

Table 5.1. Details of orchards monitored.

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted	Year monitored
Non-bearing	Riverbend: S 33°25.107 E 25°42.677	Witkrans Navel	515	2014	2015, 2016, 2017
		M7 Navel	603	2015	2015
		Orr Mandarin	606	2015	2015, 2016, 2017
		Afourer Mandarin	611	2014	2015
		Midnight Valencia	506	2014	2015, 2016, 2017
		Midnight Valencia	502	2014	2015
	Miskruier: S 33°27.427 E 25°41.431	Cambria Navel	11	2013	2016, 2017
		Midnight Valencia	71	2013	2016, 2017
	Halaron: S 33°30.591 E 25°39.704	Afourer Mandarin	75	2015	2016, 2017
Juvenile	Woodridge: S 33°28.787 E 25°41.665	Cambria Navel	300	2011	2015
		Cambria Navel	303	2012	2015, 2016, 2017

Soil texture	pH, KCL	Fungicides applied (active ingredients)
sandy clay loam	7.3	**2015 and 2016**: None **2017**: mancozeb, copper hydroxide
sandy loam	6.4	
sandy clay	5.6	
sandy clay loam	7.2	
sandy clay	6.8	
sandy loam	6.8	
sandy clay loam	5.0	**2016:** None **2017:** azoxystrobin, benomyl, copper hydroxide, mancozeb
clay loam	7.3	**2016:** None **2017:** azoxystrobin, benomyl, copper hydroxide, mancozeb
sandy clay loam	7.4	**2016 and 2017:** None
sandy clay loam	7.4	mancozeb, tebucanazole, pyraclostrobin, trifloxystrobin,
sandy clay loam	7.4	**2015, 2016 and 2017:** mancozeb, tebucanazole, carbendazim **2015:** trifloxystrobin

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted	Year monitored	Soil texture	pH, KCL	Fungicides applied (active ingredients)
								2016: pyraclostrobin **2017**: copper hydroxide, pyraclostrobin
		Midnight Valencia	301, 302	2011	2015, 2016, 2017	sandy clay loam	5.3	**2015, 2016 and 2017:** mancozeb, carbendazim **2015:** trifloxystrobin **2016:** pyraclostrobin **2017**: copper hydroxide, pyraclostrobin
	Buffelsbos: S 33°27.294 E 25°42.071	Valley Gold	650, 653	2012	2015, 2016, 2017	sandy clay loam	6.2	**2015, 2016 and 2017:** mancozeb, carbendazim **2015:** trifloxystrobin **2016:** pyraclostrobin **2017**: copper hydroxide, pyraclostrobin
	Halaron: S 33°30.591 E 25°39.704	M7 Navel	71	2012	2016, 2017	sandy clay loam	7.1	**2016 and 2017:** benomyl, mancozeb **2017:** tebucanazol
	Miskruier: S 33°27.427 E 25°41.431	Washington Navel	50	2005	2015, 2016, 2017	sandy clay loam	5.7	**2015, 2016 and 2017:** benomyl, mancozeb, azoxystrobin **2017:** tebucanazole
		Newhall Navel	49	2005	2015	sandy clay loam	7.3	benomyl, mancozeb, azoxystrobin
		Midnight Valencia	51, 52	2005	2015, 2016, 2017	sandy clay loam	7.4	**2015, 2016 and 2017:** benomyl, mancozeb, azoxystrobin, copper hydroxide
	Woodridge: S 33°28.787 E 25°41.665	Afourer	341, 342	2010	2015, 2016, 2017	sandy clay loam	6.5	**2015, 2016 and 2017:** mancozeb, pyraclostrobin **2015:** tebucanazole, trifloxystrobin, **2016:** tebucanazole, copper hydroxide **2017:** copper hydroxide
	Halaron: S 33°30.591 E 25°39.704	Bahianinha Navel	53	2007	2016, 2017	sandy loam	7.2	**2016 and 2017:** tebucanazole, benomyl, mancozeb

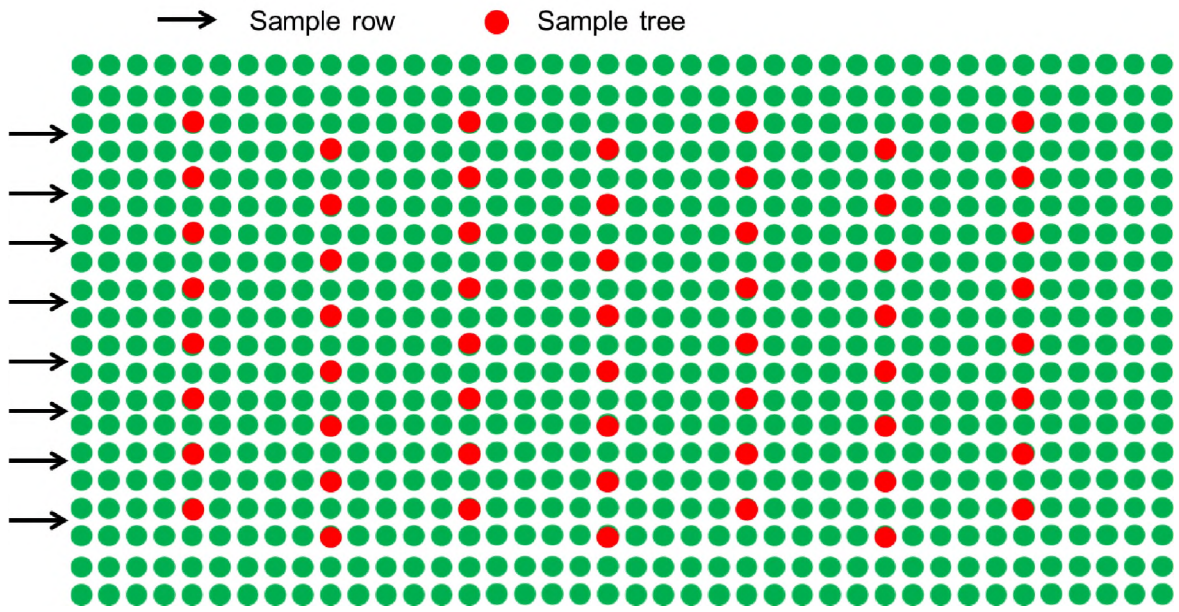

Figure 5.1: Schematic diagram of soil sampling points in citrus orchards.

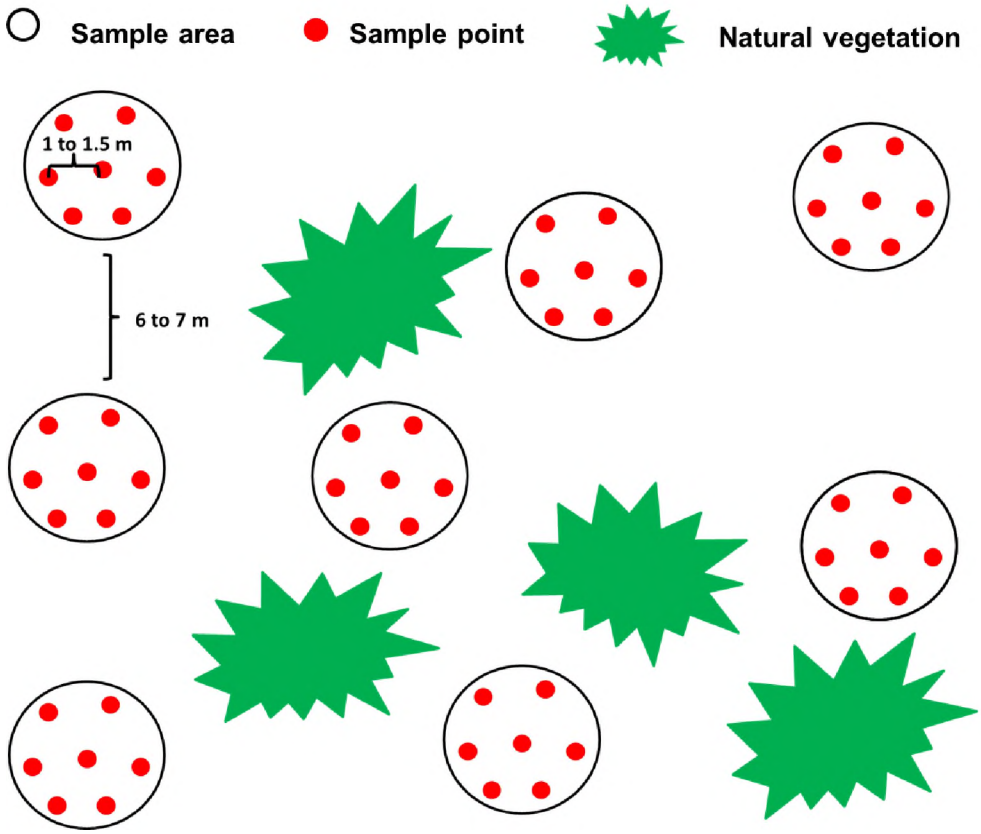

Figure 5.2: Schematic diagram of soil sampling points in field margins (refugia) adjacent to citrus orchards.

5.2.3 Baiting procedures

Entomopathogenic fungi and nematodes were recovered from soil samples by using the insect-baiting technique (Bedding & Akhurst 1975, Klingen *et al.* 2002). Each soil sample was thoroughly mixed and then sieved through a metal sieve with a mesh size of 2 mm before adding bait insects. One 250 ml curry tub was filled with soil from each sample and baited with three mealworms and three late 5^{th} instar FCM. After adding bait insects, curry tubs were sealed with a perforated lid and incubated at 25 °C in the dark. Containers were inspected every day for the first three days after baiting to remove any pre-pupating FCM from the lids of curry tubs. Soil samples were checked weekly for three weeks for the presence of dead insects. Any dead insects found were surface sterilised with 70% ethanol and then rinsed in a 1:1 ratio of sodium hypochlorite (3.5%) and distilled water solution for three seconds, followed by a rinse in distilled water only to prevent opportunistic external saprophytic fungi from growing on insect cadavers. Insects were then placed into modified White traps (Kaya & Stock 1997) to harvest any emerging EPN. Modified White traps were made by placing an open Petri dish with moistened filter paper inside a larger Petri dish or tub with a thin layer of distilled water. Emerging nematodes crawl out of the smaller Petri dish and become trapped in the layer of water surrounding it. White traps also acted as moisture chambers to promote fungal growth and sporulation.

5.2.4 Isolation and identification of fungi and nematodes

A selective growth medium, adapted from Meyling (2007) was used to isolate fungi. Sixty grams of Sabouraud Dextrose Agar (SDA), 1 ml Dodine and 1 ml 50 mg/ml Chloramphenicol were added to each litre of distilled water required to prepare the desired volume of growth medium. After being autoclaved at 120ºC for 20 min, growth medium was cooled down to 40 ± 5 °C and poured into 9 cm diameter Petri dishes. A small quantity of spores were scraped off insect cadavers, placed onto prepared media and incubated at 25 °C in the dark. All fungi which were potentially entomopathogenic were identified under a compound microscope using tape mounts, according to morphological characteristics described in taxonomic keys

and other relevant literature (Barnett 1960, Domsch *et al.* 2007). Species identification was confirmed by molecular identification of representative samples.

Deoxyribonucleic acid (DNA) was extracted from fungal isolates using a modified salting out protocol (Sunnucks & Hales 1996). Fungal spores and mycelia were scraped off SDA plates using a 1000 µl tip and added to a 1.5 ml Eppendorf tube containing 100 µl of sterile distilled water supplemented with 0.1% Tween 20. The fungal suspension was homogenised and 180 µl of ATL buffer (Qiagen) and 15 µl of proteinase K were added to the Eppendorf tube. The samples were then placed in a heatblock at 56 °C and left overnight. Samples were removed and centrifuged at 13, 000 rpm for five minutes. The supernatant of each sample was then transferred to a clean Eppendorf tube and 65 µl of 5M NaCl was added to each tube. Samples were then vortex mixed for 30 seconds and centrifuged at 13 000 rpm for five minutes, after which the supernatant of each sample was transferred to another clean Eppendorf tube. Once the supernatant was transferred to the new Eppendorf, 150 µl of cooled 98% isopropanol was added. The samples were then inverted 30 times and placed in the freezer overnight. The following day, samples were centrifuged at 13,000 rpm for five minutes. The supernatant was removed from each sample, leaving a DNA pellet behind. Samples were then supplemented with 250 µl of cooled 70% ethanol. Samples were then vortex mixed for 30 seconds and centrifuged at 13,000 rpm for five minutes, after which ethanol was removed and the previous step was repeated to extract ethanol from samples. Samples were placed in a heating block at 50 °C to ensure all ethanol was removed and that the pellet was dry. Each DNA pellet was then re-suspended in 20 µl TE buffer (elution buffer) (Qiagen). The DNA concentration for each sample was determined using a Nanodrop spectrophotometer (Thermo Scientific®). This was considered when mixing reagents for polymerase chain reaction.

Universal fungal primers were used to amplify the internal transcribed spacer region in order to identify the fungal samples (Table 5.2). The polymerase chain reaction (PCR) products were analysed by 1% agarose gel electrophoresis (AGE) to confirm whether the amplification of the ITS gene region occurred (Table 5.3 and Table 5.4). The PCR products were sequenced by integrated DNA technologies (IDT, WhiteSci). FinchTV® version 1.4.0 (Geospiza Inc. 2004-2006, Seattle,

Washington) was used to view the chromatograms. Sequences were then subjected to NCBI BLAST to identify the fungal samples. Mega version 5 (Tamura *et al.* 2011) was used to construct a neighbour joining bootsrap, Kimura 2-paramater model tree to determine closely related species.

Table 5.2: Universal oligonucleotide primers for the amplification of the internal transcribed spacer gene region (White *et al.* 1990).

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

Table 5.3: Quantity of reagents needed for the amplification of the ITS region of fungal isolates.

Reagents	Quantities of reagents needed (µl)	
	-ve Control	Samples
TopTaq	12.5	12.5
ITS1 (10 µm)	2	2
ITS4 (10 µm)	2	2
Template DNA (50 ng/µl)	2	2
$MgCl_2$	1	1
ddH_2O	5.5	7.5
TOTAL	25	25

Table 5.4: Cycling parameters for the amplification of the ITS gene region.

Conditions	Temperature (°C)	Time (minutes)	Cycles
Initial denaturation	94	5:00	1
Denaturation	94	0:30	
Annealing	47	0:45	35
Extension	72	1:00	
Final extension	72	7:00	2

All EPN isolated were sent to the department of Conservation Ecology and Entomology at Stellenbosch University, Western Cape, South Africa for morphological and molecular identification. Morphological and molecular identification was done as described by Malan *et al.* (2011). Taxonomic criteria as suggested by Hominick *et al.* (1997) and Stock and Kaya (1997) were used to confirm molecular species identification.

5.2.5 Quick screening method to detect entomopathogenic fungi

In order to confirm Koch's postulates for pathogenicity, nematodes and fungal spores were harvested and used to inoculate final instar mealworms (Steyn & Cloete, 1989). One week after nematode emergence, 1 ml of a water and nematode suspension was harvested from White traps and used to inoculate five mealworms. Insect mortality was determined after 72 h. Dead larvae were placed into White traps to determine nematode emergence. A rapid screening method, adapted from Ali-Shtayeh *et al.* (2002) was used to confirm pathogenicity of fungi. Conidia and mycelia were collected from sporulating fungal cultures and placed into sterilized 1.5 ml Eppendorf tubes supplemented with 1 ml of triple distilled water and 0.05% Tween 20 and then vortex mixed for 3 min. Five mealworms were dipped into each fungal suspension for 3 s. The larvae were then placed into moisture chambers (Petri dish with moistened filter paper) and incubated in the dark at 25 °C. Petri dishes were checked daily for the presence of dead larvae for 2 weeks. Dead larvae were placed onto SDA agar until sporulation

5.2.6 Scouting for ants

During 2017 the same orchards and sample trees which were scouted for the presence of FCM eggs were monitored for the presence of ants (Section 4.2, Table 4.1, Fig. 4.2). The orchard surface close to and under the canopy of each tree was inspected for 20 s and rated for ant activity. Ratings allocated were as follows; 0 - no ants, 1 - one to four ants, 2 - five to nine ants, 3 - 10 to 19 ants, 4 - more than 20 ants.

5.3 Statistical analyses

All statistical analyses were performed using Statistica version 13.2, 2017 (Statsoft South Africa Research (Pty) Ltd, Johannesburg, South Africa). A chi-square test (observed vs. expected) was used to compare differences in EPF occurrence in bait insects. All other data were analysed using ANOVA with post-hoc comparisons

of means using a Tukey-Kramer HSD test. Significant differences were determined at a 95% probability level.

5.4 Results

5.4.1 Fungal and nematode species isolated and bait insect

Significantly more EPF isolates (χ^2 = 499, P < 0.001) were recovered from mealworm (33.43% of soil samples) than from FCM (5.18% of soil samples) (Fig. 5.3). The majority of isolates recovered (n = 810) were *Beauveria* sp. (87.88% of all isolates) followed by *Metarhizium* sp. (11.87% of all isolates). Eighty-four percent, 91%, 89% and 85% of EPF isolated from refugia, non-bearing orchards, juvenile orchards and mature orchards respectively were *Beauveria* sp. Only one isolate of an *Isaria* sp., which has not been described yet, was isolated from a non-bearing orchard (0.25% of all isolates) (Fig. 5.4). Significantly more *Beauveria* sp. were recovered from both mealworm (n = 690, χ^2 = 890, P < 0.001) and FCM (n = 120, χ^2 = 187, P < 0.001) than *Metarhizium* sp. Only 14.06% and 5.83% of EPF isolates recovered from mealworm and FCM respectively were *Metarhizium* sp. One *Metarhizium robertsii* (Metschnikoff) Sorokin isolate and one *Metacordyceps brittlebankisoides,* (Liu *et al.*) (the teleomorph of *M anisopliae* (Liu *et al.* 2001)), isolate were recovered from a mature orchard. All other *Metarhizium* sp. were identified as *M. anisopliae.* All *Beauveria* sp. isolated were identified as *B. bassiana.*

Only five EPN isolates were recovered from soil samples (0.24% of soil samples). All isolates recovered were identified as *H. bacteriophora.* Three of the five isolates were recovered from mature orchards while the other two were recovered from juvenile orchards. No EPN isolates were recovered from non-bearing orchards. Four of the five isolates were recovered from Woodridge Farm and one from the neighbouring farm, Miskruier (Table 5.1).

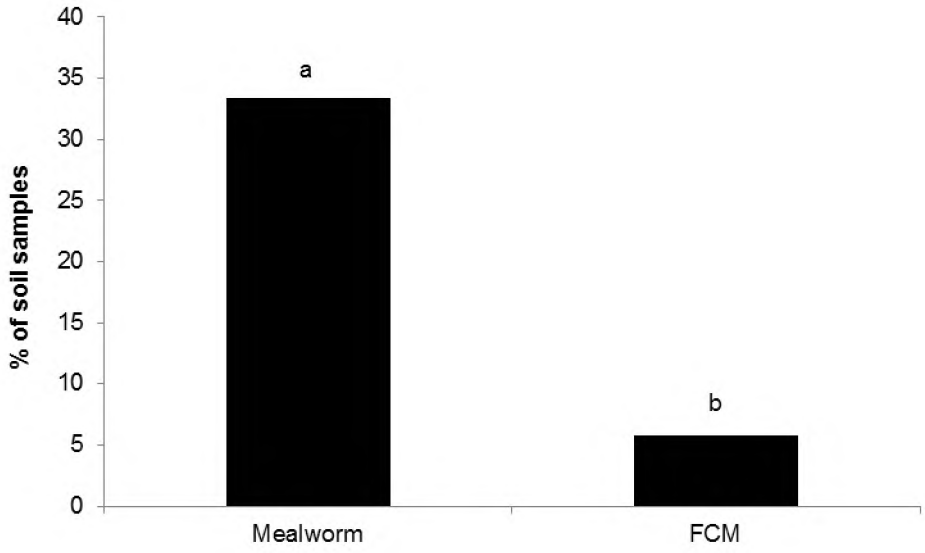

Figure 5.3: The occurrence (percentage of samples) of entomopathogenic fungi isolated from the two different bait insects used (n = 2064). Different letters above bars denote significant differences (chi-square tests, $P < 0.05$).

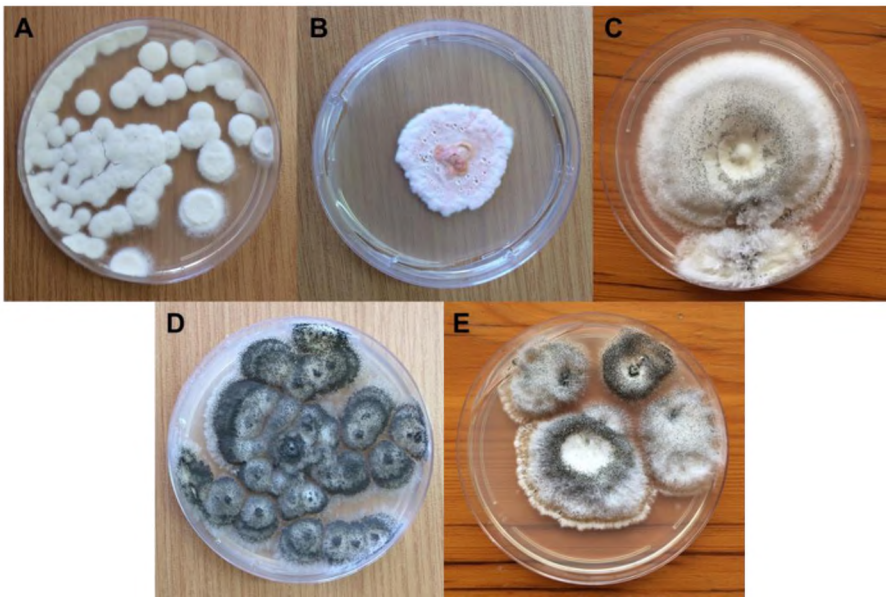

Figure 5.4: Cultures of entomopathogenic fungal species isolates. A: *Beauvaria bassiana, B: Isaria* sp. C: *Metacordyceps brittlebankisoides,* D: *Metarhizium anisopliae* and E: *Metarhizium robertsii.*

4.4.2 Orchard age and occurrence of entomopathogenic fungi

The one-way ANOVA for mean EPF occurrence (percentage of soil samples collected during April 2015) isolated from mealworms (Fig. 5.5) indicated no significant differences ($F_{(3, 20)} = 0.46$, $P = 0.71$) in EPF occurrence between refugia,

non-bearing orchards, juvenile orchard or mature orchards. The highest mean occurrence of 35.42 ± 10.91% was recorded in refugia, followed by mean EPF occurrence of 33.33 ± 4.27% and 31.25 ± 4.17% EPF recorded in mature and non-bearing orchards respectively, with the lowest EPF occurrence of 25 ± 4.56% recorded in juvenile orchards.

The one-way ANOVA for mean EPF occurrence (percentage of soil samples collected during April 2015) isolated from FCM (Fig. 5.5) indicated no significant differences ($F_{(3, 20)}$ = 1.96, P = 0.15) in EPF occurrence between refugia, non-bearing orchards, juvenile orchard or mature orchards. The highest mean EPF occurrence of 8.33 ± 2.64% was recorded in refugia, followed by mean EPF occurrence of 6.25 ± 4.27% and 2.08 ± 2.08% recorded in non-bearing and mature orchards respectively. No EPF were isolated from FCM in soil samples collected from juvenile orchards.

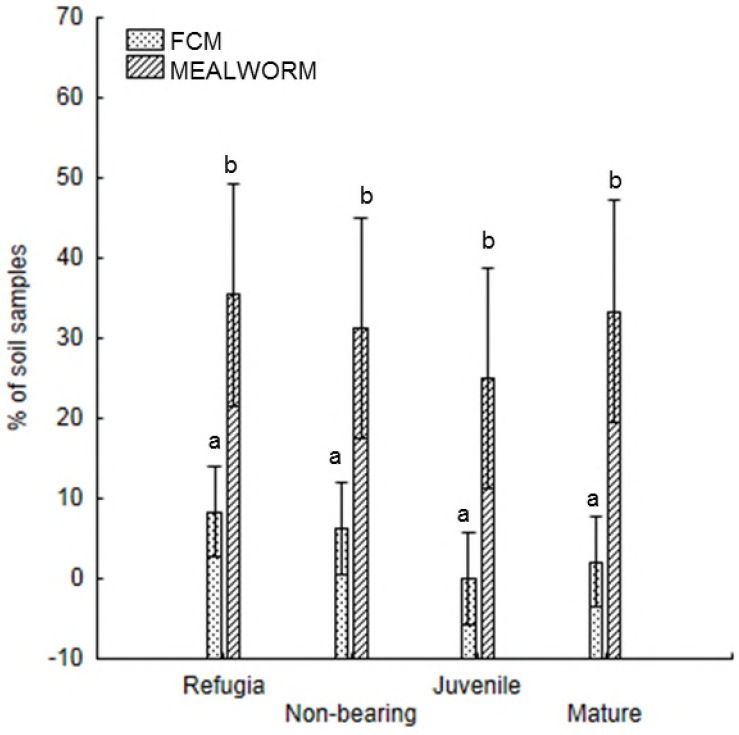

Figure 5.5: Mean occurrence (percentage of samples, n = 48) of EPF in soils sampled during April 2015 from refugia and from non-bearing, juvenile and mature orchards. Different letters above bars denote significant differences (comparison of means, P < 0.05).

The one-way ANOVA for mean EPF occurrence (percentage of soil samples) isolated from mealworms (Fig. 5.6) indicated significant differences ($F_{(2, 179)}$ = 12.24, P < 0.001) between orchards of different ages. Significantly higher EPF occurrence (P < 0.001) was recorded in both non-bearing orchards (40.33 ± 2.13% of samples) and mature orchards (36.76 ± 2.05% of samples) compared to juvenile orchards (25.30 ± 2.02% of samples). No significant difference in EPF occurrence (P = 0.92) was recorded between mature and non-bearing orchards.

Results of the one-way ANOVA for mean EPF occurrence (percentage of soil samples) isolated from FCM (Fig. 5.6) indicated significant differences ($F_{(2, 179)}$ = 5.87, P = 0.003) between orchards of different ages. Significantly higher EPF occurrence (P = 0.002) was recorded in non-bearing orchards (9.23 ± 1.18% of samples) than in juvenile orchards (3.21 ± 0.79% of samples). EPF occurrence recorded in mature orchards (5.36 ± 1% of samples) was not significantly different from both non-bearing (P = 0.44) and juvenile orchards (P = 0.61).

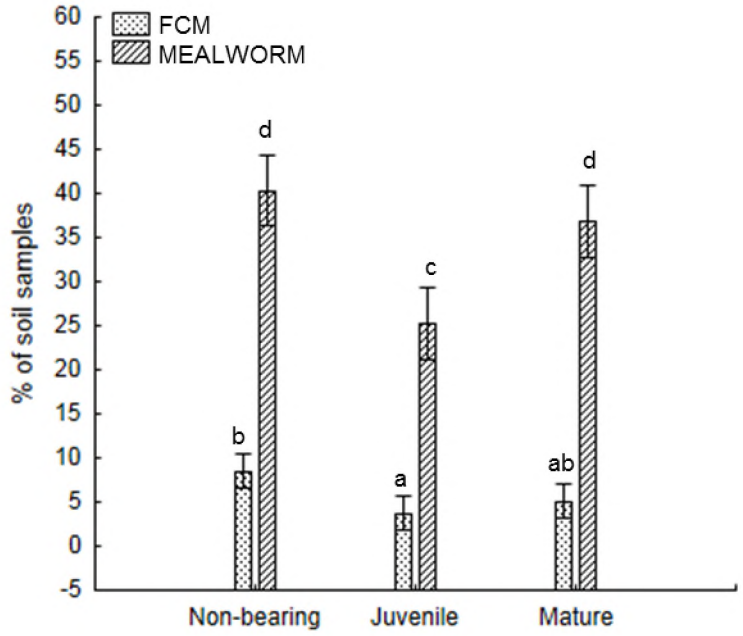

Figure 5.6: Collective mean occurrence (percentage of samples, n = 672) of EPF in soils sampled during the entire study from non-bearing, juvenile and mature orchards. Different letters above bars denote significant differences (comparison of means, P < 0.05).

The two-way ANOVA for mean EPF occurrence recovered from mealworm (Fig. 5.7) showed no interaction between main effects maturity (three levels, non-

bearing, juvenile and mature) and sample year (three levels, 2015, 2016 and 2017) ($F_{(2, 173)}$ = 1.07, P = 0.374). EPF occurrence in non-bearing orchards increased slightly from 35.58 ± 4.02% in 2015 to 39.48 ± 4.23% in 2016 (P = 1) and to 40.53 ± 6.09% in 2017 (P = 1), however not significantly so. In juvenile orchards, EPF occurrence increased insignificantly (P = 0.385) from 15.67 ± 3.22% in 2015 to 20.76 ± 3.33% in 2016, but increased significantly (P = 0.0189) from 2016 to 32.3 ± 4.07% in 2017. EPF occurrence was significantly lower in juvenile orchards than in non-bearing and mature orchards during 2015 and 2016, but was not significantly different during 2017. EPF occurrence in mature orchards increased insignificantly (P = 0.241) from 31.94 ± 4.56% in 2015 to 39.18 ± 4.11% in 2016 and decreased slightly from 2016 to 38.43 ± 6.15% in 2017.

Results of the two-way ANOVA for mean EPF occurrence recovered from FCM (Fig. 5.7) showed no interaction between main effects maturity (three levels, non-bearing, juvenile and mature) and sample year (three levels, 2015, 2016 and 2017) ($F_{(2, 173)}$ = 1.68, P = 0.158). No significant differences in EPF occurrence were recorded between sample years for any of the three maturity groups. EPF occurrence decreased slightly in non-bearing orchards from 11.02 ± 3.55% in 2015 to 8.18 ± 2.81% in 2016 and to 7.13 ± 2.67% in 2017. In contrast, EPF occurrence in juvenile orchards increased slightly from 0.84 ± 2.67% in 2015 to 1.89 ± 0.79% in 2016 and to 5.9 ± 1.79+% in 2017. EPF occurrence increased slightly in mature orchards from 3.24 ± 1.48% in 2015 to 6.54 ± 1.87% in 2016 and decreased slightly from 2016 to 5.04 ± 2.04% in 2017. EPF occurrence in non-bearing orchards was significantly higher than both mature (P = 0.012) and juvenile orchards (P = 0.001) during 2015. During 2016, EPF occurrence in juvenile orchards was significantly lower than both non-bearing (P = 0.043) and mature orchards (P = 0.049). No significant differences between orchards were recorded during 2017.

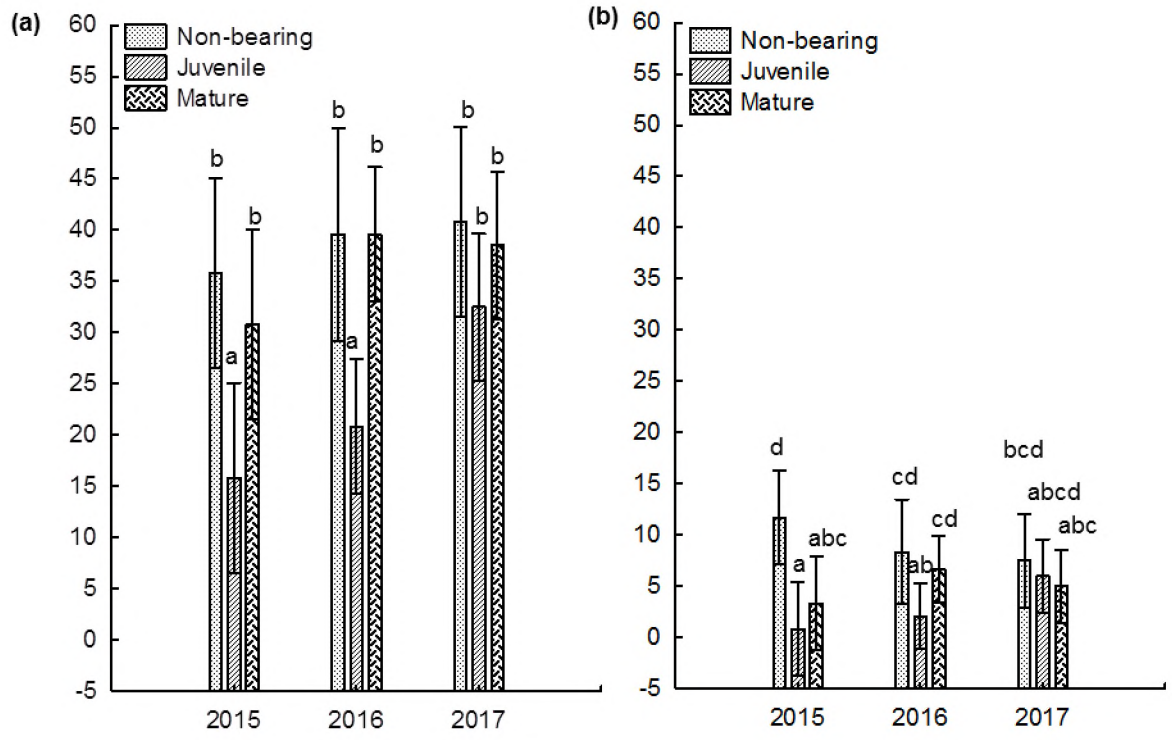

Figure 5.7: The occurrence (percentage of samples) of EPF in soils sampled from non-bearing, juvenile and mature orchards that were recovered from (a) mealworms and (b) FCM. Different letters above bars denote significant differences (comparison of means, $P < 0.05$).

4.4.3 Orchard cover and irrigation method

Results of a t-test showed no significant difference in EPF occurrence (Table 5.5) between orchards irrigated by drip or micro-sprinkler irrigation for EPF recovered from both FCM ($P = 0.619$) and mealworms ($P = 0.3$). EPF occurrence in soil samples baited with mealworms was significantly lower ($P = 0.155$) in orchards that were covered by mulch than in orchards which had no soil covering. No significant difference ($P = 0.714$) was recorded in EPF occurrence in soil samples baited with FCM in orchards that were covered by mulch and orchards that had no soil covering (Table 5.6).

Table 5.5 Comparison of EPF occurrence between orchards irrigated by drip and micro-sprinkler systems recovered from different bait insects.

Bait insect	Mean drip	Mean micro	t-value	df	P-value	SD juvenile	SD mature
FCM	5.77	5.74	0.02	250	0.619	9.69	9.15
Mealworm	32.57	35.22	-0.4	250	0.300	18.47	20.88

Table 5.6 EPF occurrences between orchards covered by mulch and orchards with no soil covering, recovered from different bait insects.

Bait insect	Mulch	None	t-value	df	P-value	SD juvenile	SD mature
FCM	6.59	6.09	0.02	208	0.714	9.85	9.66
Mealworm	31.85	38.57	-2.44	208	0.0155	19.98	19.67

4.4.2 Orchard age and ant occurrence

Results of a t-test showed no significant difference (t = -1.47, P = 0.144) in the mean rating of ant occurrence recorded in juvenile and mature orchards. The mean rating for ant occurrence was 0.76 ± 0.05 ~ 1 (one to five ants per tree per week) in mature orchards and 0.87 ± 0.06 ~1 (one to five ants per tree per week) in juvenile orchards.

5.4 Discussion

Beauveria bassiana was isolated significantly more often from both mealworm (84.94% of isolates) and FCM (94.17% of isolates) than any other fungal species. The high occurrence of *B. bassiana* recorded in this study is similar to results reported by Hatting *et al.* (2004), who conducted an extensive survey on EPF distribution in South Africa and determined that 87% of EPF species isolated were *B. bassiana.* Similar results were reported by Goble *et al.* (2010), who conducted a survey on the occurrence and distribution of EPF in soils from citrus orchards and refugia in the Eastern Cape Province of South Africa. *Beauveria bassiana* and *M. anisopliae* were the most commonly isolated species. Results of this study and

those reported by Hatting *et al.* (2004) and Goble *et al.* (2010), are similar to results reported by Chandler *et al.* (1997), who determined that EPF species diversity is low, usually with one or two dominant species occurring frequently. Similar results were also recorded in surveys conducted by Keller *et al.* (2003) and Quesada-Moraga *et al.* (2007).

A survey conducted by Malan *et al.* (2011) reported 15.4% EPN occurrence in soils collected from citrus orchards in the Eastern Cape, South Africa. However, only 0.24% of soil samples collected during this study tested positive for EPN. The low occurrence of EPN reported in this study could possibly be due to the close proximity of orchards sampled.

No difference in ant activity was recorded in juvenile and mature orchards, thus indicating that ants colonise new orchards within four to six years after planting. Although the study focused on ant diversity rather than ant abundance, similar results were recorded by Perfecto & Vandermeer (2002). Perfecto & Vandermeer (2002) recorded no difference in ant diversity in organically managed coffee plantations and conventionally managed coffee plantations. However, ant diversity was significantly lower in conventionally managed coffee plantations than in nearby forests. The organic coffee plantation surveyed by Perfecto & Vandermeer (2002) was characterised by higher shade density than the conventionally managed coffee plantation surveyed. Ant activity may be lower in non-bearing orchards younger than three years, but this was not determined and was considered irrelevant, as FCM control is not required in orchards which do not bear fruit.

In this study, FCM was shown to be significantly less susceptible to EPF infection than mealworms. EPF occurrence of 5.18% was recorded in soil samples baited with FCM compared to 33.43% occurrence in soil samples baited with mealworms. These results were similar to results recorded by Goble *et al.* (2010), who recorded significantly lower EPF occurrence of 2.1% in soil samples baited with FCM compared to 15.62% in soil samples baited with wax moth, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) larvae. According to Goble *et al.* (2010), FCM is probably less susceptible to EPF than *G. mellonella,* because they pupate in the soil and may therefore have adapted better defence mechanisms against soil

entomopathogens than *G. mellonella*, which does not have a soil inhabiting life-stage. A study conducted by Shapiro-Ilan and Mizell (2015) has shown that the pupal stages of some soil dwelling insects have antimicrobial properties that supress EPF development. Goble *et al.* (2010) also suggested that the ability of FCM to spin a cocoon prior to pupation will prevent EPF conidia from coming into direct contact with the insect cuticle, which will prevent spores from germinating. Similar to *G. mellonella*, mealworm may not have adapted defences against EPF infection because they do not have a soil inhabiting life-stage. Furthermore, mealworms were observed to actively move through the soil which increases their contact with infective conidia, while FCM only wanders a short distance before selecting pupation sites (pers. obs., Love 2015).

No significant differences in EPF occurrence were recorded between orchards under drip and micro irrigation. EPF occurrence in orchards under drip irrigation recorded in this study may be higher than expected because soil samples were taken under the tree canopy, close to the roots and irrigation source. EPF occurrence may possibly be lower in orchards under drip irrigation further away from the water source. The lower occurrence of EPF in soil samples, baited with mealworms, which were collected from orchards covered by mulch, compared to orchards with no covering is possibly because conidia stick to the surface of mulch, thus preventing them from reaching the soil. Furthermore, mulch may impair spore dispersal by restricting lateral water flow. However, the higher occurrence of EPF recorded in orchards with no covering did not have a significant effect on FCM infection, when compared to orchards covered with mulch. This is probably because FCM is less sensitive to EPF infection than mealworms, thus a larger difference in spore load is needed to have a significant impact on FCM control. In contrast to results reported by Goble *et al.* (2010), no significant difference in EPF occurrence was recorded in refugia compared to citrus orchards. However, results of this study are similar to those reported by Klingen *et al.* (2002) who found no differences in EPF occurrence between refugia and arable fields.

Contrary to the hypothesis, EPF occurrence was significantly higher in non-bearing and mature orchards compared to juvenile orchards. Non-bearing orchards may possibly have higher EPF occurrence than expected, as no fungicides were

applied during the first two sampling years (Table 5.1). No significant difference in EPF occurrence was recorded between mature and non-bearing orchards. These results are similar to results reported by Goble *et al.* (2010) who found no significant difference in EPF occurrence between organically and conventionally managed farms. The significantly higher occurrence of EPF in mature orchards than juvenile orchards is possibly because of the more favourable environmental condition (higher shade density) for EPF persistence in mature orchards than in juvenile orchards, which counteracts the negative impact of fungicide applications. EPF in mature orchards may possibly also have developed resistance to fungicides, as they were exposed to fungicides for a longer time that juvenile orchards, but this is still to be determined. Furthermore, the significant increase in EPF occurrence in juvenile orchards during the third growing season of this study supports the hypothesis that higher shade density increases EPF occurrence and persistence. However, EPF occurrence did not decrease significantly during the 2017 growing season in non-bearing orchards, even though it was the first season in which fungicides were applied in the majority of the non-bearing orchards monitored. It is possible that the adverse effects of fungicides on EPF reproduction will only be visible during the following growing season.

The results of this study show that juvenile orchards are more vulnerable to FCM infestation than non-bearing and mature orchards, most likely because of the combined negative impact of exposure to sunlight and fungicide applications on EPF persistence.

CHAPTER 6

Influence of orchard age on the tolerance of entomopathogenic fungi to fungicides

6.1 Introduction

The majority of entomopathogenic fungi (EPF) used as biological control agents of insect pests fall within the order Hypocreales, which includes well know genera such as *Beauveria*, *Metarhizium*, *Isaria* and *Lecanicillium* (Inglis *et al.* 2001). Hypocreales fall within the division Deuteromycota, also referred to as Fungi Imperfecti, which has no sexual state (Bidochka & De Koning 2001, Roy *et al.* 2006). However, according to Bidochka & De Koning (2001) many mycologists argue that all deuteromycetous fungi have teleomorphic stages which have just not yet been discovered. With recent molecular techniques, the abovementioned statement has partly been proven, as many of these asexual fungi (anamorphs) have similarities with ascomycetes and in addition could be linked to sexual states (teleomorph) (Inglis *et al.* 2001, Liu *et al.* 2001, Zengzhi *et al.* 2001).

However, although the teleomorphic stages of many deuteromycetous fungi have been discovered, asexual reproduction is still predominant (Devi *et al.* 2006). In spite of a low rate of sexual reproduction, high genetic diversity has been reported in deuteromycetous fungi (Fegan *et al.* 1993, Bidockha & De Koning 2001, Hughes *et al.* 2004, Devi *et al.* 2006), which has been attributed to the occurrence of parasexual cycles (Pontecorvo *et al.* 1953, Paccola–Meirelles & Azevedo 1991, Castrillo *et al.* 2005). In addition to the ability of deuteromycetous fungi to achieve genetic diversity by transferring genetic material through parasexual cycles, genetically identical strains of deuteromycetous EPF have shown the ability to adapt to environmental stressors.

Andersen *et al.* (2006) investigated the possibility of improving EPF performance in dry environments by physiologically manipulating fungal inoculum. Various EPF species were grown under water stressed conditions and produced conidia with increased concentrations of erythritol and accelerated germination rates were recorded. Fargues & Robert (1983) reported increased infectivity of *Cetonia*

aurata (Linnaeus) (Coleoptera: Scarabaeidae) and *Orycetes rhinoceros* (Linnaeus) (Coleoptera: Scarabaeidae) by *Metarhizium anisopliae* var. *anisopliae* (Metschnikoff) Sorokin, following multiple passages through each insect host. The ability of EPF to adapt to their environment is further illustrated by a study conducted by Rangel *et al.* (2011) who recorded increased tolerance of *Metarhizium robertsii* (Metschnikoff) Sorokin, to ultra violet (UV) radiation in conidia produced by cultures grown under high light intensity. Shapiro-Ilan *et al.* (2011) achieved enhanced fungicide resistance of *B. bassiana* and *Metarhizium brunneum* (Petch), through artificial selection. Increased resistance to two fungicides, fenbuconazole and triphenyltin hydroxide, was achieved for both *B. bassiana* and *M. brunneum* after exposure to each fungicide. However, some populations selected for enhanced fungicide resistance showed decreased germination, growth and virulence to the greater wax moth, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae). Shapiro-Ilan *et al.* (2002), recorded higher tolerance in *B. bassiana* strains collected from pecan orchards to three fungicides (dodine fenbuconazole and triphenyltin hydroxide), which are commonly used in pecan and other horticultural crops, compared to a commercial *B. bassiana* strain, and concluded that the higher tolerance recorded in wild *B.* bassiana isolates compared to the commercial strain was possibly due to previous exposure (and thus selection) in the field. The ability of EPF to develop resistance to fungicides will be very beneficial for citrus production in South Africa, especially in the Western Cape, Eastern Cape, Northers Cape, KwaZulu-natal, North-West and Limpopo citrus producing areas were citrus black spot (CBS), *Guignardia citricarpa,* (Kiely), occurs (Schutte 2009). Since the European Union regards CBS as a phytosanitary risk, strict control measures are required for fruit destined for European exports markets (Schutte 2009, SA-DAFF 2014, Kotze *et al.* 2017).

Results from Chapter 5 showed significantly higher EPF occurrence in non-bearing orchards (one to four year old) and mature orchards (nine years and older) than in juvenile orchards (four to eight years old). It was concluded that the higher occurrence of EPF recorded in non-bearing orchards than in juvenile orchards, was because no fungicides had been applied in non-bearing orchards, while the higher EPF occurrence recorded in mature orchards compared to juvenile orchards was because the larger tree size reduced EPF exposure to UV radiation. The positive effect of tree size on EPF persistence and reproduction was further illustrated by

increased EPF occurrence in soil sampled from juvenile orchards in subsequent years. EPF occurrence increased significantly in juvenile orchards from the first soil sampling year in 2015 to the third sampling year in 2017. However, the increase of EPF occurrence as orchards mature may also be attributed to the ability of EPF to develop tolerance or even resistance to fungicides after initial exposure. Therefore, the aim this study was to determine if EPF isolated from mature orchards are more tolerant to fungicides than EPF isolated from non-bearing and juvenile orchards.

6.2 Materials and Methods

6.2.1 Collection of fungal isolates

The compatibility of *B. bassiana* isolates collected from six non-bearing (one to three years old), six juvenile (four to eight years old) and six mature (nine years and older) citrus orchards with three fungicides (Table 6.2) were compared (Table 6.1). *Beauveria bassiana* were isolated from soil samples as described in section 5.2.3.

Table 6.1. Details of orchards from which *B. bassiana* isolates were collected.

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted
Non-bearing	Riverbend: S 33°25.107 E 25°42.677	Witkrans Navel	515	2014
		Orr Mandarin	606	2015
		Midnight Valencia	502	2014
	Miskruier: S 33°27.427' E 25°41.431	Cambria Navel	11	2013
		Midnight Valencia	71	2013
	Halaron: S 33°30.591 E' 25°39.704	Afourer Mandarin	75	2015
Juvenile	Woodridge: S 33°28.787 E 25°41.665	Cambria Navel	300	2011
		Midnight Valencia	301, 302	2011
	Buffelsbos: S 33°27.294 E 25°42.071	Valley Gold Mandarin	650, 653	2012
	Halaron: S 33°30.591 E 25°39.704	M7 Navel	71	2012
Mature	Miskruier:	Washington Navel	50	2005

Table 6.1. Details of orchards from which *B. bassiana* isolates were collected.

Maturity group	Farm and GPS coordinate	Cultivar and variety	Orchard no.	Year planted
	S 33°27.427' E 25°41.431			
		Newhall Navel	49	2005
		Midnight Valencia	51, 52	2005
	Halaron: S 33°30.591 E 25°39.704	Newhall Navel	54	2007
		Bahianinha Navel	53	2007

6.2.2 Fungicides

The compatibility of *B. bassiana* isolates, collected from citrus orchards of different maturity groups, with three fungicides registered for use in citrus against citrus black spot CBS were investigated (Table 6.2). Pennfluid was chosen because of the popular use of its active ingredient, mancozeb, in most bearing orchards (Section 5.2, Table 5.1). Benomyl (active ingredient, benomyl) was chosen because of high toxicity and Fungaway (active ingredient, azoxystrobin) because of its relatively low toxicity to *B. bassiana* strains isolated from the Sundays River Valley, Eastern Cape, South Africa (Coombes 2015). Fungicides were dissolved in sterile distilled water at the highest recommended field rate (Schutte 2009) for all experiments.

Table 6.2. Details of fungicides used.

Trade name	Active ingredient	Formulation	[1]Rate / 100 L	Manufacturer
Benomyl	benomyl	wettable powder	75 g	Arysta LifeScience
Flint	azoxystrobin	suspension concentrate	20 ml	Villa Crop Protection
Pennfluid	mancozeb	suspension concentrate	200 ml	Total

[1]Highest registered application rate for use against CBS in citrus orchards, South Africa (Schutte 2009).

6.2.3 Fungal suspensions

Spore suspensions were prepared by scraping a small quantity of spores from two week old fungal cultures and then adding the spores to 20 ml sterile distilled water supplemented with 0.01% Tween 20. Suspensions were then vortex mixed for three minutes. The concentrations of stock suspensions were determined using a Neubauer haemocytometer (Hirschmann®, Germany) as described in section 3.2.3. Once the concentrations of stock suspensions were determined, the dilutions necessary for the vegetative growth, spore production and spore viability experiments, detailed below, could be prepared. Suspensions were used immediately after preparation.

6.2.4 Agar medium

Sabouraud dextrose agar (SDA) supplemented with 1 ml chloramphenicol (50 mg/ml) was used as the standard growth medium (non-amended medium). Prepared media were cooled to 40 ± 5°C before being amended with fungicides (amended media). Approximately 20 ml of media were poured into 90 mm diameter Petri dishes under sterile conditions. All prepared plates (amended or non-amended) were used within one day of preparation.

6.2.5 Experimental treatments

The vegetative growth and spore production of *B. bassiana* isolates were determined on both amended media and on non-amended media (prepared as described in section 6.2.4 above). Spore viability was determined on non-amended media only. Spores were exposed to fungicides for one hour before being placed onto growth media. One ml of each isolate at a concentration of 1×10^7 (vegetative growth) or 1×10^4 (viability) spores/ml was added to a 10 ml suspension of each fungicide prepared in sterile distilled water at the highest recommended field rate.

6.2.5.1 Vegetative growth

The vegetative growth of *B. bassiana* isolates on fungicide-amended media was determined by placing a 5 mm diameter plug, taken from the growing region of

an unsporulated, four day old fungal culture, in the centre of each agar plate. Plugs from four day old fungal cultures were also placed onto non-amended media to serve as a control. For exposure experiments, spores were exposed to each fungicide for one hour at room temperature. After one hour, a 1 µl drop of the spore and fungicide mix was pipetted onto the centre of a non-amended agar plate. One µl drops of fungicide suspensions only were pipetted onto non-amended agar plates to serve as controls. Plates were sealed with Parafilm M® and incubated at approximately 26 °C in the dark. The growth of each culture was determined two weeks post-incubation. The diameter of each culture was measured along two previously drawn orthogonal lines and the average radial growth was used to calculate the growth area in cm^2. Each treatment, including the control, was replicated six times and the entire procedure repeated on three separate occasions (n = 18).

6.2.5.2 Spore production

After determining the effect of fungicides on the vegetative growth of *B. bassiana* strains, the spore production of each plate was also determined. Treatment plates on which fungi failed to grow were excluded from these experiments. Once the vegetative growth of each plate was determined, a 5 mm diameter plug was taken from a densely sporulated area on each plate. Each plug was then suspended in 10 ml sterile distilled water supplemented with Tween 20, housed within sterile 25 ml glass sample vials. Sample vials were then vortex mixed for three minutes to dislodge conidia from the agar plug. The concentration of spores was then determined, with the aid of a haemocytometer. Two counts were made per plug and the average count was used to calculate the number of spores/ml for each treatment (n=18).

6.2.5.3 Spore viability

A colony forming unit (CFU) analysis was used to assess spore viability. Similar to the vegetative growth experiment, spores were exposed to fungicides for one hour before being plated onto agar plates. A 100 µl sample of a pure 1×10^3 conidia/ml suspension (control) or 100 µl of the fungus and fungicide mix was spread onto a non-amended agar plate under sterile conditions. Plates were sealed with Parafilm M® and incubated at approximately 26 °C in the dark. The number of viable

colonies visible on each plate was determined four days post initial incubation and used to calculate the CFUs/ml. Each treatment, including the control, was replicated six times and the entire procedure repeated on three separate occasions (n = 18).

6.2.6 Compatibility of *B. bassiana* isolated from orchards of different maturity groups to tested fungicides: Alves' model

Alves *et al.* (1998)' model was used to compare the compatibility of *B. bassiana* isolated from citrus orchards of different maturity groups (non-bearing, juvenile and mature) to three fungicides (Benomyl, Fungaway and Pennfluid, Table 6.2).

The formula for the model used is as follows: $T = \frac{(20\ x\ VG)+(80\ x\ S)}{100}$

The values for vegetative growth (VG) and spore production (S) are expressed as percentages relative to the control to generate T values between 0 and 100. Chemicals with T values ranging from 0 to 30, 31 to 45, 46 to 60, 61 to 90 and > 90, are considered very toxic, toxic, moderately toxic, compatible and highly compatible, respectively.

The compatibility of fungicides with *B. bassiana* isolated from citrus orchards of different maturity groups were determined by adapting Alves' model (Alves *et al.* 1998) as sugested by Neves *et al.* (2001), who proposed that the model should also factor in the ability of spores to germinate, considering that germination is a vital first step in the infection process (Inglis *et al.* 2001). For the purpose of this study, spore viability was determined which not only shows the ability of spores to germinate but also to produce mycelia. The compatibility of fungal isolates grown on non-amended media after one hour exposure to fungicides were also compared according to an adapted version of Alves' model, where the proportion of viable spores were also factored into the equation.

The adapted model used is as follows: $T = \frac{(20\ x\ VG)+((80\ x\ S)(\frac{SV}{100}))}{100}$

The values for vegetative growth (VG), spore production (S) and spore viability (SV) are expressed as percentages relative to the control. The compatibility

of fungal isolates with chemicals are determined on the same scale as proposed by Alves *et al.* (1998).

6.3 Statistics

Treatment effects were determined by one-way ANOVA followed by Tukey's HSD post-hoc test if significant effects were found. Significant differences were determined on a 95% confidence level. In addition, the percentage expression in vegetative growth, sporulation and spore viability in response to each fungicide relative to the control was calculated.

6.4 Results

6.4.1 Non-bearing orchards

When *B. bassiana* isolates collected from non-bearing orchards were grown on non-amended media after one hour exposure to three fungicides (Fungaway, Pennfluid and Benomyl) significant treatment effects were recorded for spore viability ($F_{3, 68}$ = 91.56, P < 0.001), vegetative growth ($F_{3, 68}$ = 4.93, P = 0.004) and sporulation ($F_{3, 68}$ = 308, P = 0.033) (Table 6.3). Spore viability decreased significantly when exposed to all three fungicides. Pennfluid reduced spore viability by 61.48% followed by Benomyl (64.44% reduction) and the highest reduction was recorded with exposure to Fungaway which reduced spore viability by 75.56%. Only Pennfluid significantly reduced vegetative growth (P = 0.002) by 51.63%, but increased sporulation slightly from 0.22 x 10^7 spores/ml to 0.29 x 10^7 spores/ml. None of the three fungicides significantly reduced sporulation in comparison to the control. However, significantly lower sporulation was recorded in isolates exposed to Benomyl (P = 0.028) compared to Pennfluid, which slightly increased sporulation.

None of the *B. bassiana* isolates collected from non-bearing orchards were able to grow on Benomyl amended media. Significant treatment effects were recorded for vegetative growth ($F_{2, 51}$ = 19.96, P < 0.001) and sporulation ($F_{2, 51}$ = 30.62, P < 0.001), when the above mentioned isolates were grown on media amended with Fungaway and Pennfluid (Table 6.4). Both Fungaway and Pennfluid

significantly reduced sporulation by 52.29% ($P < 0.001$) and 73.41% ($P < 0.001$) respectively, and vegetative growth by 78.57% ($P < 0.001$) and 98.31% ($P < 0.001$) respectively, compared to the control.

6.4.2 Juvenile orchards

On non-amended media, *B. bassiana* isolates collected from juvenile orchards showed significant treatment affects for spore viability ($F_{3, 68} = 34.89$, $P < 0.001$), vegetative growth ($F_{3, 68} = 20.82$, $P < 0.001$) and sporulation ($F_{3, 68} = 17.31$, $P < 0.001$), after one hour exposure to three fungicides (Fungaway, Pennfluid and Benomyl) (Table 6.3). All three fungicides significantly reduced spore viability of *B. bassiana* isolates from all three orchards maturity groups. Fungaway, Pennfluid, and Benomyl reduced spore viability of isolates from non-bearing orchards by 24.44%, 38.52% & 35.65%, isolates from juvenile orchards by 35.81%, 31.08% and 20.27% isolates from mature orchards by 48.65%, 43.92% and 18.24% respectively.

All *B. bassiana* isolates collected from juvenile orchards were unable to grow on Benomyl amended media. When grown on media amended with Fungaway and Pennfluid, significant treatment effects were recorded for both vegetative ($F_{2,51} = 54.48$, $P < 0.001$) growth and sporulation ($F_{2, 51} = 75.08$, $P < 0.001$) (Table 6.3). Both Fungaway and Pennfluid significantly reduced vegetative growth by 41.51% ($P < 0.001$) and 94.51% ($P < 0.001$) respectively, and sporulation by 47.83% ($P < 0.001$) and 98.72% ($P < 0.001$) respectively.

6.4.3 Mature orchards

Significant treatment effects were recorded for spore viability ($F_{3, 68} = 30.01$, $P < 0.001$), vegetative growth ($F_{3, 68} = 7.30$, $P < 0.001$) and sporulation ($F_{3, 68} = 13.54$, $P < 0.001$) of *B. bassiana* isolates collected from mature orchards grown on non-amended media after one hour exposure to three fungicides (Fungaway, Pennfluid and Benomyl) (Table 6.3). All three fungicides significantly reduced spore viability. The lowest spore viability was recorded in fungicides exposed to Benomyl (18.24% spore viability compared to the control, $P < 0.001$) followed by Pennfluid (43.92% spore viability compared to the control, $P < 0.001$) and Fungaway (48.65% spore viability compared to control, $P < 0.001$). In addition to significantly reducing spore

viability, Pennfluid also significantly reduced vegetative growth (P = 0.015) by 19.65% and sporulation (P = 0.043) by 25.25%. Although Benomyl had the greatest effect on spore viability, it did not have a significant effect on vegetative growth (P = 0.622) or sporulation (P = 0.120). Fungaway significantly reduced vegetative growth (P < 0.001) by 29.52%, but significantly increased sporulation (P = 0.009) from 0.27 x 10^7 spores/ml to 0.40 x 10^7 spores/ml.

None of the *B. bassiana* isolates collected from mature orchards were able to grow on either Benomyl or Pennfluid amended media. Results of t-tests showed no significant reduction in either vegetative growth (P = 0.119) or sporulation (P = 0.252) when isolates were grown on Fungaway amended media compared to the control (Table 6.4).

Table 6.3: Influence of each fungicide on the viability, vegetative growth and sporulation of *B. bassiana* isolated from orchards of different ages when grown on non-amended media after being exposed to each fungicide for one hour. Means (± standard errors) are presented. The effect of each fungicide on each trait relative to the control is presented next in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.

Fungicide	Viability (10^3 CFUs/ml)	Vegetative growth (area in cm^2)	Sporulation (10^7 spores/ml)
Non-bearing orchards			
Control	1.35 (0.06) a[1]	11.06 (1.18) a	0.22 (0.03) ab
Fungaway	0.33 (0.03) c, **24.44%**	8.11 (1.25) a, **73.32%**	0.18 (0.02) ab, **81.82%**
Pennfluid	0.52 (0.04) b, **38.52%**	5.35 (1.36) b, **46.12%**	0.29(0.06) a, **100%**
Benomyl	0.48 (0.06) bc, **35.56%**	7.71 (1.16) a, **69.71%**	0.15 (0.01) b, **68.18%**
Juvenile orchards			
Control	1.48 (0.10) a	9.39 (0.54) a	0.32 (0.01) a
Fungaway	0.53 (0.13) b, **35.81%**	6.25 (0.39) b, **66.56%**	0.15 (0.01) b, **46.88%**
Pennfluid	0.46 (0.07) b, **31.08%**	5.41 (0.26) b, **57.61%**	0.18 (0.02) b, **56.63%**
Benomyl	0.30 (0.04) b, **20.27%**	5.66 (0.37) b, **60.27%**	0.20 (0.03) b, **62.50%**
Mature orchards			
Control	1.48 (0.11) a	6.21 (0.20) a	0.27 (0.03) b
Fungaway	0.72 (0.11) b, **48.65%**	4.30 (0.12) b, **69.24%**	0.40 (0.04) a, **100%**
Pennfluid	0.65 (0.10) b, **43.92%**	4.83 (0.17) b, **77.78%**	0.17 (0.01) c, **62.96%**
Benomyl	0.27 (0.03) c, **18.24%**	5.67 (0.56) a, **91.30%**	0.35 (0.02) ab, **100%**

[1] Different letters denote statistically significant differences within each grouping (comparisons of means, $P < 0.05$)

Table 6.4: Influence of each fungicide on the vegetative growth and sporulation of *B. bassiana* isolated from orchards of different ages when grown on fungicide amended media after being exposed to each fungicide for one hour. The effect of each fungicide on each trait relative to the control is presented next in bold. Percentages range between 0% and 100%, with 100% representing a response identical to or greater than the control.

Fungicide	Vegetative growth (area in cm^2)	Sporulation (10^7 spores/ml)
Non-bearing orchards		
Control	8.95 (1.00) a[1]	1.54 (0.17) a
Fungaway	4.27 (0.58) b, **47.71%**	0.33 (0.05) b, **21.43%**
Pennfluid	2.38 (0.62) c, **26.68%**	0.26 (0.01) c, **1.69%**
Juvenile orchards		
Control	9.95 (1.07) a	0.92 (0.10) a
Fungaway	5.82 (0.21) b, **58.49%**	0.48 (0.06) b, **52.17%**
Pennfluid	0.54 (0.19) c, **5.43%**	0.012 (0.04) c, **1.28%**
Mature orchards		
Control	5.59 (0.06) a	0.94 (0.06)a
Fungaway	4.35 (0.44) a, **77.82%**	1.08 (0.10) a, **100%**
Pennfluid	-[2]	-

[1] Different letters denote statistically significant differences within each grouping (comparisons of means, $P < 0.05$)

[2] Spore counts could not be made due to the inability of the fungus to grow and was thus excluded from statistical analysis.

6.4.4 Comparison using Alves' model

Both Benomyl and Pennfluid were highly toxic to *B. bassiana* isolates collected from all three orchard maturity groups when grown on media amended with these two fungicides (Table 6.5). On amended media, Fungaway was very toxic to *B. bassiana* isolates collected from non-bearing orchards, moderately toxic to isolates collected from juvenile orchards and highly compatible with isolates collected from mature orchards.

After one hour exposure to fungicide on non-amended media, isolates collected from non-bearing orchards were compatible with all three fungicides tested,

according to both models used (Table 6.5). However when spore viability was also factored into the model, Fungaway and Benomyl were toxic and Pennfluid was moderately toxic to isolates collected from non-bearing orchards (Table 6.6).

According to Alves' model, isolates collected from juvenile orchards were compatible with Benomyl, but only moderately compatible with Fungaway and Pennfluid on non-amended media after brief exposure. All three fungicides tested were very toxic to isolates collected from juvenile orchards when spore viability was factored into the equation.

On non-amended media, Fungaway and Benomyl were highly compatible and Pennfluid was compatible with *B. bassiana* isolates collected from mature orchards after one hour exposure to each fungicide. When compatibility of fungicides to isolates collected from mature orchards were compared according to the adapted model, Fungaway was still highly compatible, while Pennfluid and Benomyl were toxic.

Table 6.5: Compatibility (T) of tested fungicides with *B. bassiana* isolates collected from orchards of different ages on fungicide amended and non-amended media following one hour exposure, according to Alves' model (Alves *et al.* 1998).

	Non-bearing		**Juvenile**		**Mature**	
Amended media						
Fungicide	**T**	**Outcome**	**T**	**Outcome**	**T**	**Outcome**
Fungaway	26.67	Very toxic	53.43	Moderately toxic	95.56	Highly compatible
Pennfluid	6.69	Very toxic	2.11	Very toxic	0	Very toxic
Benomyl	0	Very toxic	0	Very toxic	0	Very toxic
Non-amended media						
Fungaway	80.12	Compatible	50.86	Moderately toxic	100	Highly compatible
Pennfluid	100	Highly compatible	56.83	Moderately toxic	65.92	Compatible
Benomyl	68.49	Compatible	62.05	Compatible	100	Highly compatible

Table 6.6: Compatibility (T) of tested fungicides with *B. bassiana* isolates collected from orchards of different maturity groups on non-amended media following one hour exposure, according to Alves' model (Alves et al. 1998) adapted to factor spore viability into the equation.

	Non-bearing		Juvenile		Mature	
Fungicide	**T**	**Outcome**	**T**	**Outcome**	**T**	**Outcome**
Fungaway	30.66	Toxic	26.74	Very toxic	71.45	Compatible
Pennfluid	49.85	Moderately toxic	25.15	Very toxic	37.68	Toxic
Benomyl	33.34	Toxic	22.19	Very toxic	37.17	Toxic

6.5 Discussion

The response of *B. bassiana* isolates tested in this study was variable. On non-amended media, all three fungicides significantly reduced spore viability in *B. bassiana* isolates collected from all three orchard maturity groups. No growth was recorded for any isolates grown on media amended with Benomyl. Limited growth and sporulation was recorded in isolates collected from non-bearing and juvenile orchards when grown on media amended with Pennfluid. Isolates collected from mature orchards were unable to grow on media amended with Pennfluid. Vegetative growth and sporulation was significantly reduced in isolates collected from non-bearing and juvenile orchards when grown on media amended with Fungaway while no significant reduction in vegetative growth and sporulation was recorded in isolates collected from mature orchards.

On non-amended media, brief exposure to Fungaway had no significant effect on vegetative growth or sporulation of isolates collected from non-bearing orchards. In contrast, Fungaway significantly reduced vegetative growth and sporulation in isolates from juvenile orchards. Interestingly, brief exposure to Fungaway significantly reduced vegetative growth in isolates collected from mature orchards, but significantly increased sporulation. Brief exposure to Pennfluid significantly reduced vegetative growth and sporulation in isolates collected from juvenile and mature orchards. Pennfluid had no significant effect on sporulation of isolates collected from non-bearing orchards, but reduced vegetative growth significantly

after brief exposure to the fungicide. Brief exposure to Benomyl significantly reduced vegetative growth and sporulation in isolates collected from juvenile orchards, while no significant effect on vegetative growth and sporulation was recorded in isolates collected from mature orchards. Benomyl had no significant effect on vegetative growth of isolates collected from non-bearing orchards, but did reduce sporulation significantly after brief exposure to the fungicide.

Isolates collected from the three maturity groups only responded to Fungaway, as proposed by our hypothesis, when compatibility to Fungaway was determined according to Alves' model, on fungicide-amended media. On fungicide-amended media, Fungaway was very toxic to isolates collected from non-bearing orchards, moderately toxic to isolates collected from juvenile orchards and highly compatible with isolates collected from mature orchards. Pennfluid and Benomyl were very toxic to isolates collected from all three orchard maturity groups on fungicide-amended media.

According to Alves' model, brief exposure to Fungaway was highly compatible with isolates collected from mature orchards, compatible with isolates collected from non-bearing orchards and moderately toxic to isolates collected from juvenile orchards. A similar trend was recorded when compatibility with brief exposure to Fungaway was determined according to the adapted model, which considered the effect of fungicides on spore viability. According to the adapted model, Fungaway was compatible with isolates collected from mature orchards, toxic to isolates collected from non-bearing orchards and very toxic to isolates collected from juvenile orchards. On non-amended media, Pennfluid was highly compatible with isolates collected from non-bearing orchards, compatible with isolates collected from mature orchards and moderately toxic to isolates collected from juvenile orchards according to Alves' model. According to the adapted model brief exposure to Pennfluid was moderately toxic to isolates from non-bearing orchards, toxic to isolates collected from mature orchards and very toxic to isolates collected from juvenile orchards. According to Alves' model brief exposure to Benomyl was compatible with isolates collected from non-bearing and juvenile orchards and highly compatible with isolates collected from mature orchards. However, according to the adapted model, brief exposure to Benomyl was toxic to isolates collected from non-bearing and mature orchards and very toxic to isolates collected from juvenile orchards.

From the results of this study we can conclude that *B. bassiana* isolates from mature orchards do not have higher tolerance to Benomyl and Pennfluid than do isolates from non-bearing orchards which have never been exposed to fungicides. Isolates collect from mature orchards only showed higher tolerance to Fungaway than did isolates from non-bearing orchards. However, if isolates are becoming more tolerant to fungicides after longer periods of exposure, we would expect to see the strongest response when isolates are exposed to Pennfluid, due to the widespread use of its active ingredient, mancozeb (Table 5.1, Schutte 2009, Kotze *et al.* 2017). However, results of this study showed that *B. bassiana* isolates from non-bearing orchards are actually more tolerant to mancozeb than isolates collected from mature orchards (Table 6.6).

Although exposure to fungicides has been shown to increase EPF tolerance to fungicides (Shapiro-Ilan *et al.* 2011), Shapiro-Ilan *et al.* (2002) was only able to achieve increased resistance to one *B. bassiana* strain after exposure to three fungicides, Dodine, fenbuconazole and triphenyltin hydroxide. No improvement in fungicide resistance could be achieved after exposing a mix of wild *B. bassiana* strains to the abovementioned fungicides. Shapiro-Ilan *et al.* (2002), concluded that they were possibly unable to improve fungicide tolerance in wild *B. bassiana* strains because they were already more resistant to fungicides than the commercial strain prior to selection and thus possible changes in resistance were more subtle and difficult to detect. Shapiro-Ilan *et al.* (2002) also concluded that the higher tolerance recorded in wild *B. bassiana* isolates than in the commercial strain, may be due to previous exposure (and thus selection) in the field. However, results of this study showed that *B. bassiana* isolates collected from non-bearing orchards, which have not been exposed to fungicides, have higher tolerance to mancozeb and equal tolerance to benomyl compared to *B. bassiana* isolates collected from mature orchards, which have been exposed to fungicides for multiple growing seasons.

In general, *B. bassiana* isolates from juvenile orchards were more sensitive to fungicides than isolates from non-bearing and mature orchards. It is not clear why isolates from juvenile orchards were more sensitive to fungicides and it may simply be incidental, possibly due to the relatively limited extent of the survey. The greater sensitivity of isolates from juvenile orchards, as recorded in Chapter 5, could have

contributed to the significantly lower EPF occurrence in juvenile orchards than in non-bearing and mature orchards. However, results recorded in Chapter 5 also showed that EPF occurrence increased significantly in juvenile orchards from the first sampling year in 2015 to the third sampling year in 2017. Since the results of this study show that EPF sensitivity to fungicides is not decreasing after longer periods of exposure, we confirm the conclusions made in Chapter 5. In Chapter 5 we concluded that one of the reasons why juvenile orchards are more vulnerable to FCM infestation than non-bearing and mature orchards, is the combined negative impact of exposure to sunlight and fungicide applications on EPF persistence.

CHAPTER 7
General discussion

7.1 Introduction

At the start of this study it was stated that the current knowledge of false codling moth (FCM), *Thaumatotibia (Cryptophlebia) leucotreta* (Meyr) (Lepidoptera: Tortricidae) (FCM), ecology was limited. Since FCM is a key economic pest of citrus in South Africa, the majority of studies done on FCM have been aimed at developing and improving various control methods. Only a few studies have attempted to understand the trophic interactions between FCM, its natural enemies and the environment. Understanding a pest's biology and its interactions with natural enemies is crucial for developing effective control programmes (Faust 2008). Anecdotal reports in the citrus industry claimed higher populations of FCM during the first three to five harvesting years of citrus planted in virgin soil, after which, FCM numbers seemed to decrease and remain consistent (D. Gerber, pers. comm.). Therefore the aim of this study was to determine if, and why juvenile orchards (four to eight years old) play host to higher FCM infestation than mature orchards (nine years and older). In order to achieve this aim, three objectives were set out: to determine differences in physiology between fruit from juvenile and mature orchards; to determine the above ground influence of orchard age on the ecology of FCM and its natural enemies and to determine the influence of orchard age on the subterranean natural enemies of FCM.

Laboratory trials were conducted to achieve the first objective. Choice and no choice trials were conducted to compare the attractiveness of fruit from juvenile and mature orchards for oviposition. Differences in the volatile emissions of Washington Navel oranges from juvenile and mature orchards were compared with the use of solid-phase microextraction, gas chromatography/mass spectrophotometry (SPME-GC/MS) detection. Internal fruit quality, nutritional content and susceptibility of fruit from juvenile and mature orchards to FCM damage were also compared. Since only a limited number of larvae could be reared in fruit, artificial diets simulating the nutrient composition of fruit from juvenile and mature trees were prepared to

compare FCM survival rate, growth rate, larval and pupal weight and fecundity. Artificial diets were also used to determine if differences in the chemistry of fruit from juvenile and mature trees will alter the susceptibility of FCM to Cryptophlebia leucotreta granulovirus (CrleGV), entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN). Field surveys were conducted to achieve the second and third objective. To compare FCM damage in juvenile and mature orchards, fruit infestation levels and trap catches were recorded weekly. Any dead larvae that showed signs of virus infection were recorded. Live larvae were kept and reared on artificial diet to record the possible development of latent virus infection and emergence of larval parasitoids. Weekly surveys were conducted to record and compare the abundance of ants in juvenile and mature orchards. Fortnightly fruit surveys were conducted to record FCM egg numbers and egg parasitism. Soil samples were collected every second month to compare EPF and EPN occurrence in juvenile and mature orchards.

7.2 Effect of plant physiology and architecture on false codling moth populations

Literature on the influence of orchard age on insect pest ecology is scarce. Wearing & Skilling (1975), compared the function of tree age on providing cocooning shelter for codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae), which is known to pupate in trees. Results of their study showed that, codling moth populations in young apple orchards are severely limited by a lack of protective pupation sites. Although FCM does not pupate in trees, rather in the soil (Newton 1998), juvenile orchards are more exposed to sunlight, which will reduce soil moisture. Lower eclosion success of FCM has been recorded in dry soil than moist soil (Love 2015). Soil moisture also aids in the formation of soil aggregates and cracks in the soil, which provide sheltered pupation sites for FCM (Love 2015). Since the microclimate, tree structure, management systems and soil quality change as an orchard ages, the ecological processes within the system also change. This body of work successfully illustrated what ecological changes are occurring as citrus orchards age (see below for details).

Ontogenetic changes in plant resistance and tolerance to diseases and herbivory are common in nature (Boege & Marquis 2005). According to Boege *et al.* (2007), these shifts in resistance and tolerance are due to changes in plant architecture and resource availability. Since plants prioritise growth above other metabolic activities (Tiffin 2002, Weiner 2004), plant chemical defences may remain constrained until plants reach maturity (Boege 2005). Various studies have also shown that plants are able to become more resistant to herbivory after previous exposure (Karban & Myers 1989, Karban & Niiho 1995, Kessler *et al.* 2004, Howe & Jander 2008). It is now known that fruit from juvenile citrus trees are more attractive for oviposition and more susceptible to FCM damage than fruit from mature trees. However, the degree of vulnerability varies depending on cultivar. The difference in the susceptibility of fruit from juvenile and mature trees to FCM damage will be less notable in citrus cultivars, such as Cambria Navel oranges, which have been shown to be generally less susceptible to FCM damage, than other Navel orange cultivars (Love *et al.* 2014). The difference in susceptibility of fruit from juvenile and mature citrus orchards to FCM damage will also vary depending on farm management practices. The difference in susceptibility may be less notable if optimal conditions for plant defence are met. Plants are known to be more susceptible to diseases when water stressed (Old *et al.* 1990, Lowman & Heatwole 1992, Desprez-Loustau *et al.* 2006). The mineral composition of orchard soils will also influence the vulnerability of trees to diseases. Some minerals have been shown to improve plant defence, while other will increase susceptibility to disease. For example, fertilisers high in nitrogen (N) and low in potassium (K) have been shown to increase stalk rot in maize plants (Dodd 1980). Results reported by Feeny (1970) further illustrate high N levels to increase plant susceptibility to insect damage. The study showed that high N levels in oak leaves increase growth and development of the winter moth, *Operophtera brumata* (Linnaeus) (Lepidoptera: Geometridae). In contrast, minerals such as silicon (Si), boron (B) and calcium (Ca) have been shown to improve plant defence against diseases (Bramlage 1992, Fauteux *et al.* 2005, Dordas 2008, Epstein 2009).

This study also determined that the volatile profiles of fruit from juvenile Washington Navel trees are very different from the volatile profiles of mature trees. Various studies have shown that the volatiles emitted by plants have a significant

influence on the oviposition choices of insects (Ohsugi *et al.* 1985, Renwick 1989, Bruce *et al.* 2005, Ioannu *et al.* 2012). Data recorded by SPME-GC/MS detection showed Washington Navel oranges from juvenile trees to emit significantly higher levels (pico amp) for a total of 11 volatile compounds, than were emitted by fruit from mature trees. The higher preference for fruit from juvenile trees than mature trees for oviposition could be as a result of the higher emission of such volatile compounds. The higher oviposition preference of fruit from juvenile orchards than mature orchards may be of less concern under field conditions, as FCM is a poor disperser (Newton 1998, Moore *et al.* 2004, Timm *et al.* 2010, Stotter *et al.* 2014). Thus, juvenile orchards are unlikely to pull FCM from surrounding mature orchards even though they are more attractive for oviposition. However, results of no-choice trials also showed higher oviposition on fruit from juvenile orchards than mature orchards. Therefore, results of the study suggest that the volatiles emitted by fruit from juvenile trees will stimulate higher oviposition in juvenile orchards than mature orchards. Results of field trials recorded in this study suggested that this may be the case. Higher mean egg counts of 1.56 eggs per tree per week were recorded in a juvenile Washington Navel orchard than 1.11 eggs per tree per week recorded in a nearby mature orchard, however, the difference was not significant.

The higher susceptibility of fruit from juvenile trees in comparison to fruit from mature trees to FCM damage was further demonstrated in artificial diet containing fruit powder from juvenile and mature trees. Larval survival showed a strong negative response to an increase in the percentage of fruit powder added to maize meal diets. These decreases in larval survival showed that citrus fruit contained secondary metabolites, which are not present in maize meal. In addition, higher larval survival was recorded in diets containing fruit powder from juvenile trees than mature trees. Lower survival rates of larvae recorded in mature tree diets showed that mature trees produce higher concentrations of such secondary metabolites than juvenile trees.

7.3 Effect of orchard age on natural enemies of false codling moth

Differences in plant chemistry have shown the ability to change susceptibility of insects to pathogen infection (Cory & Hoover 2006). Therefore, experiments were

conducted to determine if differences in plant chemistry between fruit from mature and juvenile citrus trees had an influence on FCM susceptibility to EPF, EPN and CrleGV. Results of this study suggest that differences in plant chemistry between fruit from juvenile and mature trees do not have a significant influence on the susceptibility of FCM to EPN and EPF. However, differences in plant chemistry between fruit from juvenile and mature trees were shown to significantly affect the ability of CrleGV to infect neonate FCM. Mortality of neonate larvae was significantly lower when placed on the mature tree diet than the juvenile tree diet, after ingesting the lowest virus concentration tested (2×10^4 OBs/ml). Secondary metabolites, such as tannins, have been shown to bind to virus particles in the midgut of insects, thus reducing the infectivity of viruses (Keating *et al.* 1990, Felton & Duffey 1990). Lower mortality of larvae recorded in the mature tree diet is probably due to higher concentrations of such virus-binding secondary metabolites present in fruit from mature trees than in juvenile trees. Although larval mortality was still higher in the mature tree diet than the juvenile tree diet, after feeding larvae suspensions with higher concentrations of virus particles, the difference was not significant. The inhibitory effect of virus-binding secondary metabolites may be reduced at higher virus doses since only a limited amount of these chemicals is obtained during feeding, which can only bind a limited number of virus particles. However, control mortality was also shown to be significantly higher in the mature tree diets than the juvenile tree diets. Thus, the lower susceptibility of larvae to low doses of virus in mature orchards is not a concern. When Abbott's formula (Abbott 1925) was not used to correct for insects that died of natural causes, the mortality achieved in CrleGV bioassays was similar for both diets. Furthermore, virus particles have been shown to be adversely affected by exposure to ultraviolet (UV) radiation (Moore 2002, Mwanza 2016). Virus particles will be less exposed to UV radiation in mature orchards than in juvenile orchards, since the canopies of juvenile citrus trees are less dense than the canopies of mature trees. Thus, virus efficacy will be higher in mature orchards than in juvenile orchards. The difference in CrleGV persistence between juvenile and mature orchards will be similar to the difference in persistence between the sunny northern and more shaded southern side of trees as recorded by Mwanza *et al.* (2016). Mwanza *et al.* (2016) showed LD_{50} values of CrleGV recovered from the northern side of trees to be 15 times higher than CrleGV recovered from the southern side of trees, 21 days after application. After 28 days virulence of virus from

the northern side of trees was insignificant, while virus from the southern side of trees still showed a clear dose response.

The higher efficacy of viruses in mature orchards than in juvenile orchards was confirmed in field surveys. Although the effect of viruses on neonate larvae could not be compared, a significantly higher proportion of larvae retrieved from mature orchards were infected by virus than were larvae retrieved from juvenile orchards. Similar results were recorded by Killick & Warden (1991) who reported higher mortality of pine beauty moth, *Panolis flammea* (Denis & Schiffermüller) (Lepidoptera: Noctuidae), due to virus infections in the lower branches of pine trees, which were less exposed to UV radiation, compared to branches higher up in the tree canopy.

This study has also shown that differences in tree architecture between juvenile and mature trees have a significant influence on egg parasitism. Lower egg parasitism recorded in juvenile orchards compared to mature orchards will cause juvenile orchards to be more vulnerable to FCM damage. Egg parasitism was consistently higher in mature orchards than juvenile orchards, with the exception of Mandarins during 2015, where egg parasitism was slightly higher in juvenile orchards than mature orchards, but the difference was not significant. Higher egg parasitism recorded in mature orchards supports results recorded by Langellotto & Denno (2004), who reported a strong negative response to simplified habitat structure in spiders, hemipterans, mites and parasitoids. Parasitoids are also more exposed to wind and dust in juvenile orchards, which can increase grooming and reduce foraging, oviposition and lengths of visits on dusty foliage (Van Driesche & Bellows 1996). In addition, egg parasitism in juvenile orchards will also be reduced by the more open canopy and smaller size of trees which will improve pesticide coverage (Simon *et al.* 2007), and various studies have shown that egg parasitoids are highly sensitive to chemical pesticides (Cônsoli *et al.* 1998, Hassan *et al.* 1998, Brunner *et al.* 2001, Grützmacher *et al.* 2004, Takada *et al.* 2001). Although higher pesticide coverage in mature trees than juvenile trees will also improve FCM control, the effect will be more severe on parasitoids which are more mobile and less cryptic than most pest species (Samways 2005), including FCM. FCM is only exposed to pesticides from oviposition until they burrow into fruit shortly after hatching, whereas

parasitoids will be exposed to these pesticide residues for the full duration of their time as an effective parasitoid (i.e. an adult).

Results of laboratory trials conducted in this study have shown that fruit from juvenile orchards are more susceptible to FCM damage. Therefore, juvenile orchards are expected to facilitate higher FCM infestation levels than mature orchards of the same cultivar when placed under similar or higher pest pressure. In addition, higher mean temperatures recorded in juvenile orchards than mature orchards will expedite larval development (Daiber 1979 a, b, c, Stibick 2010). However, significantly higher counts of viable (non-parasitised) eggs in either juvenile or mature orchards were not necessarily linked to significantly higher levels of FCM damage. For example, during 2016, FCM damage levels recorded in juvenile and mature Midnight Valencia orchards were similar, even though significantly higher counts of viable eggs were recorded in mature orchards. These results confirm those recorded in laboratory trials, which showed Midnight Valencia oranges from mature trees to be significantly less susceptible to FCM damage than fruit from juvenile trees. In contrast, significantly higher counts of viable eggs recorded in juvenile Navel orchards than mature orchards during 2016 did not result in significantly higher levels of FCM damage. As mentioned earlier in this section, the more open tree canopy of juvenile trees will improve pesticide coverage (Simon *et al.* 2007) and increase wind exposure, which could cause FCM damage levels to be lower than expected. Furthermore, temperature and humidity readings showed that FCM eggs and neonate larvae will be more exposed to temperature extremes and lower humidity levels than in mature trees, which will increase larval mortality (Daiber 1979 a, b). Therefore the higher susceptibility of fruit from juvenile orchards than fruit from mature orchards will probably not be notable under field conditions unless the difference in susceptibility is very large. For example, although it was statistically significant, the 12% higher susceptibility of fruit from juvenile Washington Navel trees than mature trees will probably not cause significantly higher FCM damage under field conditions. However, although the actual damage caused will be lower under field conditions, the 31% higher susceptibility of Nova Mandarins from juvenile trees than mature trees, recorded in laboratory trials, will probably result in significantly or at least substantially higher FCM infestation levels in the field.

Low soil moisture levels and exposure to UV radiation have been shown to significantly reduce EPF persistence (Jaronski *et. al.* 2010). This study hypothesised that EPF occurrence will be lowest in non-bearing orchards, which are most exposed to sunlight, followed by juvenile orchards, with the highest occurrence of EPF in mature orchards. However, in contrast to what the hypothesis predicted, EPF occurrence in citrus orchards did not appear to be affected by sunlight exposure alone. In fact, the highest occurrence of EPF was recorded in non-bearing orchards, followed by mature orchards, with the lowest EPF occurrence recorded in juvenile orchards. Results of this study suggest that EPF occurrence is complex and also dependent on orchard management practices. This study concluded that EPF occurrence in non-bearing orchards may have been higher than expected since no fungicides were applied in non-bearing orchards during the first two sampling years of the trial. Similar to results recorded by Goble *et al.* (2010) who found no difference in EPF occurrence between organically and conventionally managed citrus orchards, EPF occurrence recorded in non-bearing and mature orchards were not significantly different. The study further concluded that the significantly higher occurrence of EPF in mature than juvenile orchards was probably because the higher shade density in mature orchards counteracted the adverse effects of fungicides applications. This was confirmed by the significantly higher occurrence of EPF recorded during the third sampling year in juvenile orchards than recorded in the first two sampling years. Although the addition of mulch to orchards is expected to improve EPF occurrence by reducing exposure to UV and improving soil moisture levels (Mando & Stroosnjider 1999, Li 2003, Ramakrishna *et al.* 2006), this was not recorded. In contrast to what was expected, EPF occurrence was significantly lower in orchards covered by mulch than orchards with no covering. Mulch may reduce EPF occurrence by restricting the lateral flow of water, thus preventing spores from spreading. Mulches will also reduce wind dispersal of fungal spores. Although mulches have been shown to increase microbial activity (Tiquia *et al.* 2002), Coombes (2015) suggested that an increase in biological activity associated with mulches may also increase antagonistic effects, causing a reduction EPF in efficacy.

Differences in tree architecture and orchard microclimate between juvenile and mature orchards did not have a significant effect on ant abundance. Due to the low occurrence of EPN in orchards sampled, the influence of differences in orchard

microclimate between non-bearing, juvenile, and mature orchards could not be determined. However, similar to EPF, EPN persistence is also negatively affected by low soil moisture levels (Wright *et al.* 2005) and UV radiation (Gaugler *et al.* 1992), but unlike EPF, various studies have shown that EPN are highly compatible with most agrochemicals (Rovesti & Deseö 1990, Van Niekerk & Malan 2014), with the exception of nematicides (Manrakhan *et al.* 2014). Therefore EPN occurrence is still expected to be lowest in non-bearing orchards, followed by juvenile orchards, with the highest occurrence in mature orchards.

Laboratory trials conducted by Shapiro-Ilan *et al.* (2011) achieved enhanced fungicide resistance of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium brunneum* (Petch), after exposure to fungicides. Therefore, laboratory trials were conducted to determine if the higher occurrence of EPF recorded in mature orchards than juvenile orchards may also have been because EPF isolates occurring in mature orchards had developed some resistance to fungicides, as opposed to isolates from non-bearing and juvenile orchards, due to the more prolonged exposure of EPF in mature orchards to fungicides. Six *B. bassiana* isolates were collected from each of six non-bearing, juvenile and mature orchards to compare the tolerance of isolates collected from each maturity group to three fungicides (Fungaway, Pennfluid and Benomyl). However, isolates collected from the three orchard maturity groups did not respond to fungicides as the hypothesis had predicted. Due to the variation in response of isolates from the three orchard maturity groups to the three fungicides tested, this study concluded that EPF isolates are not necessarily becoming more tolerant to fungicides after being exposed to fungicides for longer periods of time. Similar results were recorded by Shapiro-Ilan *et al.* (2002), who was unable to achieve increased resistance in a mix of wild *B. bassiana* strains after exposure to three fungicides, dodine, fenbuconazole and triphenyltin hydroxide.

7.4 Recommendations for future research

The qualities of juvenile orchards which will improve or decrease their susceptibility to FCM in comparison to mature orchards are summarised in Table 7.1. Now that these differences are known, adjustments for improving FCM control can be made according to the age of the orchard. Future research should be

conducted to determine if juvenile citrus trees are possibly also more susceptible to other citrus pests. For example, citrus fruit from juvenile orchards may also be more susceptible to post-harvest decay than fruit from mature orchards. According to Bramlage (1992), fruit from juvenile pome fruit trees are highly susceptible to a variety of post-harvest disorders. If this is also the case with citrus fruit, changes in post-harvest treatments of fruit from juvenile orchards may be required to optimise shelf life. Although research should still be conducted to confirm this, juvenile trees of other tree crops are probably also more attractive for oviposition and more susceptible to insect damage.

FCM has been shown to be a poor disperser (Newton 1998, Moore *et al.* 2004, Timm *et al.* 2010, Stotter *et al.* 2014). Therefore, the higher attractiveness of juvenile orchards for oviposition may not be as problematic under field conditions. Results recorded by Stotter (2009) indicate that juvenile orchards are unlikely to pull FCM from more mature orchards which are further than 600 m away, even though they are more attractive for oviposition. If the size of the farm allows it, the vulnerability of juvenile orchards to FCM infestation will be greatly reduced by planting new orchards more than 600 m away from established orchards. To optimise land use, the influx of FCM from more mature orchards into juvenile orchards can be reduced by planting lemons between juvenile and mature orchards. Lemons are not considered as a host for FCM (Moore *et al.* 2015) and can act as a barrier between susceptible orchards. Preventing the influx of highly mobile insects such as *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) (De Villiers *et al.* 2016) from mature orchards to more attractive juvenile orchards will be more challenging. Juvenile trees of crops such as mangos and bananas, which are highly susceptible to *B. dorsalis* damage, will be especially vulnerable (Rwomushana *et al.* 2008). To improve pest control in juvenile orchards of crops which have been shown to be more attractive for oviposition than mature orchards, future research could aim at developing control products which repel the desired pest insect. Covering juvenile orchards with nets will also restrict pest mobility. The use of nets is becoming more common around the world (Solomakhin & Blanke 2007). Nets are primarily used to prevent hail damage (Solomakhin & Blanke 2007, Kührt *et al.* 2006), but are also used to exclude, insects, birds and bats from orchards (Tanny *et al.* 2009). Citrus

producers also use nets to ensure seedlessness of citrus varieties such as Afourer Mandarin (Gambetta *et al.* 2013).

In terms of fruit susceptibility, further studies should also be conducted to determine at what age trees reach peak immunity. Citrus producers will then know how susceptible trees are at what age and adjust control measures accordingly. Studies should also be conducted to determine if tree immunity can be improved by optimising fertilisers to enhance plant defence. Plant defence of juvenile trees may be improved by limiting N (Feeny 1970, Dodd 1980) and increasing, K, B, Ca and Si (Dodd 1980, Bramlage 1992, Fauteux *et al.* 2005, Dordas 2008, Epstein 2009). The ability of plant defence hormones such as salicylic, jasmonic and azelaic acid (Spoel & Dong 2012) to improve resistance of juvenile trees to diseases should also be determined. Determining which defence chemicals are responsible for increased immunity in fruit from mature orchards may also be beneficial. The chemicals identified can then be mass produced and used to improve FCM control in general.

As mentioned, the higher susceptibility of fruit from juvenile trees to FCM damage than fruit from mature trees recorded in laboratory trials will be reduced under field conditions. The smaller size of juvenile trees will increase pesticide coverage and improve pest control in juvenile orchards. FCM is also more exposed to temperature extremes and low humidity levels, which will increase larval mortality (Daiber 1979 a, b). Similar results were recorded in dwarf apple tree orchards. A study conducted by Kührt *et al.* (2006) reported that mean air temperatures within the canopy of dwarf apple trees were 0.7 °C higher during daytime and 0.4 °C lower at night than tall trees. In the case of codling moth control, population numbers will also be severely limited in juvenile and dwarf apple orchards by a lack of protective pupation sites (Wearing & Skilling 1975). The evidence given above may also be applicable to stone and pome fruit trees which have been manipulated by training systems, such as the centrifugal training system, which creates a more open, aerated canopy (Simon *et al.* 2006).

From this study we can conclude that the greatest challenge for FCM control in juvenile citrus orchards (and possibly also pests of other tree crops, such as codling moth control in apple orchards), is lower efficacy of parasitoids and UV

sensitive microbial control agents. The negative impact of higher dust exposure in juvenile than mature orchards on parasitoids can be reduced by regularly wetting unpaved roads adjacent to juvenile orchards. Wetting roads to reduce dust in orchards is not an unusual practice in citrus production (pers. obs.). Since egg parasitoids and EPF are less abundant in juvenile orchards than mature orchards, inundative releases of these biological control agents will improve FCM control in juvenile orchards considerably. However, adjustments in the frequency and timing of applications in juvenile orchards will be necessary as both these biological control agents have been shown to be more sensitive to pesticide applications in juvenile orchards than in mature orchards. Adverse effects of chemicals on EPF may be avoided by applying EPF asynchronously with fungicides (Jaros-Su *et al.* 1999). Genetic improvement of both EPF and parasitoids, through artificial selection for pesticide resistance, will benefit FCM control in both juvenile and mature orchards. However, care must be taken that artificial selection for improved resistance to pesticides does not reduce other qualities necessary for efficiency in the field (Shapiro-Ilan *et al.* 2011). The negative impact of insecticides on parasitoids can be avoided by applying alternative methods for FCM control. A suite of effective alternative options are available for FCM control, which includes sterile insect technique (SIT), mating disruption and various microbial control agents, such as EPN, EPF and CrleGV. However, UV sensitive microbial control agents will have to be applied more frequently in juvenile orchards than in mature orchards, as juvenile orchards are more exposed to UV radiation.

Future trials should be conducted to compare persistence of UV sensitive microbial control agents in juvenile and mature orchards. Results can then be used to determine application intervals for microbial control agents according to orchard age. Results reported by Mwanza *et al.* (2016), showed that virus persistence on the sunny northern side of orchards is significantly lower than on the southern shaded side of orchards. Mwanza *et al.* (2016) used a model to determine how frequently CrleGV should be reapplied. Factors considered in the model included the registered field rate of CrleGV (in Cryptogran), the volume of virus suspension remaining on trees after application, the relative surface area of fruit, approximate density of OBs on fruit surfaces, the surface area on which larvae feed and the influence of molasses on larval feeding behaviour. The model calculated that CrleGV should be

reapplied every 2-3 weeks if applied without molasses and every 3-4 weeks if applied with molasses. However, the model also considered the shady southern side of the tree to be more reflective of virus persistence throughout the tree, as virus on fruit inside the tree canopy would be similarly or even more thoroughly protected against UV. Therefore, CrleGV will have to be reapplied more frequently in juvenile orchards, which are more exposed to UV. Results reported by Cherry *et al.* (2000), determined persistence of baculovirus on crops with an open architecture to be less than a day. If found to be necessary, application of CrleGV more frequently than once a week in juvenile orchards will be considered impractical and too costly. Therefore, future studies on virus and other UV sensitive microbial control agents should focus on improving pathogen persistence by adding cost-effective UV protectants to product formulations. Various studies have shown UV protectants to increase persistence of microbial control agents such as baculovirus (Burges & Jones 1998, Shapiro 1992, McGuire *et al.* 2001), EPF (Inglis *et al.* 1995, Thompson *et al.* 2006, Cohen & Joseph 2009) and entomopathogenic bacteria (Burges & Jones 1998, Schisler *et al.* 2004, Ratnakar *et al.* 2013). However, according to Grzywacz & Moore (2017), the added expense and low field efficacy of some UV protectants is the major reasons why UV protectants are not used in commercial product formulations.

The ability of UV sensitive control agents to control pests in orchards with more open, aerated canopies, such as juvenile trees, dwarfing trees and trees manipulated by centrifugal training systems, will also be improved by covering orchards with nets. Covering orchards with nets will reduce exposure to UV radiation, wind and dust (Tanny & Cohen 2003, Tanny *et al.* 2009). Nets will also prevent insect pests, which prefer fruit from juvenile orchards for oviposition, from reaching fruit. However, studies have shown that covering orchards with nets will have a significant impact on the microclimate of orchards, which include higher mean humidity levels (Solomakhin & Blanke 2007) and cooler mean temperatures (Kührt *et al.* 2006, Solomakhin & Blanke 2007, Tanny *et al.* 2009). Future studies should be conducted to determine the influence of nets on the ecology of tree crop pests and their natural enemies. Higher humidity levels in juvenile orchards may increase fruit susceptibility to FCM since larval mortality will be lower (Daiber 1979 a, b). Juvenile orchards under nets may also be less exposed to temperature extremes, which will

further improve FCM survival (Daiber 1979 a, b, c). In addition, higher humidity levels will improve growth and development of citrus black spot (CBS), *Guignardia citricarpa,* (Kiely), which is considered a quarantine pest in Europe (SA DAFF 2014). Therefore, it is not recommended to use nets to improve FCM control in either juvenile or mature orchards, until the effect of nets on the ecology of citrus pests and their natural enemies is determined. Trials should also be conducted to determine if the adverse effects of nets on orchard microclimates can be reduced by only covering the roof of orchards. Only covering the roof of orchards will also reduce the cost of nets, making it a more viable option for improving FCM control in juvenile orchards.

Table 7.1 List of factors in juvenile orchards which increase or decrease susceptibility to FCM in comparison to mature orchards.

Increase	Decrease
Fruit more susceptible to FCM damage	Smaller tree size improves pesticide coverage
Fruit more attractive for oviposition	Lower average humidity increases mortality of FCM eggs and neonate larvae
Less protection against UV radiation for UV sensitive microbial control agents	Higher exposure to temperature extremes increases FCM mortality
Less suitable microclimate for parasitoids *viz.* lower egg parasitism	
Higher average temperatures expedite FCM larval development	

7.5 Conclusion

Through this study it is concluded that great advances have been made towards understanding the physiological and ecological differences between juvenile and mature citrus orchards. The knowledge gained from this study shows that juvenile and mature orchards are significantly different. Therefore, changes in pest management are required to improve FCM control (and possibly also control of other pests) in juvenile citrus orchards. However, in order to improve FCM control in juvenile citrus orchards, further trials are required to determine exactly what those changes should be. Since juvenile orchards will deliver a lower yield than mature orchards, citrus producers should also determine if the cost involved in improving FCM control in juvenile orchards is financially viable. For example, it may be more financially rewarding to improve FCM control in high value citrus varieties such as Mandarins than Navel oranges. This study suggests that FCM control in juvenile orchards will be improved by releasing parasitoids and applying UV sensitive microbial control agents more frequently than in mature orchards. The efficiency of these biological control agents may also be improved by covering juvenile orchards with nets. However, the use of nets to improve FCM control is not recommended until the effect of nets on the ecology of FCM and other citrus pests is determined.

CHAPTER 8

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