

**Using the larval parasitoid, *Agathis bishopi* (Nixon)
(Hymenoptera: Braconidae), for early detection of False
Codling Moth, *Thaumatotibia leucotreta* (Meyrick)
(Lepidoptera: Tortricidae) infested fruit**

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Abstract

Thaumatotibia leucotreta (Meyrick) (Lepidoptera: Tortricidae) is one of the major citrus pests of economic importance for South Africa's citrus industry. It is endemic to Africa, and therefore a phytosanitary pest with zero tolerance by most export markets. The cryptic nature of *T. leucotreta* makes visual inspection an inefficient method for detecting neonate larvae in fruit in the packhouse. Therefore, a more accurate method for sorting infested fruit at the packhouse, particularly for newly infested fruit could ensure market access. A recent study showed that fruit infested by *T. leucotreta* emit a chemical profile different from that of a healthy fruit. Several studies provide evidence that parasitoids locate their hosts feeding on fruit by exploiting the novel chemical profiles produced due to host herbivory. The aim of this study was to evaluate the potential of using the naturally occurring behaviour of a larval parasitoid *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) for detection of *T. leucotreta* infested fruit, by determining which compound in infested fruit is attractive to parasitoids. Y-tube olfactometer and flight-tunnel bioassays with healthy and *T. leucotreta* infested fruit showed a significantly stronger response of *A. bishopi* female parasitoids to infested fruit. Among the volatile compounds associated with *T. leucotreta* infested fruit, D-limonene elicited the strongest attraction to *A. bishopi* female parasitoids. Attraction of mated *A. bishopi* female parasitoids to *T. leucotreta* infested fruit and D-limonene significantly increased after oviposition experience. Behavioural responses of *A. bishopi* female parasitoids that were associated with *T. leucotreta* infested fruit were investigated to determine which behaviours are distinct and interpretable. Probing and oviposition behaviours were the most noticeable and were only elicited on infested fruit when parasitoids contacted *T. leucotreta* frass, indicating that chemical compounds in frass are short-range cues used for final host location. Since production of D-limonene by fruit is elevated due to herbivory by different pests including mechanical injury on fruit, response of *A. bishopi* female parasitoids to compounds in frass offers a more specific and potentially useful mechanism for development of a detection system for *T. leucotreta* infested fruit. Chemical analysis of *T. leucotreta* frass and conditioning *A. bishopi* parasitoids to respond behaviourally to compounds in frass is proposed.

Declaration

The following thesis has not been submitted to a university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

Signed: Date:

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List of Abbreviations

' – Minutes	GPS – Global positioning system
% – Percent	H₂O – Water
°C – Degrees celsius	HCl – Hydrochloric acid
µl – Micro litre	HIPVs – Herbivore induced plant volatiles
µm – Micron	hrs – Hours
° – Degrees	i.e. – <i>id est</i> (that is)
CGA – Citrus growers association	IGR – Insect growth regulator
cm² – Square centimetre	IPM – Integrated pest management
CRI – Citrus research international	Kr – Kilorad
CrleGV – <i>Cryptophlebia leucotreta</i> granulovirus	L – Light
d – Days	L – Litre
d.f. – Degrees of freedom	m – Metre
DAFF – Department of agriculture, fisheries and forestry	Medfly – Mediterranean fruit fly
DRC – Democratic Republic of Congo	min – Minutes
e.g. – Example	ml – Millilitre
et al. – <i>et alia</i> (and others)	mm – Millimetre
FCM – False codling moth	MS – Mass spectroscopy
Fig. – Figure	NH₃OH – Ammonia hydroxide
	P – Significance level
	PER – Proboscis extension response

g – Gram

GC – Gas chromatography

SD – Standard deviation

SE – Standard error

SIT – Sterile insect technique

sp. – Species

SPME – Solid phase microextraction

T – Treated

RH – Relative humidity

UV – Ultraviolet

Vs – Versus

W/V – Weight per volume

χ^2 – Observed chi-square value

ZAR – South African Rand

CHAPTER 1: General Literature Review

1.1 Citrus Production in South Africa

Citrus is one of the major components of the horticultural industry in South Africa. By gross value, the industry ranks third after vegetable and deciduous industries respectively. Oranges are the highest contributor to the citrus industry contributing about 67% by gross value in 2011 (DAAF, 2011). The total area under citrus production in South Africa is about 58 581 hectares and major producing areas include; Limpopo, Eastern Cape, Mpumalanga, Western Cape, KwaZulu-Natal and Northern Cape (CGA, 2012). The citrus industry is highly export oriented with over 70% of annual production being exported (CGA 2012). Major export destinations by volume include; United Kingdom 36%, Northern Europe 19%, Middle East 12%, Russia 12%, Far East 6%, Canada 5%, United States of America 5% and others 5% (CGA, 2012). Further, the industry is an important employer in the country with an estimate of more than a million households depending on this industry for their livelihoods (CGA, 2012).

1.1 False Codling Moth

1.1.1 Taxonomy

In South Africa, False Codling Moth *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), was first recorded as a citrus pest in Kwazulu-Natal (Fuller 1901). Meyrick (1912) described it as *Argyroploce leucotreta* (Lepidoptera: Tortricidae). Later, Clarke (1958) re-assigned it to the genus *Cryptophlebia* but eventually it was moved to the genus *Thaumatotibia*.

1.1.2 Distribution

Thaumatotibia leucotreta is endemic to sub-Saharan Africa, including the islands of the Atlantic and Indian Oceans (Newton 1998) and has been reported to be endemic in Angola, Benin, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Chad, Democratic Republic of Congo (DRC), Cote D'Ivoire, Eritrea, Ethiopia, The Gambia, Kenya, Madagascar, Malawi, Mali, Mauritius, Mozambique, Niger, Nigeria, Rwanda, Saint Helena, Senegal, Sierra Leone, Somalia, South Africa, North Sudan, South Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia and Zimbabwe (Newton 1998; Stibick 2008).

1.1.3 Life Cycle

1.1.3.1 Egg

The egg of *T. leucotreta* is approximately 1mm in diameter (Fig 1.1A). It is flat and oval in shape, white and translucent with a shiny surface and reticulate sculpture (Newton 1998; Stibick 2008). The egg is white at oviposition but progressively turns reddish after a few days and gets darker as the embryo develops (Daiber 1979; Newton 1998). The larva hatches and burrows into the fruit rind (Moore 2002).

1.1.3.2 Larva

Thaumatotibia leucotreta has five larval instars determined by size and width of the head capsule (Daiber 1979). The first instar larva has a brown to black head capsule with average head width of about 0.21 mm and about 1-1.3 mm body length (Daiber 1979; Newton 1998). At this stage, the body colour is creamy white with minute black spots and short hairs (Fig 1.1B). The average head capsule width measurements for subsequent larval instars (2nd, 3rd, 4th and 5th) are 0.37, 0.61, 0.94 and 1.37 mm respectively (Daiber 1979). As the larva develops, the body colour changes progressively from cream white to pinkish-red (Newton 1998). Under field conditions, duration of larval development is highly variable, taking 25-67 days (Stofberg 1954; Daiber 1979). The growth variation is mainly due to food quality and quantity, temperature and humidity (Daiber 1979).

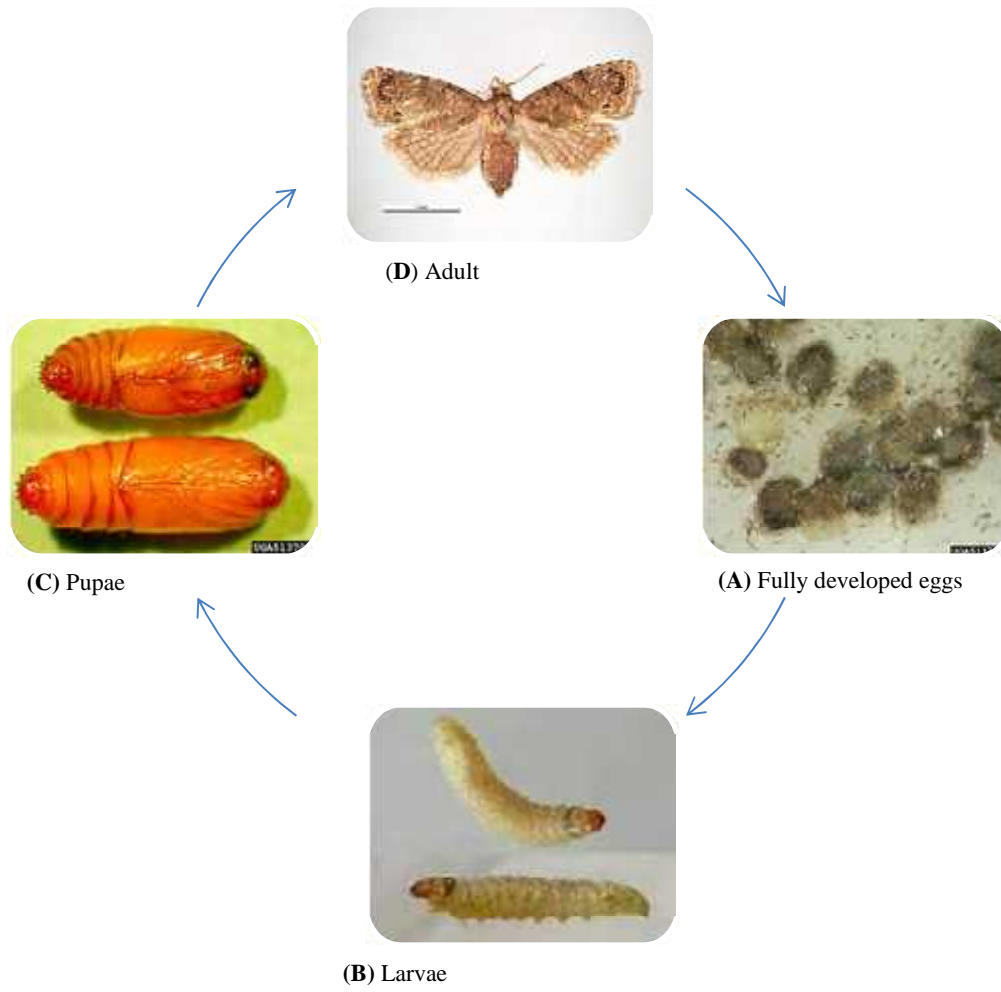


Figure 1.1 The life cycle of *Thaumatotibia leucotreta* (Pictures source: www.invasive.org)

1.1.3.3 Pupa

By the end of fifth instar, the larva exits the fruit from which it has been feeding and drops to the ground, where it spins a cocoon from silk and soil particles (Daiber 1979). The fully grown fifth instar larva in the fresh cocoon (now a pre-pupa), pupates in the cocoon, usually under the soil surface (Moore 2002). Initially the pupa is cream coloured and soft skinned but its chitin hardens progressively giving it a dark brown colour (Fig 1.1C) (Daiber 1979; Newton 1998). The pupa is segmented with transverse row spines. Newton (1998), reported that the average length of the pupa is about 7 mm when fully developed, with males being smaller than females. Duration of development under field conditions is influenced by many factors including temperature and humidity (Daiber 1979).

1.1.3.4 Adult

The adult *T. leucotreta* has a greyish brown to dark brown or black colour with average body length and width of 6-9 mm and 2.5 mm respectively (Fig 1.1D) (Daiber 1979). The forewings are broader, elongated, mottled with black triangular patches and fringed with hairs (Newton 1998). Conversely, the hind wings have a lighter greyish brown uniform colouration. Wing span ranges from 16-20 mm, with males having a smaller wing span than females (Newton 1998). Further, adult male *T. leucotreta* are distinguished from females by hind wings having a deep circular pocket, hind tibia with tufts and dense brush of greyish white hairs on hind legs (Newton 1998). In addition, males have a shorter lifespan than females (Daiber 1980). Soon after eclosion from the pupa, females mate and proceed to a pre-oviposition period for 5-6 days under field conditions (Newton 1979). Daiber (1980) reported that female *T. leucotreta* can lay up to 57.6 eggs per day and with consequent reduction in laying as the females undergo reproductive senescence.

1.1.4 Economic Importance of False Codling Moth

1.1.4.1 Host Range

Thaumatotibia leucotreta is highly polyphagous and has a broad range of wild and cultivated host plants (Newton 1998; Venette *et al.* 2003; Stibick 2008) (Table 1.1).

Table 1.1 Some of the major cultivated plants that have been recorded as hosts for false codling moth in sub-Saharan Africa. However, many of the crops below have been observed as forced associations under laboratory conditions

Common Name	Species Name	Family Name
Avocado	<i>Persea americana</i> Mill.	Lauraceae
Castor bean	<i>Ricinus communis</i> L.	Euphorbiaceae
Citrus	<i>Citrus</i> spp L.	Rutaceae
Coffee	<i>Coffea arabica</i> L.	Rubiaceae
Cotton	<i>Gossypium</i> spp L.	Malvaceae
Cowpea	<i>Vigna unguiculata</i> (L) Walp.	Fabaceae
Guava	<i>Psidium guajava</i> Mill.	Myrtaceae
Litchi	<i>Litchi chinensis</i> Sonn.	Sapindaceae
Macadamia	<i>Macadamia</i> spp (F) Muell.	Proteaceae
Maize	<i>Zea mays</i> L.	Poaceae
Mango	<i>Mangifera indica</i> L.	Anacardiaceae
Okra	<i>Abelmoschus esculentus</i> L.	Malvaceae
Olive	<i>Olea europaea</i> L.	Oleaceae
Peach	<i>Prunus</i> spp L.	Rosaceae
Pepper	<i>Capsicum</i> spp L.	Solanaceae
Pomegranate	<i>Punica granatum</i> (L) Moench.	Lythraceae
Sorghum	<i>Sorghum bicolor</i> L.	Poaceae
Tea	<i>Camellia sinensis</i> (L) Kuntze.	Theaceae

1.1.4.2 Economic Impact on Citrus Production

Thaumatotibia leucotreta was initially discovered as a pest of citrus *Citrus sinensis* L. in 1899 and has ever since been considered an important pest of citrus cultivars throughout southern Africa (Stofberg 1954). Female *T. leucotreta* lay its eggs on fruit and after hatching, neonate larvae penetrate the fruit and complete their larval development within the fruit (Daiber 1978). This infestation causes premature fruit drop from trees prior to harvest, rendering the fruit unmarketable (Georgala 1969; Newton 1998). Further, holes in fruit due to larval feeding and penetration serve as entry points for secondary infestation by fungi and bacteria resulting in fruit decay (Georgala 1969; Moore 2002). After larval penetration, it takes a few days for the minute penetration punctures to be visible (Georgala 1969). Therefore fruit harvested shortly after infestation can potentially be packed with healthy fruit destined for export markets (Moore 2002).

This condition not only causes high postharvest losses due to fruit decay but also increases the chances of accidental introduction of *T. leucotreta* into South Africa's main fruit importing countries outside Africa (Moore 2002). *Thaumatotibia leucotreta* being endemic to Africa is considered a phytosanitary pest in all export markets outside Africa. This situation has resulted in increasing demand for infestation free fruit from citrus importing countries. Therefore detection of *T. leucotreta* in export markets could result in rejection of fruit consignments and consequent huge loss of income for the South African citrus industry (Moore 2002).

1.1.5 Control of False Codling Moth

1.1.5.1 Monitoring

In agricultural pest management, pest population monitoring is vital for the success of an Integrated Pest Management (IPM) programme (Campbell *et al.* 2002). Pest control decisions (pesticide use, biological control, cultural control) are based on sampling pest populations to establish whether economic threshold levels have been reached to warrant initiation of a control measure (Higley & Pedigo 1993). However, this does not apply to a phytosanitary pest like *T. leucotreta*, where management decisions are not based on economic thresholds but on zero tolerance by export markets (Moore 2011). Traps containing synthetic sex pheromones of female *T. leucotreta* are used for monitoring the population and activity in citrus orchards (Hofmeyr 2003). Such a monitoring system is important for accurate determination of the necessity and timing of control measures for *T. leucotreta* (Higley &

Pedigo 1993). Three pheromone based monitoring systems are available and registered, including Lorelei, FCM PheroLure and Chempac FCM Lure (Moore 2011).

1.1.5.2 Chemical Control

Conventional insecticides are probably the most common pest management activity mainly due to chemicals being readily available, rapid acting and highly reliable (Rodriguez-saona & Stelinski 2009). A single application of chemicals may control a number of pest species and its effects may persist for several hours or days following application (Haynes 1988). Examples of chemical products (pesticides) registered for use against *T. leucotreta* in South Africa include pyrethroids (Cypermethrin and Fenpropathrin), Spinetoram and Chlorantraniliprole (Moore 2002; Moore 2011). Chemical control has however not proved very effective against *T. leucotreta* due to the pest's ability to develop resistance to toxicity of insecticides and having many alternative hosts (Hofmeyr & Pringle 1998). Further, the larvae feeding inside the fruit are protected from the chemical toxicity (Reed 1974), meaning that the timing of application must be accurate. In addition, overuse and misuse of pesticides has resulted in widespread criticism of chemical control due to its environmental impact and risk to human health (Rodriguez-saona & Stelinski 2009). Several studies have demonstrated the various side effects associated with the use of pesticides (Haynes 1988; Desneux *et al.* 2007). Most important to the agro-ecosystem are the non-target effects of most pesticides which lead to decimation of beneficial insects (Newton 1998; Desneux *et al.* 2007). Pesticide residues on fruit also pose a health risk to consumers, thus leading to an increase in demand for pesticide free fruit and other produce (Pimentel *et al.* 1992).

Chitin synthesis inhibitors have been used against *T. leucotreta* with considerable success (Newton 1998). Products that have been registered in South Africa for use against *T. leucotreta* on citrus include; Alsystin (Triflumuron), Nomolt (Triflubenzron) (Moore 2011). However, it was noted that *T. leucotreta* developed resistance to chitin synthesis inhibitors (Hofmeyr & Pringle 1998) and also showed detrimental effects to *Trichogrammatoidea cryptophlebiae* (Nagaraja) (Hymenoptera: Braconidae), an important egg parasitoid of *T. leucotreta* (Newton 1989; Moore 2002).

1.1.5.3 Mating Disruption

Utilization of *T. leucotreta* sex pheromone for mating disruption has proved to be a potentially effective technique for the control of *T. leucotreta* in the fruit industry (Hofmeyr 2003). The female *T. leucotreta* produces a species specific pheromone which is exploited by

males for mating. The male *T. leucotreta* locates females by moving against an increasing concentration gradient of the pheromone until a mate is found. The mating disruption technique works by the saturation of the orchard with synthetic sex pheromone of *T. leucotreta*, limiting the ability of the male *T. leucotreta* to find females. Consequently, the mating disruption technique delays or prevents mating resulting in lower population size and reduced crop damage (Moore 2011). The principle behind this control method is that the competitive attraction to synthetic pheromones causes the male *T. leucotreta* to follow false sources at the expense of finding a mate, thus delaying or preventing mating (Hofmeyr 2003). Two products containing liquid sex pheromone are currently registered for FCM control i.e. Isomate FCM and Checkmate FCM-F (Moore 2011).

1.1.5.4 Cultural Control

The use of cultural control methods such as orchard sanitation has a long and rich history in the control of *T. leucotreta* in citrus orchards (Georgala 1969). The practice involves weekly collection of dropped fruit from beneath fruit trees, including infested fruit on trees and destroying them to reduce the next season's population of *T. leucotreta* (Newton 1998). Destruction of *T. leucotreta* is achieved by submerging infested fruit in water for a week or disposing infested fruit in to a 30 cm deep trench and covered with soil (Hepburn & Bishop 1954; Georgala 1969). Alternatively, larvae can be destroyed by pulping fruit into small pieces using a hammer mill and later spreading it out on the ground to dry in the sun. Studies have revealed that up to 75% of *T. leucotreta* larvae could be removed by implementing a weekly sanitation programme (Georgala 1969). Further, Georgala (1969) recommended commencement of sanitation programme by December but Schwarts (1974) later proposed that sanitation programme commence earlier as pupae of *T. leucotreta* were observed in some orchards earlier than December. However, Ulyet (1939) demonstrated that such sanitation programmes could have adverse effects on the population of egg and larval parasitoids, as parasitoids in parasitized larvae and eggs are destroyed along with *T. leucotreta* through fruit burying and pulping.

1.1.5.5 Sterile Insect Technique

The Sterile Insect Technique (SIT) approach to pest control is designed to suppress the pest population through male insect's sterilisation, reducing its reproductive potential (Blomefield *et al.* 2010). Insects are sterilized by exposure to non-lethal levels of gamma radiation. Moore (2002) reported that a dose of 30 kr (gamma rays) was enough to completely sterilize the ova of female *T. leucotreta* while complete sterilization of male *T. leucotreta* sperm ranged

between 60-70 kr. Partial sterility is however preferred as it maintains the male moth's competitiveness (Moore 2002; Bloem *et al.* 2003).

The principle behind this technique is that cells in the chromosomes that are damaged due to radiation cannot divide correctly and thus do not form normal gametes or produce viable offspring (Bloem *et al.* 2010). Sterile male *T. leucotreta* are released into the orchards in large numbers (i.e. 10:1 partially sterile to wild male ratio). This condition increases the probability of wild female moths mating with sterile males resulting in few or no viable eggs (Bloem *et al.* 2003). Sterile insect technique is an environmentally friendly method of pest control and is also species specific (Blomefield *et al.* 2010). SIT has been used in citrus orchards in South Africa and has proved to be an effective area wide control for *T. leucotreta*. However, the success of this technique depends on a number of pest population factors that include ease of mass production of the pest, females mating only once, males being sterilized without losing their competitive vigour, low initial pest population levels and a large geographical area (Bloem *et al.* 2001; Moore 2002).

1.1.5.6 Biological Control

Biological control involves the utilization of a pest's natural enemies in the management of the pest (Opoku-Debrah *et al.* 2013). In principle, biological control involves rearing and release of natural enemies such as parasites, predators and pathogens (Opoku-Debrah *et al.* 2013; Moore 2002) and these natural enemies help in maintaining the ecological balance by regulating the population of their hosts or prey (Balmer *et al.* 2014). It is the disruption of this ecological balance that results in certain insect species becoming pests (Desneux *et al.* 2007). Therefore, biological control seeks to correct the ecological balance in one of the following ways:

- a) Classical Biological Control: It involves the reunion of natural enemies with pests that have moved to new geographical areas (Zeddies *et al.* 2001; Kipkoech *et al.* 2006).
- b) Augmentation Biological Control: This involves activities aimed at increasing the population of natural enemies through mass-culture, periodic inoculative or inundative release, and consequently suppression of pest population (Opoku-Debrah *et al.* 2013).
- c) Conservation Biological Control: This technique involves the enhancement of biodiversity in an agro-ecosystem to protect and optimize the survival and effectiveness of natural enemies (Straub *et al.* 2008).

An entomopathogenic virus, the *Cryptophlebia leucotreta* Granulovirus (CrleGV) (Baculoviridae), has been used against *T. leucotreta* in citrus (Opoku-Debrah *et al.* 2013). Once the virus is ingested by a *T. leucotreta* larva on a treated fruit, the virus is absorbed by the microvilli and consequently spreads throughout its body inducing appetite loss, morbidity, flaccidity and finally death (Moore 2002; Opoku-Debrah *et al.* 2013). Three virus based products (Cryptogran, Cryptex and Gratham) are currently available for use against *T. leucotreta* (Opoku-Debrah *et al.* 2013).

Some examples of natural enemies for *T. leucotreta* include; *Trichogrammatoidea cryptophlebiae* which is a small egg parasitic wasp. *Trichogrammatoidea cryptophlebiae* is endemic to southern Africa and occurs in all citrus producing areas. It has been demonstrated that under field conditions, *T. cryptophlebiae* can parasitize up to 80% of *T. leucotreta* eggs (Moore 2002). It has been widely reared and released in most citrus producing areas of South Africa (Newton 1998; Moore & Fourie 1999). *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) the subject of this study, is a larval parasitoid attacking *T. leucotreta* (Ulliyet 1939; Sishuba 2003; Gendall 2007). Gendall (2007) reported up to 34% parasitism of *T. leucotreta* larvae in the Sundays River Valley.

1.2 The Parasitoid *Agathis bishopi*

1.2.1 Taxonomy

The parasitoid *A. bishopi* belongs to a fairly large subfamily, Agathidinae of the Braconidae family, consisting of about 54 genera and 1000 described species (Sharkey *et al.* 2006). Members of this subfamily mostly dwell in terrestrial habitats (i.e. desert, tropical rain forests, temperate forests, savannah, alpine and sub-arctic habitats) and have a worldwide distribution (Sharkey *et al.* 2006). Most of the known species are solitary koinobiont endoparasitoids of lepidopteran larvae and attack first and second instar host larvae concealed in microhabitats (i.e. leaf rows, stems, flowers).

1.2.2 Biology

1.2.2.1 Pre-Adult Development

The female *A. bishopi* lays its eggs in the body cavity of *T. leucotreta* larvae (2nd or 3rd instar) just as they penetrate the fruit rind (Gendall 2007). After hatching inside the body of the host larva (FCM), the parasitoid larva feeds on the host's body fluid and tissue until it emerges (Odebiyi & Oatman 1972). Under laboratory conditions of 27°C and 60% relative humidity, the development period of *A. bishopi* from egg to pupation ranges from 16-20 days,

with males emerging earlier than females (Sishuba 2003). After completing larval development, the parasitoid larva cuts its way out of its host's cocoon (Dondale 1954; Odebiyi & Oatman 1972).

After emergence from its host, the fully grown *A. bishopi* larva spins its cocoon inside the host's cocoon (Odebiyi & Oatman 1972). Only one adult per host emerges suggesting that superparasitism rarely yields multiple offspring in *A. bishopi* (Odebiyi & Oatman 1972; Sishuba 2003; Gendall 2007). Virgin females only produce male progeny and mated females produce both male and female offspring (Sishuba 2003; Gendall 2007) suggesting that *A. bishopi* is parthenogenetic (Gendall 2007). The development of *A. bishopi* is well synchronized with its host as it emerges about 1-2 days after emergence of unparasitized *T. leucotreta* (Sishuba 2003; Gendall 2007). The exarate pupa is initially light yellow but gradually darkens as development proceeds.

1.2.2.2 Adult

The body of adult *A. bishopi* is highly sclerotized (Gendall 2007). The female *A. bishopi* is distinguished by a long ovipositor (Fig 1.3) which is almost the length of its body, measuring about 4 mm (Sishuba 2003; Gendall 2007). Conversely, males are smaller than females and have darker colouration (Fig 1.3). Average longevity for male and female *A. bishopi* is about 8 and 18 days respectively. Sishuba (2003) gave a detailed morphological description of *A. bishopi*.



Figure 1.3 Male (left) and female (right) *Agathis bishopi* parasitoids. The female parasitoid is larger, lighter colored and has a pronounced ovipositor

1.3 Mediation of Insect –Host Interaction by Plant Volatiles

Plant and insects have co-existed for a very long time and have in the process evolved a range of beneficial and deleterious interactions (Hare 2011). Plant may respond to insect herbivory by way of induced direct and indirect defence responses (Meiners *et al.* 2003; Onzo *et al.* 2012). Under direct responses, the plant emits chemicals that are targeted at the herbivore and result in retarded growth or death (Onzo *et al.* 2012). Conversely, indirect response strategy involves production of volatile chemicals (semiochemicals) by the plant in response to herbivory (Pare & Tumlinson 1999). These volatile chemicals attract predators and parasitoids that attack the herbivorous insects (Fatouros *et al.* 2005). This interaction between the plant, herbivore and third trophic level is known as a tritrophic relationship (Hare 2011). These volatile phytochemicals increase herbivore mortality through recruitment of natural enemies of the herbivore responsible for the damage (Yu *et al.* 2008).

Further, the production of semiochemicals from a plant under herbivore attack can trigger defensive responses in neighbouring plant (Pare & Tumlinson 1999). The volatile phytochemicals produced by plant are therefore key in mediation of interactions between plant and insects (Hare 2011). The Herbivore Induced Plant Volatiles (HIPVs) not only benefit the plant through attraction of natural enemies of herbivores, but also serve as critical cues for host location to parasitoids (Hare 2011). For example the pollination process in plant is beneficial to both the plant and insect, as while collecting nectar from plant flowers insects facilitate cross pollination/fertilization on the plant (Klein *et al.* 2003).

1.4 Herbivore Induced Plant Volatiles

Generally plant are known to release a small amount of volatile chemicals from accumulated storage sites in the leaf and other plant parts (Pare & Tumlinson 1999). Herbivore damage on plants results in increased production of volatile chemicals (Pare & Tumlinson 1999; Kendra *et al.* 2011). These chemical volatiles produced as a result of herbivore feeding are referred to as Herbivore Induced Plant Volatiles (HIPVs). HIPVs vary depending on the plant and insect species involved but generally include monoterpenes, sesquiterpenes, aromatics, saturated alcohols, aldehydes and esters (Tollsten & Muller 1996; Pare & Tumlinson 1999). Plant attacked by herbivores release specific HIPVs different from those emitted by undamaged and mechanically damaged plant (Tollsten & Mijller 1996; Khan *et al.* 2011; Chamberlain *et al.* 2012).

Bais *et al.* (2006) pointed out that HIPVs serve as “caution” signals to the rest of the plant and neighbouring plant about the impending herbivore attack. In chemical labelling experiments, Pare & Tumlinson (1999) provided evidence that herbivore damage initiates a defence response in leaves closer to the herbivore damaged ones and claimed that the defence response in undamaged leaves is due to translocation of a chemical messenger from herbivore damaged leaves. HIPVs are a distress signal for the plant and natural enemies exploit them to locate their respective hosts (Pare & Tumlinson 1999; Yu *et al.* 2010; Goubert 2013). D’Alessandro & Turlings (2006) demonstrated that natural enemies are able to recognize, respond and differentiate volatiles from undamaged, mechanically damaged and herbivore damaged plant. This led to his conclusion that there are elicitors (i.e. oral secretions) associated with insect feeding that are absent from other forms of leaf damage. Pare & Tumlinson (1999) further confirmed previous findings by showing that there is a time lag between insect feeding and emission of HIPVs which suggested that a number of biochemical activities i.e. enzyme induction, protein assembly and gene expression are responsible for the production and emission of HIPVs.

1.5 Host location by Parasitoids

The reproduction success of parasitoids is influenced by many factors that include the availability of mate, food and natural enemies (Segoli & Rosenheim 2013). The female parasitoid must locate and choose an appropriate host for successful development of her offspring (Hilker & Meiners 2002). This is especially important as the pre-adult stages of parasitoids have limited mobility and live in close association with their host either as ectoparasitoids or endoparasitoids (Roberts *et al.* 2004; Rahman *et al.* 2011).

The reproductive success of the parasitoid thus depends on the ability of the female to locate its host by exploiting a variety of physical and chemical cues associated with their hosts (Hare 2011). It is well known that host plant and herbivore cues can guide female parasitoids to their hosts (Pare & Tumlinson 1999; Canale & Benelli 2011; Goubert *et al.* 2013). Key stages in host finding and selection include orientation towards the host at long range (Host habitat location), discrimination of host plant from other plant at close range (Host location) and finding a suitable host for oviposition (Host finding) (Steidle & van Loon 2003).

Due to the high levels of biomass in plant as compared to herbivorous insects, plant produce far higher amounts of volatiles that are believed to play a key role as long range cues in the orientation of parasitoids during host searching (Pare & Tumlinson 1999). Conversely, host

cues i.e. odour of oral secretions and excreta, including feeding vibrations or sounds are believed to act as close range cues in host finding (Pare & Tumlinson 1999). The parasitoid's foraging environment is described as highly complex (Lewis *et al.* 1998), due to a myriad of other volatiles released by the many herbivorous insects, host plant and dead organic matter (Beyaert *et al.* 2010). In addition, most hosts are usually hidden or concealed in various plant parts i.e. leaves, fruit, flowers, galls (Mattiacci *et al.* 1999; Graziosi & Rieske 2013).

1.6 Learning in Parasitoids

Studies have shown that parasitoids have been able to overcome the challenges posed by the complex foraging environment by developing abilities to learn the cues associated with their hosts (Pare & Tumlinson 1999; Yu *et al.* 2008). Host location in parasitoids is aided by odour specificity which is usually provided by the chemical volatiles released from a particular plant or host including specific blending ratios of volatile chemicals (Pare & Tumlinson 1999). For example, the aphid parasitoid *Diaeretiella rapae* (McIntosh) (Hymenoptera: Aphidiidae) is attracted by the volatile chemical Isothiocyanate produced due to its host feeding on Brassicaceae plant (Pope *et al.* 2008). Further, volatiles associated with herbivory provide information to the parasitoid regarding the host age and quality i.e. whether the parasitoid is parasitized or not (Fatouros *et al.* 2005; Chu *et al.* 2014). For example, *Cotesia spp* were able to discriminate between chemical volatiles released by parasitized and unparasitised *Pieris* caterpillars (Fatouros *et al.* 2005).

Associative learning is an important attribute as it enables parasitoids to cope with the variable nature of their foraging environment (van Alphen *et al.* 2003). However, while most parasitoids are able to learn a variety of cues, others are only able to learn few specific signals (Mumm & Hilker 2005). This variation in learning abilities has been found to be linked to dietary characteristics of parasitoids (Ngumbi *et al.* 2012). Generalist parasitoids have to adapt to a higher variation of cues from their polyphagous hosts, and are thus believed to have higher abilities in learning odours associated with their various hosts than using innate abilities (Vet & Dicke 1992; Ngumbi *et al.* 2012). Conversely, specialist parasitoids are known to have lower learning abilities and mainly depend on innate abilities to perceive their host related cues (Geervliet *et al.* 1998).

Variation in cue perception and learning abilities in parasitoids is believed to be among other factors influenced by phenotypic and genotypic factors (Vet & Dicke 1992). Gu & Dorn (2000) demonstrated that the ability of parasitoids to perceive cues related to their hosts is

heritable. Further, cue perception in parasitoids is also influenced by biotic factors i.e. age, hunger, reproductive state (Greiner *et al.* 2002), which influence the physiological state of the insect (Gadenne & Anton 2000). For example, a naive mated female parasitoid, *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae), responded positively to chemical cues from host frass while the unmated female parasitoid did not (Magro & Parra 2004).

1.7 Using Insects in Chemical Detection Systems

The first attempts in using arthropods (insects and arachnids) as chemical detectors were carried out in 1963 by the United States Army (Rains *et al.* 2008). At the time, the principle behind the idea was to harness the arthropods' (lice, mosquitoes and ticks) innate abilities to respond to carbon dioxide and lactic acid concentration gradients, which were assumed to indicate the proximity and location of hidden enemies (Rains *et al.* 2008). Results from these initial attempts were however not very satisfactory until the mid-1990's when an ambitious programme was launched to further investigate the abilities of *Microplitis croceipes* (Cress) (Hymenoptera: Braconidae), *Manduca sexta* (L.) (Lepidoptera: Sphingidae) and the honey bee, *Apis mellifera* (L.) (Hymenoptera: Apidae) to learn chemicals associated with explosives and toxins, including possible development of a biological chemical detection system (Tomberlin *et al.* 2008).

Karl Van Frish, through his work with *A. mellifera* demonstrated that insects are able to learn and remember odours (Rains *et al.* 2008). Further studies reviewed that besides insects learning odours in their foraging environment, they can also be trained using Pavlovian conditioning to learn and remember odours (Tomberlin *et al.* 2008). The conditioning techniques used were simple and they involve the exposure of wasps to target odours immediately before providing them with unconditioned stimuli. The wasps were then starved for a specified period of time (i.e. 48 h) prior to examination of their response to the stimulus associated with food (Rains *et al.* 2004). The conditioned wasps exhibited food or host searching responses i.e. antennating, when odours alone were subsequently encountered (Tomberlin *et al.* 2008).

This extraordinary sense of smell and the ability of insects to learn and remember odours and consequently responding or moving towards the odour sources has been harnessed to detect chemicals (Rains *et al.* 2008; Wäckers *et al.* 2011). For instance use of *A. mellifera* as a biological sensor has been demonstrated (Chamberlain *et al.* 2012) in which the honey bee proboscis extension response (PER), coupled with gas chromatography in the laboratory has

been used to condition the bee to pick out volatile components associated with orange fruit infested with Mediterranean Fruit Fly, *Ceratitis capitata* (Wiedemann), larvae, and has been used for detection of fruit infested by fruit fly (Chamberlain *et al.* 2012).

Tomberlin *et al.* (2008) succeeded in developing a portable device called “Wasp Hound[®]” that used five conditioned *M. croceipes* wasps to detect the odour to which the wasps had been conditioned. The wasp hound is a negative and positive odour detector. Positive response result when the insect aggregates around the volatile exhaust port. Conversely, negative response results when the insect wanders about in the wasp hound when exposed to a non-target odour. Volatiles sampled from the respective treatments are delivered to the wasp hound via tubing. Using visual cortex software installed on a computer, the wasp searching behaviour is analysed and transduced into quantifiable data (i.e. graphs showing response curves of insect behaviour vs. time). A sound signal can also be produced upon encountering a positive odour (Tomberlin *et al.* 2008). This device has been used to detect chemicals associated with aflatoxin in corn, explosives and plant diseases (Tomberlin *et al.* 2008). Further, research on insect ecology has reviewed that *M. croceipes* is highly sensitive to odour and has been found to be 100 times more sensitive than electron odour sensors (Rains *et al.* 2004). Use of insects in chemical sensing systems presents several advantages, some of which include high sensitivity to odour, ease of the conditioning process and lower cost of feeding and maintenance relative to canines (Tomberlin *et al.* 2008).

1.8 Rationale for study

In South Africa, *T. leucotreta* is a major pest of citrus, particularly oranges and mandarins occurring in all citrus producing areas (Moore 2002). The adult female moth lays eggs on the surface of the fruit. The newly hatched larva then burrows into the fruit, leaving a small hole on the rind of the fruit (Moore 2002; Gendall 2007). This hole becomes an entry point for microorganisms (i.e. fungi and bacteria) which cause fruit drop and postharvest fruit decay (Newton 1998; Moore 2002). This condition renders the fruit unmarketable and undesirable for consumption. Further, it takes several days for the penetration hole of the larva on the fruit surface to become clearly visible (Georgala 1969). Therefore, *T. leucotreta* infestation occurring close to harvest is often not seen during harvest and packhouse sorting. This increases the risk of packing infested fruit together with healthy fruit for export markets. Most importantly *T. leucotreta* is a major phytosanitary pest in all South Africa’s major export markets and its detection in the market could lead to great loss of income through

interception of fruit consignments (Moore 2002). Due to possible income loss and phytosanitary risk, there is great need for a more efficient screening method for early detection of *T. leucotreta* infestation.

Literature shows that insect herbivory can elicit changes in host plant chemistry and volatile emissions (Howe & Jander 2008). The identification of such volatile compounds would allow the possibility of monitoring the fruit for early signs of deterioration using biological means i.e. parasitoids and other hymenopteran insects (Tomberlin *et al.* 2008; Chamberlain *et al.* 2012). Gendall (2007) reported that *A. bishopi* is the dominant species of larval parasitoid attacking *T. leucotreta*. It is particularly predominant in the Eastern Cape Province, with parasitism rates of up to 34% of larvae in fruit and has thus been considered to possess meaningful biocontrol potential for *T. leucotreta*. The behavioural response of this parasitoid to host cues could indicate *T. leucotreta* infested fruit. If found effective, the study will demonstrate an innovative way of timeously detecting FCM infested fruit before shipment to export markets, potentially saving the citrus industry considerable income.

1.9 Research Objectives

The ultimate objective of this study was to investigate the potential of using the larval parasitoid, *A. bishopi* for early detection of *T. leucotreta* infested fruit postharvest. The process involved the assessment of the parasitoid's (specifically female) innate or conditioned behavioural responses in the presence of *T. leucotreta* infested fruit. However, several tentative objectives were to be met in order to realize the ultimate objective.

Firstly, a laboratory culture for *A. bishopi* parasitoids had to be established in order to provide sufficient numbers of parasitoids for bioassay trials and experiments aimed at improving the existing rearing protocol.

The second objective involved olfactometer bioassays with female *A. bishopi* parasitoids using fruit and volatile compounds identified by van der Walt (2012) in *T. leucotreta* infested fruit. This investigation enabled the determination of specific compound(s) in infested fruit that were attractive to *A. bishopi* female parasitoids.

The third objective was to determine whether there were distinct and interpretable behavioural responses from female *A. bishopi* female parasitoids that could be associated

with *T. leucotreta* infested fruit. This enabled confirmation of specific behavioural responses in *A. bishopi* that were exclusively elicited in presence of infested fruit. Within the context of this investigation, the host searching behavioural sequence in *A. bishopi* female parasitoid was elucidated.

The fourth objective was to condition *A. bishopi* females to respond behaviourally to volatiles associated with *T. leucotreta* infested fruit. However, execution of this objective will only be necessary if the parasitoids do not respond naturally to volatiles from *T. leucotreta* infested fruit.

The final objective was to develop a system where *A. bishopi* parasitoids can be practically used for early identification of *T. leucotreta* infested fruit postharvest particularly in the packhouse.

CHAPTER 2: Rearing of *Agathis bishopi* on *Thaumatotibia leucotreta* larvae

2.1 Introduction

Rearing of parasitoids is a very important requirement not only for inundative or inoculative releases in biological control programmes (Wang *et al.* 2014), but also for laboratory biological studies such as ethology, physiology and taxonomy, that may require large numbers of test insects (Jervis 2005; Powell & Hartley 1987).

During the rearing process, quality management is paramount as it directly relates to the general quality of the parasitoids reared (Gandolfi *et al.* 2003). Several factors affect the quality (i.e. fecundity, growth rate and survival) of parasitoids during the rearing process. (Powell & Hartley 1987). The following are some of the major factors that affect the quality of parasitoids being reared;

- a) Host feeding: After parasitism, hosts of koinobiont parasitoids continue feeding, growing and developing (Li & Mills 2004). This continued feeding and growth of hosts after parasitism represents a very important food resource for the parasitoid developing inside (endoparasitoid) or on (ectoparasitoid) the host's body (Jervis 2005). Several studies have shown the positive relationship between the host nutrition and parasitoid quality (Jervis *et al.* 2008; Bukovinszky *et al.* 2012), but Rivero & West (2005) reported that growth of an endoparasitoid was constrained by lack of certain nutrients in its host's body tissue. Furthermore, studies on parasitoids reared on artificial diets have showed that diets devoid of the host's natural food compounds leads to deterioration of native behaviour in parasitoids (Bautista & Harris 1997; Foster & Harris 1997; Gandolfi *et al.* 2003). It is therefore important that the host diet contains all the key nutrients necessary for the optimal growth and development of both the host and parasitoid, including maintenance of natural host searching behavioural traits in parasitoids (Gandolfi *et al.* 2003).

- b) Parasitoid feeding: Nutrition is an important requirement in the rearing of parasitoids as it influences their growth, fecundity and survival (Winkler *et al.* 2006). Most hymenopteran parasitoids are known to feed on honeydew, nectar and pollen in their natural habitat (Irvin & Hoddle 2007; Géneau *et al.* 2013) and can also feed on food substitutes such as diluted honey (Gendall 2007) and sugar

solution (Belda & Riudavets 2012). Reduced food and water intake in parasitoids results in reduced egg load (Ozkan 2007) and longevity of parasitoids (Azzouz *et al.* 2004; Winkler *et al.* 2006). It is therefore important to ensure that enough food supplement and water is made available and replenished at appropriate intervals.

- c) Mating: The mode of reproduction in most hymenopteran parasitoids is through parthenogenesis (Beukeboom & Pijnacker 2000). This involves production of offspring from unfertilized eggs (Makatiani *et al.* 2013). Most braconid parasitoids including *Agathis spp* (Odebiyi & Oatman 1972; Gendall 2007) are known to be haplodiploidous, in which males are haploid and females diploid and reproduce parthenogenetically by arrhenotoky (Odebiyi & Oatman 1972; Gendall 2007). Arrhenotokous reproduction involves development of male offspring from unfertilized eggs and female progeny from fertilized eggs (Odebiyi & Oatman 1972; Jervis 2005). Therefore unmated female individuals only lay haploid eggs yielding only male offspring (Kant *et al.* 2012). Furthermore, delayed mating has been observed to reduce fitness (i.e. parasitism rate and sex ratio) in parasitoids (Kant *et al.* 2013). It is therefore important to ensure that parasitoids mate timeously and successfully before they are allowed to parasitize their hosts, in order to get an optimal sex ratio of offspring.
- d) Host preference: The instar at which the host larva is parasitized by the female parasitoid has an influence on the resulting sex ratio of offspring (Hopkinson *et al.* 2013; Chu *et al.* 2014). Haplodiploidy, arrhenotokous reproduction in which male progeny arise from unfertilized eggs and female from fertilized eggs enable female parasitoids to control the sex of their offspring (Jervis 2005). Mated females are able to store sperm in the spermatheca and control the sex of their offspring by regulating release of sperm to eggs passing through the oviduct (Jervis 2005). In general, most parasitoids tend to lay haploid eggs in smaller hosts and diploid eggs in larger hosts (Jervis 2005; Chu *et al.* 2014). It is therefore important when rearing such insects, to identify the host instar most preferred by parasitoids in order to realise an optimal sex ratio of progeny (Chu *et al.* 2014). A female biased sex ratio is a much desired outcome in the rearing process as it is the females that sustain the culture (Ode & Heinz 2002).

- e) Biotic and abiotic factors: The physical factors (i.e. temperature, humidity and photoperiod) on which the parasitoids are exposed to during the rearing process have an effect on their fecundity (Prasad *et al.* 2002; Mawela *et al.* 2013; Lu *et al.* 2014). Exposing parasitoids to higher than optimal temperatures increase the general activity and metabolic cost with consequent reduction in egg production (Prasad *et al.* 2002; van Baaren *et al.* 2005). Seal *et al.* (2002) reported reduced oviposition and longevity in *Catolaccus hunteri* (Crawford) (Hymenoptera: Pteromalidae) when reared at higher than optimal temperature. Light intensity and photoperiod have a profound effect on the biology and behaviour of some parasitoids (Lu *et al.* 2014). Increase in light intensity increases the flight activity in diurnal parasitoids (Ozkan 2007). Further, high temperature and humidity is known to exacerbate microbial contamination of the rearing media by fungus (Powell & Hartley 1987). On the contrary, low humidity has been shown to reduce host searching efficiency and longevity in parasitoids (Lu *et al.* 2014). Therefore, care must be taken during the rearing process to ensure provision of optimal amounts of light, photoperiod, temperature and humidity in order to maximise the quality of parasitoids being reared. The rearing programme should aim at preventing microbial (bacterial, viral and fungal) contamination of the insect culture, particularly the host rearing medium. Contamination of the rearing medium with entomopathogenic fungi, virus or bacteria could lead to high host mortality consequently reducing parasitoid yield from the culture (Gendall 2007).

The most common method for establishing a larval parasitoid culture is to first recover parasitoids from naturally parasitized hosts (Ghimire & Phillips 2010; Belda & Riudavets 2012; Benelli *et al.* 2013; Lu *et al.* 2014). This technique has previously been used to rear *A. bishopi* parasitoid on *T. leucotreta* larvae (Sishuba 2003; Gendall 2007), and several other hymenopteran larval parasitoids (Eben *et al.* 2000). The ease of rearing larval parasitoids depends to a larger extent on the host development stage encountered by the parasitoids (Jervis 2005). In general, it is easier to rear parasitoids on non-feeding host stages such as eggs or pupae, as these do not require provision of a food supplement (artificial diet), than on larvae that require feeding (Jervis 2005). Further, it is easier to rear idiobiont parasitoids in which the attacked host stage does not feed, grow or develop beyond the stage attacked, than it is to rear koinobiont parasitoids in which the host continues feeding, growing and developing after parasitism (Li & Mills 2004; Jervis *et al.* 2008).

Gendall (2007) succeeded in rearing *A. bishopi* parasitoids on *T. leucotreta* larvae. However, low parasitism rates and fungal infection of *T. leucotreta* diet were among the challenges encountered. This study describes the field collection of parasitized *T. leucotreta* larvae and establishment of an *A. bishopi* culture to facilitate subsequent bioassay experiments. During the collection of parasitized larvae from the field, trends in fruit infestation rates and larval parasitism rates were examined. Furthermore, this study aimed to improve the existing rearing protocol through adjustment of quantity of diet in rearing jars and improvement of parasitoid feeding.

2.2 Materials and Methods

2.2.1 Fruit Collection

The culture of *A. bishopi* was initiated from parasitized *T. leucotreta* larvae in infested orange fruit collected from farms in the Sundays River Valley (South Africa, Eastern Cape Province) (Table 2.1). The orchards sampled from each farm were selected based on their history of high *T. leucotreta* infestation. Five mass collections of fruit were done from April to August in 2013 and 7 mass collections in 2014 from January to July. A total of 5441 fruit were collected in 2013 while 18309 fruit were collected in 2014. Initially, infested fruit were picked from the orchard floor but later in the season, infested fruit were picked off the trees in order to recover younger instars of *T. leucotreta* larvae. The fruit collected from the orchard (either from trees or orchard floor) were selected based on obvious signs of infestations such as the presence of a frass filled penetration holes, premature ripening around a penetration hole and discolouration (i.e. yellow or brown) around the penetration hole.

2.2.2 Recovering *Agathis bishopi* from field collected *Thaumatotibia leucotreta* larvae

In the laboratory, the fruit were dissected to recover *T. leucotreta* larvae. This was done by cutting the fruit into thin slices around the penetration hole and pulling the slices away until the larva was found. Care was taken not to cross the hole with the knife as doing so could slice or injure the larva. The recovered larvae ranging from first to fifth instar were then reared on *T. leucotreta* artificial diet as described by Moore *et al* (2014) (Table 2.2). The diet was prepared by mixing equal amounts of diet and distilled water (1 g diet: 1 ml H₂O).

Table 2.1 Fruit collection sites in the Sundays River Valley Area (2013-2014 fruit seasons) in Eastern Cape Province, South Africa

Farm	Location	Orange cultivar	Geographical coordinates
Kleinplaas	Addo	Navels	33° 29' 34.34" S, 25° 41' 42.47" E
Dunbrody Estates	Kirkwood	Navels	33° 28' 01.18" S, 25° 31' 04.69" E
Atmar	Kirkwood	Navels	33° 28' 11.07" S, 25° 31' 04.69" E
Halaron	Addo	Navels	33° 29' 07.92" S, 25° 40' 35.00" E
Eluhlaza	Kirkwood	Navels	33° 28' 56.79" S, 25° 39' 18.14" E

The diet paste was then placed in a baking tray, covered with aluminium foil and baked in an oven at 180°C for 30 minutes and later cooled under a laminar flow hood for 1 hour. The baked diet was then transferred into glass vials by pushing the vial upside down into the diet, forcing about 30 g of diet into each vial. The diet was then pressed down to the bottom of the glass vial using a sterile marker pen. This process was carried out under the laminar flow hood in order to minimize fungal contamination of diet. *Thaumatotibia leucotreta* larvae recovered from fruit were then transferred individually into the vials containing baked diet. A cotton wool plug was immediately inserted into the opening of each vial to serve as a pupation medium for *T. leucotreta* larvae as well as a barrier to microbial contaminants (particularly fungi). Date and instar of *T. leucotreta* larvae were labelled on the vials accordingly. The vials were then transferred into a climate controlled room maintained at 25°C ±2, 50-60% RH.

Vials were monitored daily until the larvae died, pupated or yielded parasitoid cocoons. All the parasitoid cocoons identified were carefully removed from the cotton plugs and placed in emergence jars to facilitate easy emergence of *A. bishopi*. Adult parasitoids (12 hours old) collected from the emergence jars were immediately transferred in pairs (male and female) into plastic vials to mate for 24 hours. A piece of cotton wool soaked in dilute honey (36% w/v) was placed through a hole on the vial top to serve as a food source for the parasitoids. After 24 hours of mating, parasitoids were subsequently used to start a culture.

Table 2.2 Composition of artificial diet (without water) used for rearing *Thaumatotibia leucotreta* (Moore *et al.* 2014)

Ingredient	Quantity (g)
Maize meal	2000
Wheat germ	200
Brewer's yeast	100
Milk powder	36.5
Nipagen	15
Sorbic acid	6.5
Total	2358

Monthly trends in parasitism and fruit infestation during the two seasons were estimated by calculating parasitism and fruit infestation rates for each batch of fruit collected from the field using the formulae below (a and b):

(a) % Parasitism = (total number of parasitoid / total number of larvae reared) × 100

(b) % Fruit with larvae = (total number of fruit with larvae / total number of fruit collected) × 100

2.2.3 Improving the parasitism success of *Agathis bishopi* during rearing

2.2.3.1 Rearing host larvae on different amounts of artificial diet in jars

Thaumatotibia leucotreta larvae were reared on artificial diet in honey jars as described above. Three sets of jars with different quantities of diet (10 g, 25 g and 40 g) were prepared with proportionate amounts of distilled water added accordingly. A tightly fitting piece of cotton wool was then inserted onto the mouth of each honey jar. The honey jars were then autoclaved at 120°C for 15 minutes and later allowed to cool under a laminar flow for 30 minutes. *Thaumatotibia leucotreta* eggs were collected from an established culture maintained at Rhodes University (Waainek Research Laboratory). A piece of egg sheet with approximately 100 eggs was cut out and sterilized by dipping it in 30% formalin for 1

minute. The egg sheet was then placed in the honey jar with artificial diet and the cotton plug replaced. This process was done under a laminar flow to prevent fungal contamination. Honey jars were then placed in a climate controlled room maintained at a temperature of $25^{\circ}\text{C}\pm 2$ and 50-60% relative humidity. The jars were monitored daily until larvae hatched with 1st instar larvae observed.

Mated parasitoids with a varying male/female sex ratio were transferred into honey jars containing 1st or 2nd instar *T. leucotreta* larvae. Food was supplied to the parasitoids by soaking pieces (3 cm²) of paper towelling in diluted honey (36% w/v) and sticking them on the side in the honey jar. After the parasitoids had spent 2 days in the jars with larvae, they were later transferred into a new jar with 1st to 2nd instar larvae. This process continued until a female parasitoid died. After transferring parasitoids from honey jars with larvae, the jars were once again plugged with a tightly fitting piece of cotton wool to serve as a pupation medium for *T. leucotreta* larvae. After most larvae had pupated on the cotton wool, the cotton wool plug was removed and placed into a 2 litre emergence jar. A ventilated lid was used to close the honey jar with diet to secure any parasitoid that could have pupated in the diet. Both the honey jars and emergence jars were monitored daily for emergence of *A. bishopi*. After emergence, the date of emergence, sex and number of parasitoids per jar were recorded. Parasitoids were then checked for deformities before they were allowed to mate and parasitize the next stock of *T. leucotreta* larvae.

2.2.3.2 Changing the carrier medium and placement of dilute honey in the rearing jars

In an attempt to sustain the quality (palatability) and enhance accessibility of dilute honey to parasitoids in the rearing jars, its carrier medium and placement in the jar was changed. A piece of cotton wool dipped in dilute honey was placed through a hole (4 cm diameter) in the metal lid of the honey jar as opposed to using paper towelling as described above. In order to reduce the risk of fungal contamination due to the higher humidity in the jars created by the moist cotton wool (with dilute honey), the duration for which parasitoids were exposed to *T. leucotreta* larvae was reduced from 4 days as previously recommended (Gendall 2007) to 2 days. A sex ratio of 1:2 (male: female) for *A. bishopi* parasitoids was used for all the jars exposed to parasitism. The jars were kept in a controlled environment room ($25^{\circ}\text{C}\pm 2$ and 50-60% relative humidity). After parasitoid emergence, parasitism rates and sex ratio of offspring were noted and recorded accordingly.

2.2.4 Development duration and longevity of adult *Agathis bishopi* parasitoids

The development duration of *A. bishopi* parasitoids from egg to adult was measured as the period from parasitism of *T. leucotreta* larvae in the jars to emergence of adult parasitoids. Development duration (number of days) for 30 male and 30 female parasitoids emerging from jars were measured and recorded. Soon after emergence (< 24 hrs) in the jars, parasitoids (1:2, male: female) were placed in plastic vials and allowed to mate for 2 days. The parasitoids were then exposed to *T. leucotreta* larvae in jars (as described above) for a period of 2 days per jar until they died. Longevity (number of days lived) for 30 male and 30 female parasitoids when exposed to a mate, 36% w/v honey and hosts were recorded. Parasitoids were kept in a controlled environment room (25°C±2 and 50-60% relative humidity).

2.2.5 Influence of parasitoid experience on parasitism success

In order to examine the influence of experience on parasitism success for *A. bishopi* females, parasitoids (1:2, male: female) were exposed consecutively to 4 sets of jars with *T. leucotreta* larvae (1st to 2nd instar). Honey jars with 40 g of artificial diet were prepared as described above. Furthermore, parasitoids were allowed to parasitize *T. leucotreta* larvae for 2 days after which they were moved into the next jar with larvae as described above. Female parasitoids were exposed to *T. leucotreta* larvae 2 days after emergence. Preliminary observations showed that most parasitoids did not oviposit on the first day after their emergence. Parasitoids were considered as 'naïve' when exposed to the first set of jars and 'increased experience' when exposed to the 2nd, 3rd and 4th set of jars respectively. After most *T. leucotreta* larvae had pupated on the cotton wool plugs, the cotton wool was removed from the honey jars and placed in 2 litre emergence jars in which both the parasitoids and moths emerged. After the moths and adult parasitoids had emerged and died in the honey jars and corresponding emergence jars, total numbers of parasitoids and moths per jar in each set were recorded. Parasitism rate in each jar was calculated using the formula below:

% Parasitism = (total number of parasitoid / total number of parasitoids + total number of moths) x 100

2.2.6 Chemical composition of *Thaumatotibia leucotreta* frass when reared on artificial diet

Artificial diet (40 g per jar) was prepared in honey jars as described above. Two categories of diet ('uninfested diet' - without *T. leucotreta* larvae and 'infested diet' - with 1st or 2nd instar larvae) with 2 jars per category were prepared for volatile chemical analysis. The two sets of diet were kept under the same conditions (25°C±2 and 50-60% relative humidity) until they were taken to Nelson Mandela Metropolitan University, Port Elizabeth, for chemical analysis. As it was difficult to isolate frass from the compounds in artificial diet, infested diet with frass and *T. leucotreta* larvae was analysed and compared to results from uninfested diet. It was assumed that the difference in the chemical profiles between the two categories of diet would only be attributed to the chemicals in frass and larvae. In the laboratory, vented metal lids on the honey jars were replaced with a septum to facilitate volatile sampling by the Solid Phase Microextraction (SPME) method. Head space volatiles from uninfested and infested diet were collected using SPME with a 70 µm CAR/PDMS, fused silica 23 Ga fibre. Volatiles in the jars were sampled by inserting the SPME fibre through the septum and exposing the fibre to the diet headspace for 20 min. The chemical volatiles adsorbed on the fibre were then injected into the Gas Chromatography /Mass Spectrometry unit (Agilent 6890N GC, 5973 MS Detector) with an HP-5 medium polarity 29 m length column. The oven temperature was initially set at 45°C and increased at 15°C/min to 200°C followed by a second ramp from 200°C to 240°C at 15°C/min for 20 min. Chemical compounds in the head space volatiles were identified by comparing the chromatogram peaks and retention time using the mass spectral library.

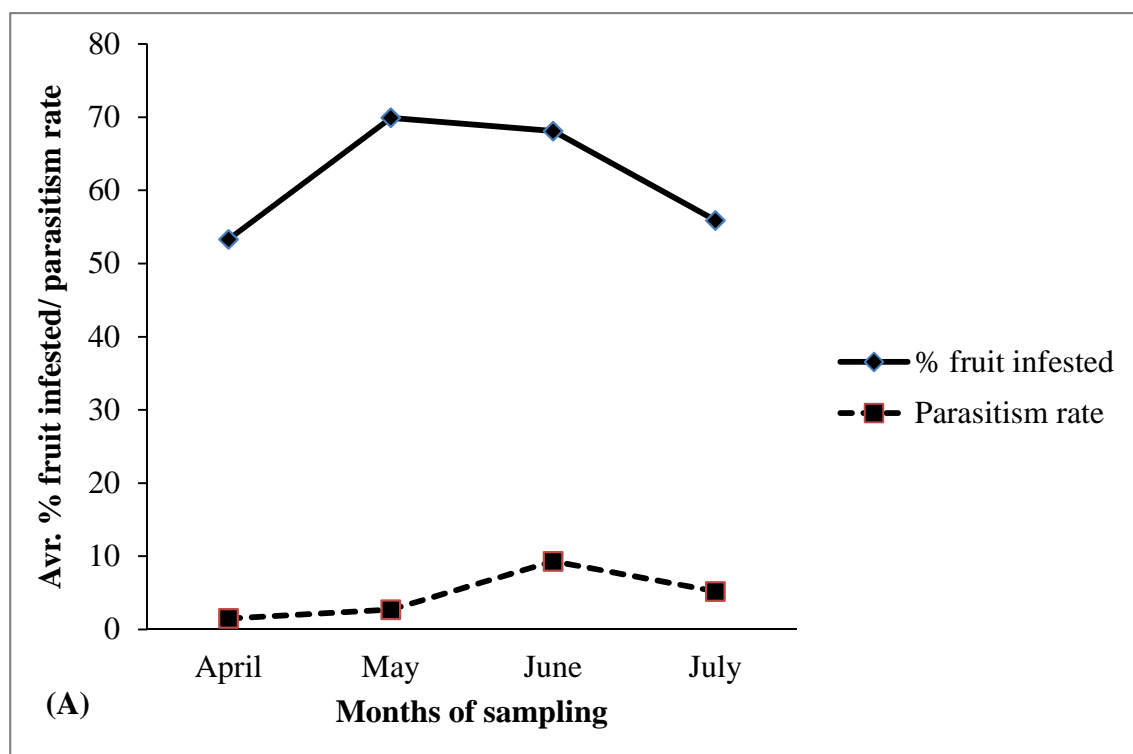
2.2.7 Statistical analyses

R-statistical package version 3.0.2 was used for all statistical analyses in this study. Data for development duration and longevity of adult parasitoids was initially tested for normality and homoscedasticity. Development duration data met the normality and homoscedasticity assumptions and thus, a Welch two sample *t*-test ($P < 0.05$) was used to compare the mean development duration between male and female parasitoids. Since the data for parasitoid longevity was not normally distributed, a non-parametric statistical test, Wilcoxon rank sum test ($P < 0.05$) was used to compare the longevity (median) between male and female parasitoids. Furthermore, a Kruskal-Wallis one-way ANOVA was used to test the differences in parasitism (median) across the different levels of parasitoids oviposition experience.

2.3 Results

2.3.1 Trends in parasitism and fruit infestation during the 2013 and 2014 fruit seasons

The monthly average percentage of fruit infested with *T. leucotreta* and percentage of larvae in fruit parasitized with *A. bishopi* for 2 fruit seasons (April-July 2013 and Jan-July 2014) are presented below (Fig 2.1 A and B). During the 4 months of sampling in the 2013 season, fruit infestation by *T. leucotreta* ranged from 53.3% to 69.9%. From April (53.3%), fruit infestation increased to 69.9% in May and later began to steadily drop to 68.8% and 55.9% in June and July respectively (Fig 2.1 A). Overall, 61.8% of fruit sampled in 2013 season were infested by *T. leucotreta*. The percentage of larvae in fruit parasitized by *A. bishopi* ranged from 1.5% to 9.3%. From April (1.5%), parasitism rate increased to 2.7% in May and reached its highest in June (9.3%) and later dropped to 5.2% in July (Fig 2.1 A). In the 2014 season, sampling began earlier in the year (Fig 2.1 B) and fruit infestation ranged from 13.8% to 37.7%. There was generally a steady increase in fruit infestation from April (13.7%) to July (37.7%) with a season average of 20.6% (Fig 2.1 B). The parasitism rate in the 2014 season ranged from 6.2% to 42.4% with a season average of 22.6%. Except for a drop in April (9.3%), there was a steady increase in the parasitism rate from February to July (Fig 2.1 B).



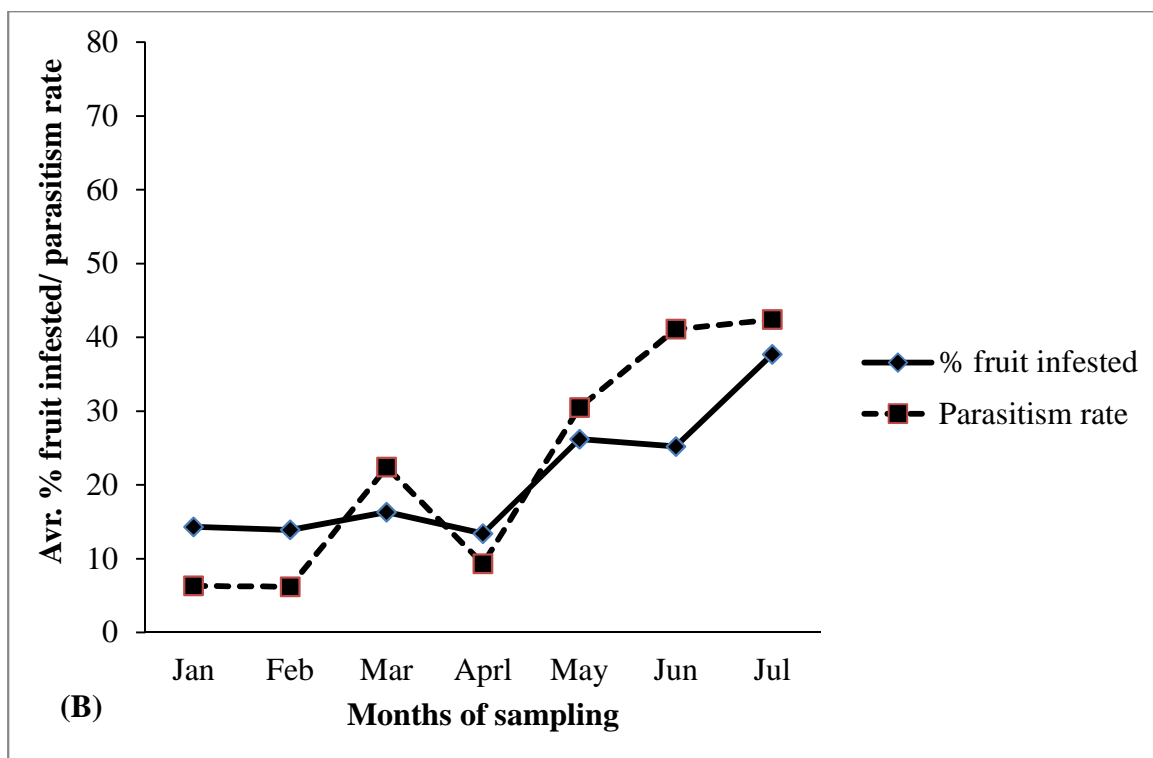


Figure 2.1 average percentage infestations of fruit with *T. leucotreta* and *A. bishopi* parasitism rates during 2013 (A) and 2014 (B) seasons. Avr. = average.

2.3.2 Rearing host larvae on different amounts of artificial diet in jars

Results for parasitism of *T. leucotreta* larvae by *A. bishopi* parasitoids when *T. leucotreta* was reared on 3 different amounts of artificial diet are presented below (Table 2.3 A, B and C). It was observed that 1 out of 15 jars (6.7 %) with 10 g diet (Table 2.3 A) yielded parasitoids while 7 out of 59 jars (11.9%) with 25g diet (Table 2.3 B) produced parasitoids. No parasitoid emergence was observed in jars with 40 g of diet (Table 2.3 C). Parasitism rates in the jars with 10 g, 25 g and 40 g diet were 2%, 8.3% and 0% respectively. A male biased sex ratio of parasitoid offspring was observed from the 10 g diet jar (2:0, male: female) and 25 g diet jars (3.8:1, male: female). Furthermore, higher mortality of *T. leucotreta* larvae was measured in all jars with 10 g diet and very few larvae survived to pupate.

2.3.3 Changing the carrier medium and placement of dilute honey in the rearing jars

Results for the trial in which the honey carrier medium and its placement in the rearing jars were changed are presented in Table 2.4. Of the 38 jars with *T. leucotreta* larvae that were exposed to parasitoids for parasitism, 31 jars (81.6%) yielded parasitoid offspring. The average parasitism rate for all the jars that yielded parasitoid offspring was 30% with a female biased total sex ratio (1:1.4, male: female).

Table 2.3 *Agathis bishopi* parasitism results when *Thaumatotibia leucotreta* larvae were reared on 10 g, 25 g and 40 g of diet in jars

Measurement	Number/percentage		
	10 g	25 g	40 g
Number of jars exposed for parasitism	15	59	12
Number of jars yielding parasitoids	1	7	0
% of jars yielding parasitoids	6.7	11.9	-
Average number of male parasitoids per yielding jar	2	7	-
Average number of female parasitoids per yielding jar	0	2	-
Average number of parasitoid per yielding jar	2	9	-
% parasitism	2	8.3	0
Maximum number of parasitoids per yielding jar	2	22	-
Minimum number of parasitoids per yielding jar	-	3	-

2.3.4 Development duration and longevity of adult *Agathis bishopi* parasitoids

The development duration from egg to adult for male and female *A. bishopi* parasitoids ranged from 25-32 days and 24-40 days respectively. The mean number of days required for male and female parasitoids to develop from egg to adult was 27.3 ± 0.3 and 30.5 ± 0.6 days respectively (Fig 2.2). Furthermore, it was observed that the development duration for female parasitoids was significantly longer than for males ($t = -4.96$, d.f = 44.68, $P = 1.058e^{-5}$). The longevity for male and female *A. bishopi* parasitoids in the presence of mate, 36% w/v honey and host (~100 larvae per jar) ranged from 15-27 days and 11-28 days respectively. Under the stated conditions, male parasitoids lived significantly longer (24.2 ± 0.7 days) (Fig 2.3) than females (19.3 ± 0.8 days) (Wilcoxon rank sum test, $W = 700.5$, $P = 0.00017$).

Table 2.4 *Agathis bishopi* rearing results after changing the carrier medium for honey and its placement in the rearing jar

Measurement	Number/percentage	
	Cotton wool	Paper towelling
Number of jars exposed for parasitism	38	12
Number of jars yielding parasitoids	31	0
% of jars yielding parasitoids	81.6	-
Average number of male parasitoids per yielding jar	12	-
Average number of female parasitoids per yielding jar	17	-
Average number of parasitoid per yielding jar	30	-
% parasitism	30	0
Maximum number of parasitoids per yielding jar	88	-
Minimum number of parasitoids per yielding jar	2	-

2.3.5 Influence of parasitoid experience on parasitism success

There were no significant differences in the parasitism rates across the different levels of oviposition experience tested (Kruskall-Wallis one-way ANOVA, d.f = 5, $P = 0.293$) (Fig 2.4). However, the optimal parasitism (24.1%) was achieved at the fourth level of oviposition experience (when parasitoids were exposed to the fourth jar with larvae), after which parasitism began to decline (Fig 2.4).



Figure 2.2 Mean development duration for male and female *Agathis bishopi* parasitoids when their host, *Thaumatotibia leucotreta*, was reared on artificial diet. Duration is significantly different ($t = -4.96$, $P = 1.058e^{-5}$). Bars = SE

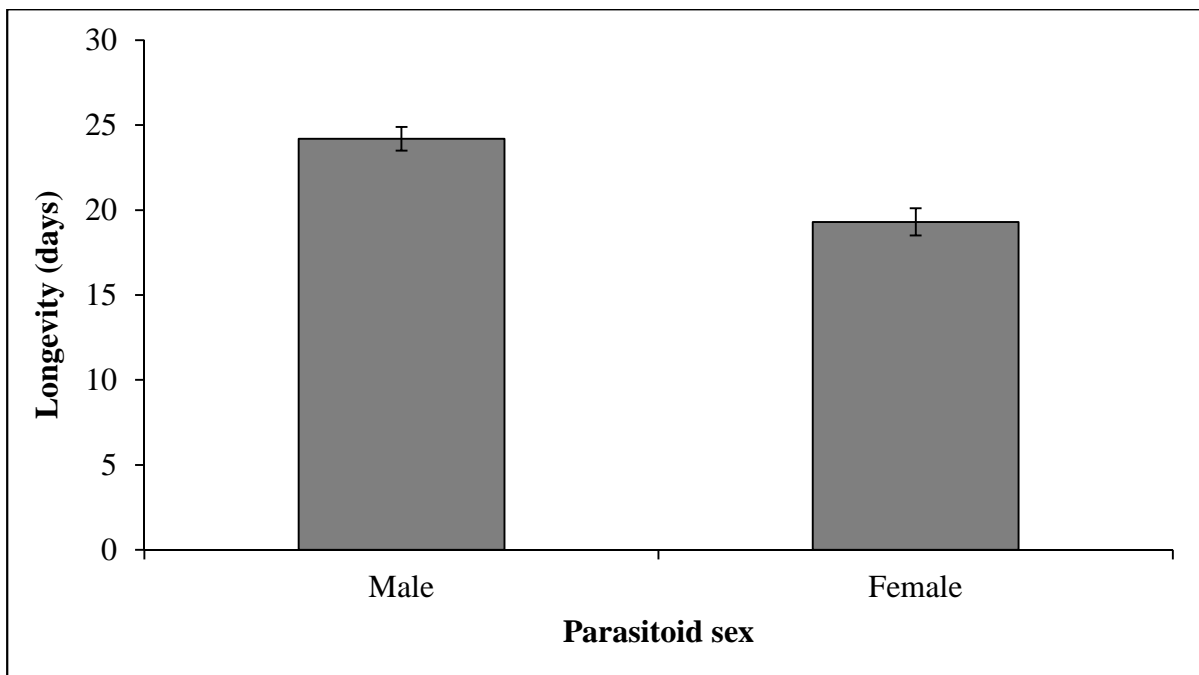


Figure 2.3 Mean longevity for male and female *Agathis bishopi* parasitoids in the presence of a mate, fed 36% w/v honey and the host (~100 larvae). Longevity of male and female parasitoids is significantly different (Wilcoxon rank sum test, $P = 0.00017$). Bars = SE

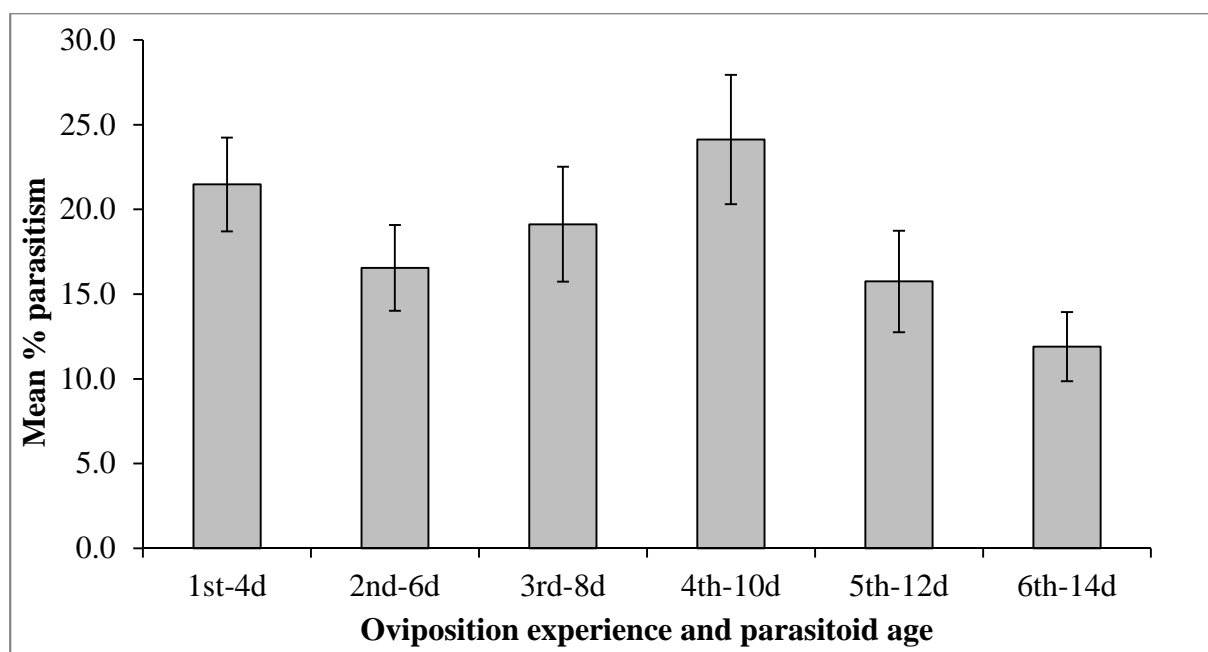


Figure 2.4 Influence of oviposition experience on parasitism success. Numbers (1st – 6th) represents number of oviposition experiences while numbers/letter (4d-14d) shows the parasitoid age at every level of oviposition experience. Bars = SE

2.3.6 Chemical composition of *Thaumatotibia leucotreta* frass when reared on artificial diet

A total of 11 major compounds were identified in uninfested diet while 15 compounds were found in diet infested with *T. leucotreta* larvae (Table 2.5). Furthermore, 3 compounds were found exclusively in uninfested diet while 7 compounds were found only in infested diet and 8 compounds were present in both uninfested and infested diet. Of the compounds that were found present in both uninfested and infested diets, carbon dioxide and hexane were elevated substantially in infested diet relative to uninfested diet. Similarly, octane decreased noticeably in infested diet compared to uninfested diet.

Table 2.5 Volatile compounds identified in the head space of uninfested artificial diet and diet infested by *Thaumatotibia leucotreta* larvae using SPME-GC technique. Shaded rows indicate 7 major compounds that were detected exclusively in infested diet.

Compound	% of total abundance	
	Artificial diet only	Artificial diet + larvae + frass
Carbon dioxide	20.71	27.04
Methylene chloride	21.89	-
Hexane	4.99	9.39
3-Buten-1-ol	1.59	1.79
Acetic acid	11.19	11.07
Toluene	1.59	1.42
Octane	2.27	0.58
Butanoic acid, 3-methyl-	-	3.93
Cyclopentasiloxane, decamethyl-	0.84	0.67
2-Propanol	-	0.81
Acetone	-	17.60
Limonene	1.59	1.96
Methane, dichloro-	-	18.41
Propane, 1-methoxy-	-	3.07
Cyclotetrasiloxane, octamethyl-	1.25	-
Pentasiloxane, dodecamethyl-	0.65	-
.alpha.-Caryophyllene	-	0.62
1,11-Dihydro gendodecamethylhexasiloxane	-	0.65

2.4 Discussion

In this study, rearing of *A. bishopi* parasitoids for use in bioassay experiments and improvement of the existing rearing protocol was attempted. Monthly trends in parasitism and fruit infestation % were ascertained from the fruit collections during the 2013 and 2014 fruit seasons. In addition, the effect of artificial diet, honey and parasitoid experience on parasitism success was elucidated as well as development duration, longevity of adult parasitoids and chemical composition of *T. leucotreta* frass.

Generally, high fruit infestation and low parasitism % were recorded during the sampling period (April-July) in the 2013 season. Most of the fruit sampled in the 2013 season were collected from the orchard floor which resulted in the majority of larvae collected being older and unparasitized (4th -5th instar) (Gendall 2007). This resulted in the relatively higher infestation and lower parasitism % recorded in 2013 season. The highest fruit infestation and parasitism % in 2013 were recorded in May (69.9%) and June (9.3%) respectively. Compared to 2013, higher mean parasitism (22.6%) and lower fruit infestation (20.6%) were recorded during the sampling period (January – July) in the 2014 season. Unlike 2013, most of the infested fruit in the 2014 season were picked from trees from which the majority of larvae recovered were younger and a higher proportion of them were parasitized (Gendall 2007). Sampling newly infested fruit from the trees resulted in the relatively higher parasitism and lower infestation % recorded in the 2014 season. The highest fruit infestation and parasitism % in 2014 were recorded in July (37.7%) and August (42.4%) respectively. Results from this study including previous work (Gendall 2007) suggest that *A. bishopi* parasitoids in the Sundays River Valley are most abundant and active from May to July. However, more useful deductions could be made if sampling is done for the entire fruit season. Other factors that could potentially affect parasitoid-host population structures such as management practices (pesticide application, fruit harvesting), abiotic and biotic factors in the orchards should be considered too. Although most of these factors were not examined in this study, the sampling results show some evidence of reduced abundance of *T. leucotreta* with increased parasitism %. It should be noted that the percentage fruit infestation and parasitism reported in this study is overly estimated as only fruit showing typical symptoms of *T. leucotreta* infestation were sampled while dead parasitized larvae were not accounted for. In addition, fruit collected from the ground in which larvae had exited were not included in the calculation.

Reducing the amount of artificial diet in the rearing jars did not show any improvement in parasitism success. *Agathis bishopi* female parasitoids are known to attack younger instars (1st – 3rd) of *T. leucotreta* larvae (Sishuba 2003; Gendall 2007). Furthermore, the older the larva, the deeper it burrows into the artificial diet, making it unreachable to the female parasitoid's 4 mm long ovipositor. It was therefore hypothesised that reducing the amount of artificial diet in the rearing jars would reduce the depth burrowed by larvae and consequently increase their exposure to female parasitoids for parasitism. This adjustment did not yield any improvement in oviposition success of *A. bishopi* females. High mortality of *T. leucotreta* larvae was observed in the lowest rate (10 g) tested, probably due to the artificial diet drying out quickly causing desiccation of larvae. It was however, evident through visual observations that female parasitoids did not search or oviposit in *T. leucotreta* larvae in the rearing jars in which honey was offered to parasitoids on paper towelling. Parasitoids were seen mostly walking on the surface of diet without any clear searching behaviour or oviposition attempts observed. It was quite clear that besides artificial diet, there were other factors affecting the oviposition behaviour of parasitoids in the jars. On the basis of this finding, it was suggested that other factors such as quality, quantity and accessibility of food (honey) to parasitoids in the jars be investigated. Previous studies have shown that food availability is one of the major factors affecting oviposition behaviour in parasitoids (Lewis *et al.* 1998; Azzouz *et al.* 2004).

Changes in the carrier medium of honey and position in the rearing jars resulted in a dramatic improvement of parasitoid searching behaviour, oviposition behaviour, and offspring yield. A 30% parasitism rate and female biased sex ratio (1: 1.4, male: female) was recorded. It is suggested that the use of cotton wool as a honey carrier medium and positioning it through the lid in the rearing jars improved the reproduction success of female *A. bishopi* parasitoids for two reasons; (1) Cotton wool soaked in honey improved feeding in parasitoids as the honey remained moist for a longer period than on the paper towelling, (2) preliminary observations showed that parasitoids spent more time resting under the lid, probably due to their natural behaviour of flying up the tree in the orchards to look for food after emergence from the soil where *T. leucotreta* larvae pupate (Goble *et al.* 2010). Therefore, positioning the honey through the lid could have improved its accessibility to parasitoids. Similar studies have also reported the negative effects of food deprivation to parasitoid host searching behaviour (Azzouz *et al.* 2004). Therefore feeding is an important factor for a successful rearing programme. This finding further suggests that the efficacy of *A. bishopi* in biological

control programme for *T. leucotreta* could be improved by planting nectar producing plant in citrus orchards. Increased parasitoid longevity and parasitism rates through incorporation of non-crop flowering (nectar producing) plant in agro-ecosystems has been reported (Amaral *et al.* 2013; Géneau *et al.* 2013).

Experiments on the development duration for the *A. bishopi* parasitoid showed that female parasitoids require a longer period (30.5 days) to develop from egg to adult than the males (27.3 days). This difference in development duration between male and female *A. bishopi* is consistent with results from previous studies (Sishuba 2003; Gendall 2007), including studies on other species of *Agathis* wasps (Odebiyi & Oatman 1972). Female parasitoids emerged about 9 days after emergence of *T. leucotreta* adults. This variation in emergence between parasitoids and moths should be studied further as it presents a potential for separation of moths and parasitoids from emergence jars which is still one of the challenges with the current rearing protocol. The difference in emergence between parasitoids and moths also reflects a desirable synchronisation of *A. bishopi* with the *T. leucotreta* development cycle, as female parasitoids emerge after moths have oviposited and most of their larvae have hatched. This difference in development time is consistent with previous studies on *A. bishopi* and other *Agathis* species (Odebiyi & Oatman 1972; Sishuba 2003; Gendall 2007). It is suggested that due to the consistently larger body size of female *A. bishopi* parasitoids (Sishuba 2003; Gendall 2007), female larvae require a relatively longer duration of feeding which could have led to the observed longer development duration in female *A. bishopi*. Larger body size is known to have reproductive benefits in females due to relatively more resources needed in egg production than sperm production in male parasitoids (Harvey 2005). Results from the longevity experiment showed that male *A. bishopi* parasitoids lived longer (24.2 days) than female parasitoids (19.3 days). Gendall (2007) equally reported this trend in longevity of *A. bishopi* parasitoids. It is suggested that there is a higher reproductive cost (egg production, host searching and oviposition) in female parasitoids than male reproductive costs (sperm production, mate finding) which could have resulted in the observed longer survival in males (Jervis *et al.* 2008).

Although there was no significant difference in parasitism rates between mated naïve and experienced parasitoids, an interesting trend was observed. There was generally an increase in parasitism rate from the naïve state (21.5%) of parasitoids until the fourth level of oviposition experience (24.1%). This trend suggests that oviposition experience improves the host searching and oviposition efficiency in *A. bishopi* parasitoids and thus supports the

findings from several previous studies with parasitoids (Geervliet *et al.* 1998; Canale & Benelli 2011). Experienced parasitoids were observed attacking larvae in the jars in the absence of frass, a behaviour that was never displayed by naïve parasitoids. The seemingly higher parasitism rate observed in naïve parasitoids could be attributed to host deprivation of parasitoids as females were only exposed to larvae after 2 days, which could have led to higher egg load and the urge to oviposit (Kant *et al.* 2013). Beyond the fourth oviposition experience, parasitism rate began to decline with each subsequent level of experience. This reduction in parasitism rate could be attributed to ageing effects as previously documented for several other species of parasitoids (Honda & Kainoh 1998; Kant *et al.* 2013). Results from this experiment show that *A. bishopi* females are highly fecund between 1-10 days of age. Knowing the high reproductive range age in parasitoids is important as it aids decision making on which ages of parasitoids to use in rearing and field releases for biological control purposes.

The chemical analysis of artificial diet samples showed that more compounds were found in artificial diet infested by *T. leucotreta* larvae than uninfested diet, suggesting their larval and frass origin. In particular, the seven compounds that were found exclusively in infested artificial diet were most likely derived from the larvae or their frass (Gandolfi *et al.* 2003). Compounds that were present in both uninfested and infested diet were most likely derived from the diet itself. A possible explanation for three compounds that were only found in uninfested diet could be that the compounds were metabolised by the larvae into one of the compounds that were only present in infested diet (Gandolfi *et al.* 2003).

It is suggested that the seven compounds that were exclusively found in infested diet are potential sources of the kairomonal activity of *T. leucotreta* frass to *A. bishopi* parasitoids. However, more replications of diet samples and possibly testing the bioactivity of the compounds found exclusively in infested diet to female *A. bishopi* parasitoids would provide more depth to this finding. Chemical compounds from *T. leucotreta* larval silk or frass that are responsible for the kairomonal activity of frass could be used as oviposition stimulants to enhance parasitism success in the rearing process for *A. bishopi* (Rutledge 1996).

This study demonstrated that during the sampling periods in 2013 (April-July) and 2014 (January-July), parasitism rates were consistently higher between May and July indicating periods of high activity of *A. bishopi* parasitoids in orchards. Furthermore, *A. bishopi* parasitoids were reared successfully on *T. leucotreta* larvae. It was found that feeding,

particularly using cotton wool soaked in dilute honey improved the parasitism rate to 30% of larvae in the rearing jars with a female biased sex ratio (1: 1.4, male: female). Although not significant, oviposition experience resulted in increased oviposition success but only from 1-10 days of age beyond which parasitism rate began to decline. Further, the development duration for male and female *A. bishopi* parasitoids were 27.3 and 30.5 days respectively. In addition, the longevity of males and females in the presence of mate, food and host were found to be 24.2 and 19.3 for male and females respectively. Chemical analysis of diet showed that seven compounds were potentially of *T. leucotreta* larval or frass origin and could therefore be responsible for the apparent kairomonal activity of frass. Future research should consider improvement of artificial diet to resist fungal contamination which is currently still a challenge. Furthermore, using larger rearing containers including developing a system for separating parasitoids and moths after emergence could help minimise parasitoid handling and general rearing costs.

CHAPTER 3: Olfactory response of *Agathis bishopi* adult females to *Thaumatotibia leucotreta* induced fruit volatiles

3.1 Introduction

Resource searching in parasitoids involves exploitation of a wide range of stimuli such as visual, chemical and vibratory cues (Beneli *et al.* 2013). Olfaction is however the main modality used by parasitoids in host location (Thompson 1999). For example, in braconid parasitoids of several lepidopteran moths, voluminous literature exists that highlights the importance of olfaction in host location (see, for example, Mattiacci *et al.* 1999; Rains *et al.* 2003; Ghimire & Phillips 2010; Obonyo *et al.* 2010; Belda & Riudavets 2012). Many studies have demonstrated that plant feeding by herbivorous insects induces production of volatiles that consequently attract parasitoids (Hare 2011; Kaplan 2012; Benelli *et al.* 2013). These volatiles are a combination of cues from the host plant and the herbivore and/or its products (frass, regurgitate or silk) (Hare 2011; Kessler *et al.* 2001). Furthermore, many plant are known to have evolved induced resistance in which herbivore feeding activates the synthesis of HIPVs (Herbivore Induced Plant Volatiles) that aid parasitoids to locate their specific hosts (Gols *et al.* 2011; Hare 2011).

As previously mentioned (Chapter 1), *Thaumatotibia leucotreta* is a phytosanitary pest with zero tolerance by many of South Africa's major export markets. This potentially high economic impact posed by the phytosanitary status of *T. leucotreta* has in the recent past stimulated considerable research, particularly aimed at developing effective control and preventive technologies such as; identification of resistant citrus cultivars (Love *et al.* 2014), improved pre-harvest (Li & Bouwer 2012; Coombes *et al.* 2013) and postharvest (Johnson & Neven 2010; Boardman *et al.* 2012) control options for *T. leucotreta* on citrus. However, very little work has been done to improve the postharvest detection of *T. leucotreta* infested fruit, particularly in the packhouse where fruit is finally processed and packaged for export. The only detection option available to date is by visual examination of fruit for external symptoms (discoloration, frass) of damage caused by *T. leucotreta*. However, the efficacy of this method is quite low ~40% (unpublished work), particularly for early infestation in which first instar larvae are as small as 1-1.3 mm in body length (Newton 1998). Therefore, development of a reliable technique for detection of infested fruit in the packhouse would be beneficial.

Van der Walt (2012) reported that infestation of orange fruit by *T. leucotreta* elicits the production of volatile compounds which are quantitatively and qualitatively different from those produced in healthy fruit. Further, several larval parasitoids attacking citrus fruit fly larvae (*Anastrepha* spp., *Bactrocera* spp., *Ceratitis* ssp.) such as; *D. longicaudata*, *Doryctobracon areolatus* (Szepligeti) (Hymenoptera: Braconidae), *P. concolor* (Szepligeti), *Utetes anastrephae* (Viereck) (Hymenoptera: Braconidae) are known to locate infested fruit by responding to the volatile compounds produced by their hosts while feeding on fruit (Eben *et al.* 2000; Benelli *et al.* 2013; Dias *et al.* 2014). This ability of parasitoids to locate infested fruit as they search for their hosts provides an opportunity for developing a detection system for *T. leucotreta* infested fruit. However, nothing is known about the natural behavioural response of adult female *A. bishopi* to volatiles associated with *T. leucotreta* infested fruit.

The hypothesis tested here was that volatile chemical profiles or specific compounds within the profile produced by *T. leucotreta* infested fruit play a crucial role in attracting the parasitoid to infested fruit. Therefore, the main objective of this study was to examine what volatiles emitted by *T. leucotreta* infested fruit are attractive to female *A. bishopi* parasitoids. Specifically we evaluated: (1) the attractiveness of volatile compounds (olfactory cue) from *T. leucotreta* infested fruit, (2) the attractiveness of authentic standard compounds (individual and blended), and (3) the attractiveness of combined cues (olfactory and visual) from *T. leucotreta* infested fruit.

3.1 Materials and Methods

3.1.1 Insects

A culture of *A. bishopi* parasitoids was established and maintained at Rhodes University (Waainek Research Laboratory). The parasitoids were reared on their natural host *T. leucotreta* using a method previously described by Gendall (2007) with the improvements and changes outlined in chapter two. After eclosion and before the bioassays, male and female parasitoids were kept in different glass jars (250 ml). To produce mated females, male and female parasitoids (sex ratio 1:2 male: female) were kept in the same jar for at least 24 hours before the bioassay. A small piece of cotton wool soaked in water was placed in the jar as a water source for the parasitoids. Food was provided by soaking a piece of cotton wool in dilute honey and placing it through a hole on the metal lid of the jar. The water and dilute honey was replenished daily. The glass jars containing parasitoids were kept in the rearing

room ($25^{\circ}\text{C} \pm 2$, $50\% \text{ RH} \pm 10$ and 12:12 (Light: Dark) photoperiod. All bioassays were carried out using 2-5 day old female parasitoids.

Thaumatotibia leucotreta larvae used for fruit infestation were obtained from the established colony maintained at Rhodes University (Waainek Research Laboratory). The larvae were reared on artificial diet using a method described by Moore *et al* (2014), as explained in chapter two. Until the larvae (1st instar) were used for fruit infestation, the moths were kept under the following conditions; $25^{\circ}\text{C} \pm 2$, $30\% \text{ RH} \pm 10$ and 12:12 (Light: Dark) photoperiod.

3.1.2 Volatile sources

3.1.2.1 Orange fruit *Citrus sinensis* L, *Rutaceae*; cv *Navel*

Because *A. bishopi* females attack the early instars (1st & 2nd) of *T. leucotreta* (Gendall 2007), volatiles from infested fruit were tested in the bioassays. Orange fruit of fairly uniform size were collected from farms in Sundays River Valley (South Africa, Eastern Cape Province) and stored in a cold room at 4°C until they were used in the trials. Before infestation with *T. leucotreta* larvae, fruits were removed from the cold room and kept at room temperature for 24 hours. In order to obtain uniformly infested fruit, ten newly emerged 1st instar *T. leucotreta* larvae were placed individually on the fruit rind. Both infested and healthy fruit were kept in the rearing room ($25^{\circ}\text{C} \pm 5$, $30\% \text{ RH} \pm 10$ and 12:12 (Light: Dark) photoperiod) for 3-4 days before their use in bioassays. Based on preliminary observations under the above conditions, 1st instar *T. leucotreta* larvae placed on fruit reached 2nd instar between 3-4 days after inoculation.

3.1.2.2 Individual authentic standard compounds

Chemical standards for four major volatile compounds released by *T. leucotreta* infested fruit as previously identified in the fruit headspace by van der Walt (2012) were each used in the Y-tube olfactometer bioassays. Individual standard compound treatments were prepared by diluting each pure chemical standard in a volume of hexane, culminating to concentrations equal to average concentrations of each compound detected in naturally infested fruit (cv Late Valencia) (Table 3.1). The following concentrations were prepared for each of the chemical standards; D-Limonene ($15.0 \mu\text{g/ml}$), 1,3, 6 Octatriene 3,7 Dimethyl or Ocimene ($8.9 \mu\text{g/ml}$), Caryophyllene ($0.3 \mu\text{g/ml}$) and Naphthalene ($3.3 \mu\text{g/ml}$). The prepared chemical solutions were stored in small air tight glass vials (5 ml) and kept in a freezer (-4°C) until their use. About 24 hours before the Y-tube olfactometer bioassay, the chemical compound

solutions were removed from the freezer and kept at room temperature. Prior to the bioassay, 2 ml of chemical solution was applied to a filter paper (Qual, 150 mm diameter, grade 3 HW, Munktell, Sweden) and placed on a glass petri dish (8 cm diameter) under a fume hood for 1 minute to ensure complete evaporation of the solvent. Filter paper treated with the chemicals solution (treatment) and solvent treated filter paper (control) was immediately placed in the odour containers before running the Y-tube bioassays.

3.1.2.3 Infested fruit volatile simulation

The volatile profile of infested fruit was simulated by blending the four chemical standard compounds in ratios corresponding to concentrations of individual compounds as detected in *T. leucotreta* infested fruit in a previous study (van der Walt 2012) (Table 3.1). The four component chemical blend was obtained by initially preparing individual chemical solutions as described in the previous section and later mixing the four solutions. The blended chemical solution was stored in glass vials (10 ml) and placed in a freezer (-4°C) until the bioassay was conducted. Filter paper used as an odour carrier was treated using the method described above.

3.1.3 Y-tube olfactometer setup

The attraction of *A. bishopi* to volatiles from fruit infested by *T. leucotreta* was examined using a glass Y-tube bioassay (Fig. 3.1) adapted from the design used by Yu *et al.* (2010). The olfactometer used was a Y-shaped tube with cylindrical arms (inner diameter: 250 mm and length of arm: 150 mm). The angles between the two upper arms and between the upper arm and base arm were 75° and 140° respectively. The upper arms of the olfactometer were each connected to an air pump which pumped the air through the perimeter of the system. First the air was pumped through the plastic tubing (diameter: 10 mm), purified by passing through two conical flasks with activated charcoal and later moistened by bubbling through conical flasks filled with distilled water (500 ml). After being moistened, tubing was connected to two flow meters that regulated the air flow at 250 ml/min. This metered flow of air was then pumped through two plastic jars (1000 ml) with an odour source (either treatment or control) and finally into the two upper arms of the olfactometer. A square cage with each side measuring 600 mm made of white Perspex material and clear glass top was used to isolate the Y-tube olfactometer from the external stimuli.

Table 3.1 Concentration ($\mu\text{g/ml}$) of volatile compounds identified in *Thaumatotibia leucotreta* infested oranges (cv; Late Valencia) using SPME-GC technique (van der Walt 2012). Text and numbers in bold indicate the four major volatile compounds that were used in bioassays with *Agathis bishopi* females

Compound	Control (healthy fruit)	Infested fruit	Concentration of compound used in Y-tube bioassay ($\mu\text{g/ml}$)
Acetaldehyde	0-0.01	0.06-0.13	
2- propanol	0.01-.25	0.06-0.13	
2-ethyl-1-hexanol	0-0.01	0-0.01	
D-limonene	0.54-2.83	1.41-28.61+	15.01
1,3,6 Octatriene 3,7 dimethyl (Z)	0.24-3.26	1.22-16.56	8.89
1,6 Octadiene-3-ol 3,7 dimethyl	0-0.01	0.01-0.06	
Hexanoic acid	0.01-0.06	0.01-0.06	
(E)- 4,8 Dimethyl 1,3,7 nonatriene	0.01-0.06	0.01-0.25	
1-Undecanol	0.01-0.06	0-0.01	
Hexyl butanoate	0-0.01	0.01-0.5	
Octanoic acid	0-0.01	0-0.13	
Dodecane	0-0.01	0-0.01	
2,6 Dihydroxyacetophenone bis (trimethylsilyl) ether	0-0.01	0.01-0.06	
Caryophyllene	0.01-0.06	0.06-0.5	0.28
Alloaromadendrene	0.01-0.06	0.06-0.25	
Humulene	0-0.01	0.01-0.06	
alpha.-Panasinsen	0.01-0.06	0.01-0.06	
Naphthalene	0.123-0.785	0.266-6.30	3.28
2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	0.25-0.5	0.25-0.5	
Propanoic acid	0.01-0.06	0.13-0.25	

Two holes (110 mm apart) were made in the middle of the glass top to facilitate connection of tubing to the upper arms. The upper arms were covered with a fabric (0.2 mm mesh) to prevent parasitoids from entering the tubes. The Y-tube olfactometer, conical flasks, plastic jars and plastic tubing were washed with detergent solution, rinsed with distilled water and 70% ethanol before being dried in an oven at 60°C for 24 hours after every five replications. During assembly, all the components were connected by tight fitting joints to ensure an air tight system. Absence of air leaks and uniformity of air flow into two arms of the olfactometer was confirmed by carrying out a ‘smoke test’. After setting up the system,

ammonia fumes (produced by adding HCl to NH₃OH in the two conical flasks) were pumped into the two arms of the Y-tube and fume flow monitored. Prior to testing the insects, the odour sources (treatment or control) were placed into the odour jars and the Y-tube was allowed to run for about 10 min to stabilise the air flow.



Figure 3.1 Olfactometer setup. A=Air pump; B=Activated charcoal filter; C=distilled water; D=Flow meter; E=Odour source container; F=Perspex cage housing the Y-tube; G=Base arm of Y-tube; H=Starting line; I=Y-tube olfactometer; J=Choice line; K=Upper arm of Y-tube; L=Air flow tubes.

3.1.3.1 Olfactometer trials

Preliminary tests with parasitoids in a horizontally oriented Y-tube bioassay yielded a poor response as parasitoids mostly circled around the base arm of the olfactometer and did not move towards the upper arms. Based on this observation and previous similar studies (Steidle & Scho 2002; Desouhant *et al.* 2005; Belda & Riudavets 2012), a vertical orientation of the Y-tube was used in this study and resulted in a marked improvement in parasitoid response.

The following combinations of treatments were tested with female *A. bishopi* parasitoids in the Y-tube bioassay; infested fruit vs healthy fruit; each of the four chemical standard vs blank-control; infested fruit volatile simulation vs blank-control. In total, seven treatments

were tested. During each trial, a mated female parasitoid was introduced into the base arm and allowed to walk up towards the two arms. Each parasitoid was observed and scored as having made a choice if it crossed the choice line (drawn at 50 mm from the arm junction) on either arm. The choice of the arm and time elapsed after crossing the choice line were noted and recorded. Parasitoids that did not respond within 15 min were recorded as ‘no choice’. After each trial, the parasitoid was removed from the Y-tube olfactometer using a mouth aspirator and each insect was used only once. After every five trials, the treatment arms and tubes were switched to prevent positional effects. Twenty five female parasitoids were tested for each treatment. In order to avoid circadian variation in parasitoid behaviour, all experiments were carried out between 08h00 and 17h00. The bioassays were conducted in a laboratory ($22^{\circ}\text{C} \pm 3$, 40-50% RH) and the laboratory was illuminated by two fluorescent tubes with a light intensity of 5 lux.

3.1.4 Flight tunnel setup

The preference of *A. bishopi* females for *T. leucotreta* infested or healthy fruit was tested using a flight tunnel design (Fig. 3.2) adapted from (Segura *et al.* 2012). The flight tunnel (length 50 cm, width 15 cm and height 15 cm) was made of white poly (methyl methacrylate) sides with a clear top. The two distal ends of the flight tunnel each had a circular hole (diameter 10 cm) covered with white fabric to facilitate natural air flow and prevent build-up of fruit volatiles within the arena. In this bioassay design, the female wasps were simultaneously exposed to a combination of cues (olfactory, visual and contact). For each choice test, a pair of options (infested fruit vs healthy fruit) placed at opposite ends of the arena were offered to the parasitoids. Evaluation of the parasitoid’s preference was done by dividing the flight tunnel into three regions; middle region-considered as no-choice region (middle 20 cm) and two opposite distal regions considered as choice regions (15 cm each).

3.1.4.1 Flight tunnel trials

Similar to the Y-tube olfactometer trials, both naive and experienced mated parasitoids were tested. Only one treatment (infested fruit vs healthy fruit-control) was used with twenty five female parasitoids tested in each category (naïve or experienced) and each parasitoid was used only once. For each test, an individual female wasp was placed in a plastic vial and gently lowered and placed at the centre on the base of the arena. The parasitoid was allowed to walk out of the vial and fly around the arena until it settled on one of the two regions within 5 min, and was scored as a choice for a treatment in the chosen region. Parasitoids that did not make a choice (i.e. settled on the no-choice region) were excluded from the data.

After testing five wasps, the fabric on the flight tunnel was washed with detergent in warm water and rinsed in distilled water

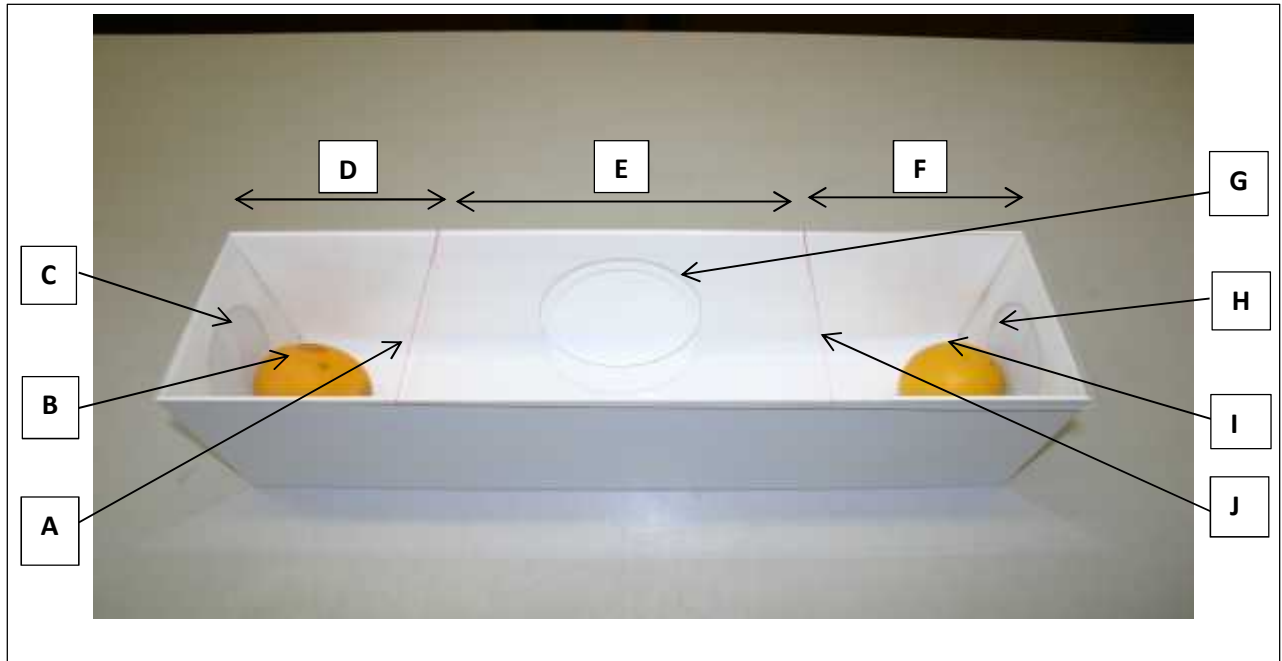


Figure 3.2 Flight tunnel setup. A and J=Lines dividing three choice regions; B and I=Healthy and infested fruit as cue sources; C and H=Circular holes covered with fabric; D and F = Choice regions; E = No choice region; G = Entrance hole

. In addition, the flight tunnel was washed with detergent in warm water, rinsed with distilled water followed by 90% ethanol. Further, the position of the flight tunnel was switched (rotated 90°) after every five trials to minimise positional effects on parasitoid choice. All experiments were carried out between 08h00 and 17h00 due to the diurnal nature of parasitoids. The bioassays were conducted in a laboratory (22°C ± 3, 40-50% RH) and the laboratory was illuminated by two fluorescent tubes with a light intensity of 5 lux.

3.1.5 Statistical analyses

R-statistical package version 3.0.2 was used for analysis of both Y-tube olfactometer and flight tunnel data. Response of *A. bishopi* to selected odour sources versus controls was analysed using chi-square tests (50:50 distribution). The analysis was based on the assumption that the number of parasitoids responding to either the odour source or control is

equal to 50%. Thus the analysis evaluated whether the cumulative responses of parasitoids in each treatment pair differed significantly from the 50%: 50% distribution. In both bioassays (Y-tube and flight tunnel), probability levels of; $P < 0.05$, $P < 0.01$ and $P < 0.001$ were used to show the level of significance in parasitoid preference for odour source. Only parasitoids that made a choice were recorded and used in this analysis.

3.2 Results

3.2.1 Attractiveness of *Thaumatotibia leucotreta* infested fruit to mated naïve and experienced female *Agathis bishopi* parasitoids in a Y-tube olfactometer (olfactory cue)

The attraction of mated naïve and experienced parasitoids to *T. leucotreta* infested fruit and healthy fruit volatiles in a Y-tube olfactometer are shown below (Fig. 3.3 A and B). Relatively more naïve parasitoids (68%) were attracted to *T. leucotreta* infested fruit as compared to healthy fruit (32%) (Fig. 3.3 A). However, there were no statistical differences in the preference of naïve parasitoids between the two odour sources offered ($X^2 = 3.24$, d.f. = 1, $P > 0.05$). For experienced *A. bishopi* females, highly significant differences were observed in the parasitoids' choice for odour sources ($X^2 = 17.64$, d.f. = 1, $P < 0.001$). Only 8% of parasitoids preferred healthy fruit while 92% chose *T. leucotreta* infested fruit (Fig. 3.3 B).

3.2.2 Attractiveness of four individual authentic standard compounds to *Agathis bishopi*

3.2.2.1 D-Limonene

The olfactory response of mated naïve and experienced *A. bishopi* to d-limonene versus hexane (control) in a Y-tube olfactometer is shown below (Fig. 3.4 A and B). Besides mated naïve female parasitoids being more attracted to d-limonene (64%) than the control (36%), there was no significant difference in preferences between the two options ($X^2 = 1.96$, d.f. = 1, $P > 0.05$) (Fig. 3.4 A). Conversely, highly significant differences in preferences between the two options were observed for mated experienced parasitoids ($X^2 = 6.76$, d.f. = 1, $P < 0.01$) (Fig. 3.4 B). Only 8% of parasitoids were attracted to the control while 92% preferred d-limonene.

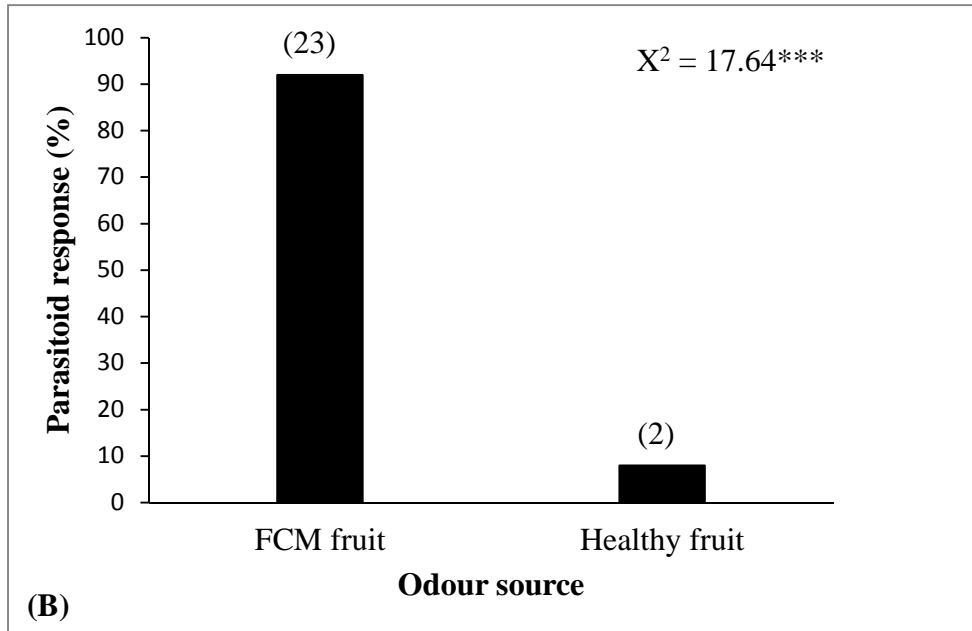
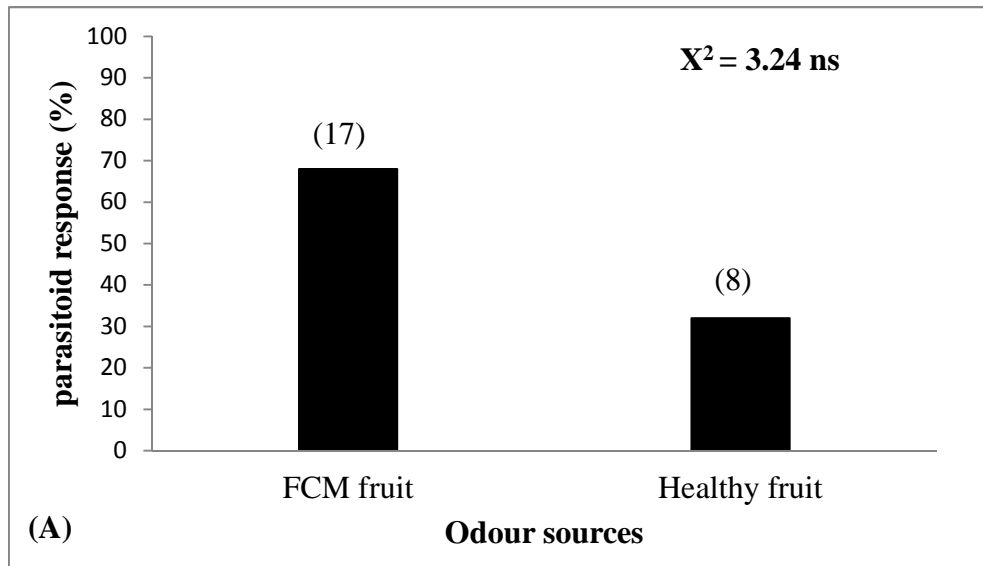


Figure 3.3 Attraction of naïve (A) and experienced (B) mated female *Agathis bishopi* to odour from *Thaumatotibia leucotreta* infested fruit versus healthy fruit in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to odour in each arm per treatment was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for odour per treatment were analysed using the X^2 statistical test. (* denotes $P <$

0.05, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *Thaumatotibia leucotreta*.)

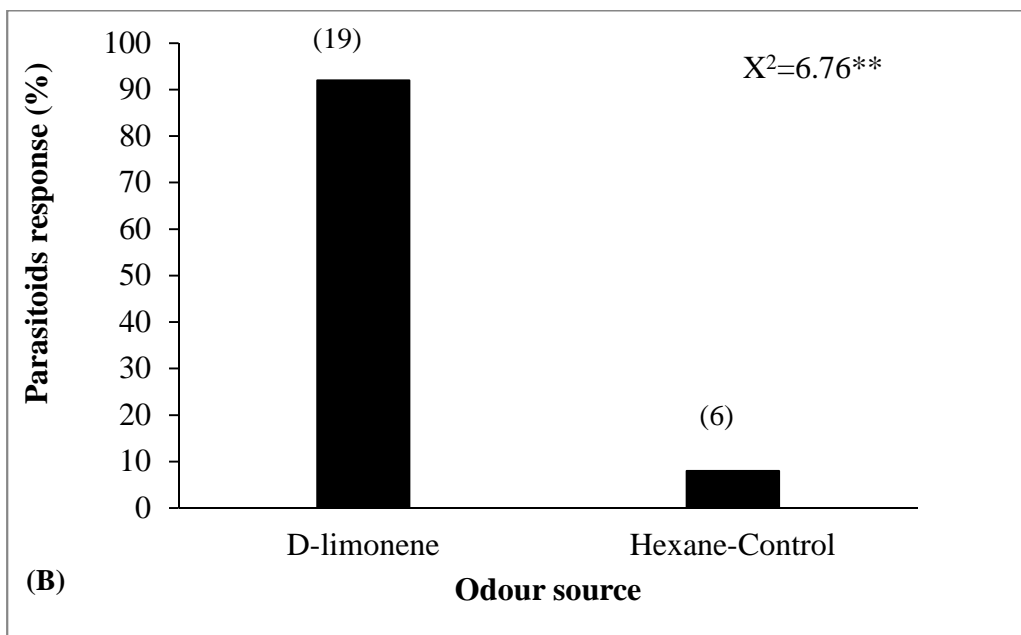
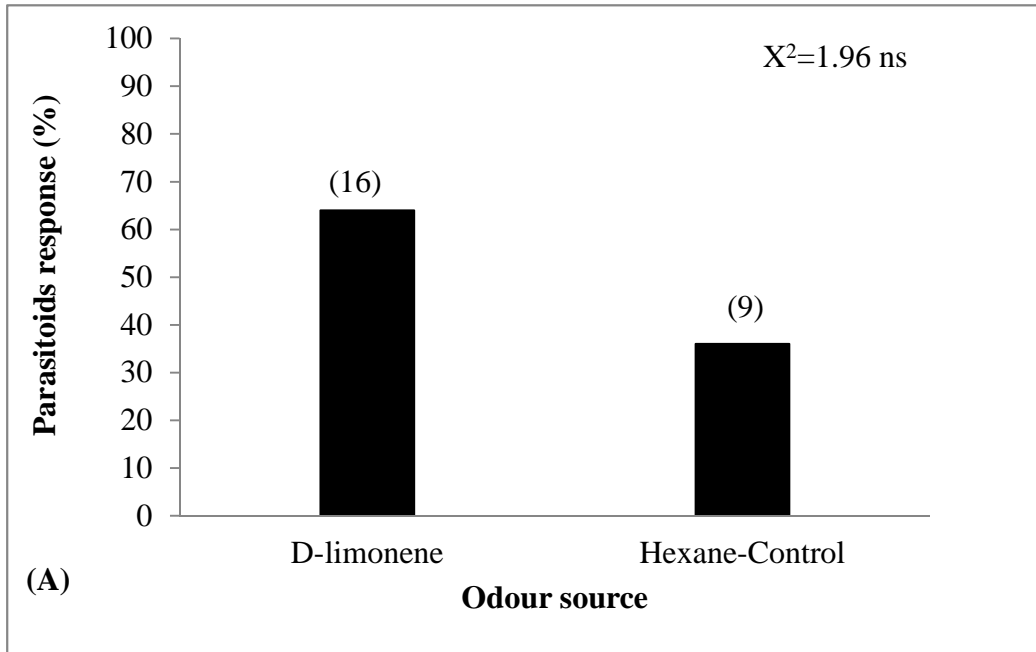


Figure 3.4 Preference of mated naïve (A) and experienced (B) female *Agathis bishopi* to d-limonene versus control in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to the treatment (d-limonene versus control) was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant, $P > 0.05$; FCM fruit – fruit infested by *Thaumatotibia leucotreta*

3.2.2.2 Ocimene

The response of mated naïve and experienced female *A. bishopi* to ocimene versus hexane (control) in a Y- tube bioassay is shown below (Fig 3.5 A and B). There was no statistical difference in the olfactory response of mated naïve parasitoids between ocimene and the control ($X^2 = 0.04$, d.f. = 1, $P > 0.05$) (Fig. 3.5 A). Preferences for ocimene and the control for mated naïve parasitoids were 52% and 48% respectively. For the mated experienced parasitoids, significant difference in olfactory response between ocimene and the control were recorded ($X^2 = 4.84$, d.f. = 1, $P < 0.05$) (Fig. 3.5 B). Only 28% of parasitoids were attracted to the control while 72% preferred ocimene.

3.2.2.3 Caryophyllene

Statistically, more mated naïve parasitoids were attracted to caryophyllene than the control ($X^2 = 4.84$, d.f. = 1, $P < 0.05$) (Fig. 3.6 A) and their response to caryophyllene and the control were 72% and 28% respectively. For the mated experienced *A. bishopi* females, more parasitoids were attracted to the control (64%) than caryophyllene (36%) (Fig. 3.6 B). However, this was not statistically different ($X^2 = 1.96$, d.f. = 1, $P > 0.05$).

3.2.2.4 Naphthalene

Significant differences were recorded in the preference of mated naïve parasitoids between naphthalene and the control ($X^2 = 6.76$, d.f. = 1, $P < 0.05$) (Fig. 3.7 A). More parasitoids (76%) were attracted to the control while only 24% were attracted to naphthalene. Similarly, more mated experienced parasitoids were attracted to the control (68%) than naphthalene (32%). Despite the apparent repulsion from naphthalene, no statistical differences in the parasitoids' choices between the two options were observed ($X^2 = 3.24$, d.f. = 1, $P > 0.05$) (Fig. 3.7 B).

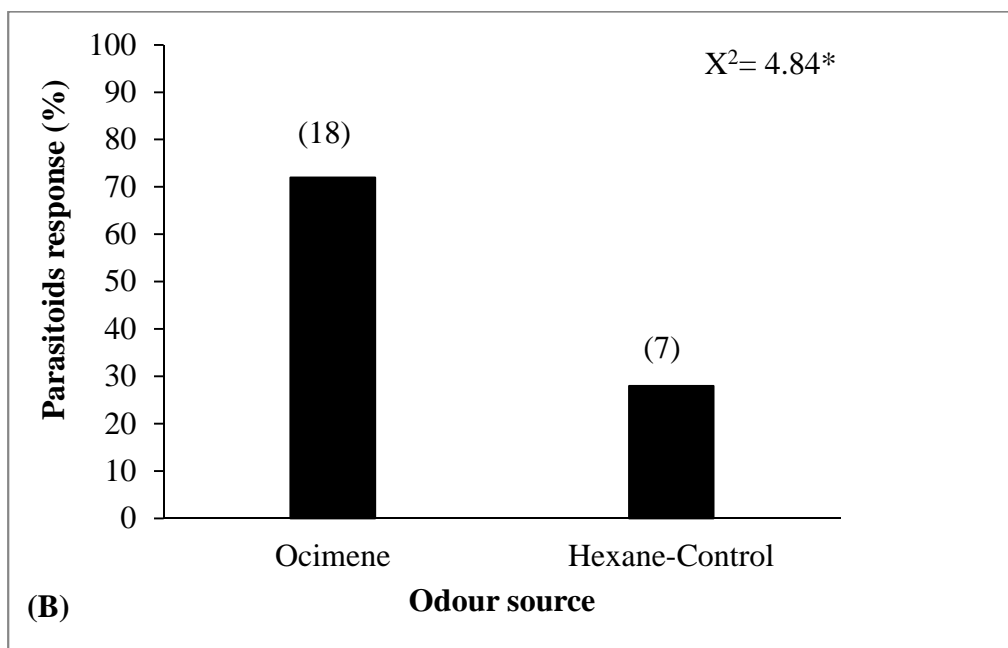
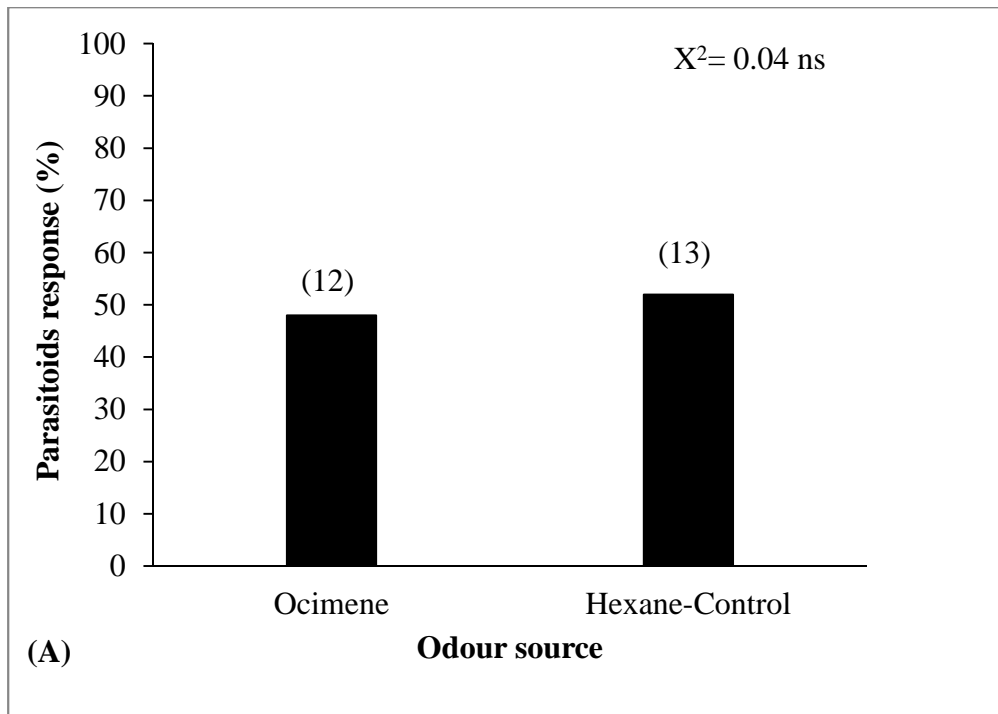


Figure 3.5 Preference of mated naïve (A) and experienced (B) female *Agathis bishopi* to ocimene versus control in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to the treatment (ocimene versus control) was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *Thaumatotibia leucotreta*.)

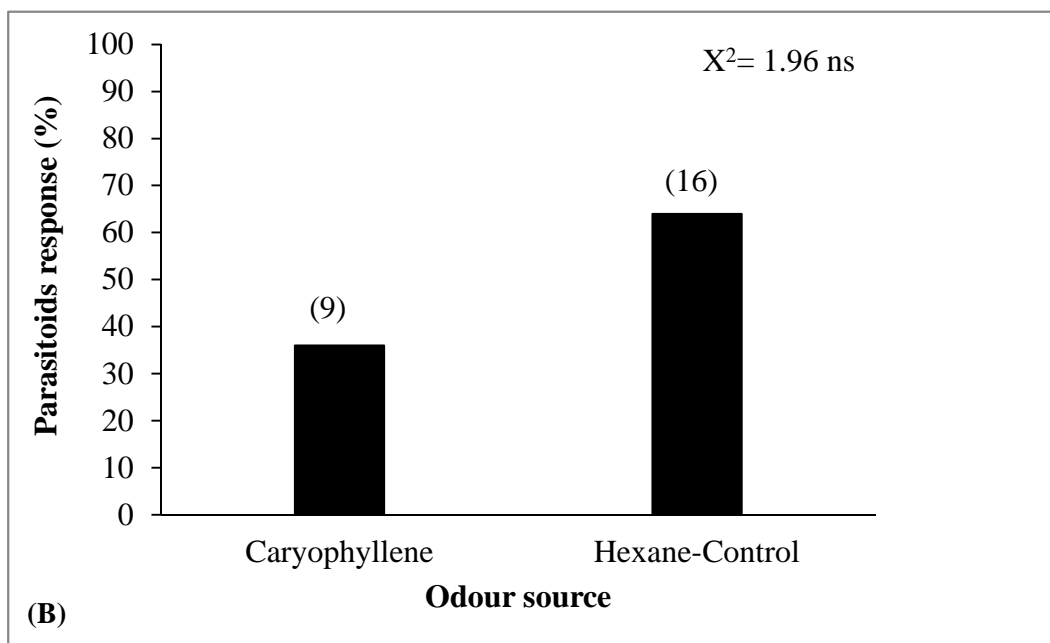
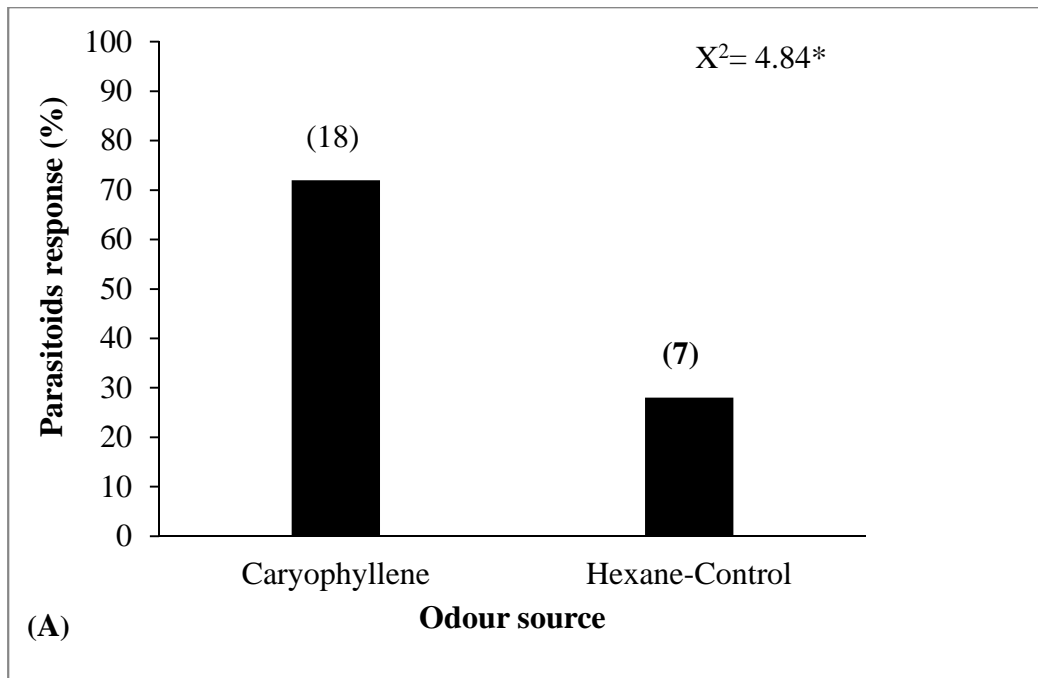


Figure 3.6 Preference of mated naïve (A) and experienced (B) female *A. bishopi* to caryophyllene versus control in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to the treatment (caryophyllene versus control) was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *T. leucotreta*.)

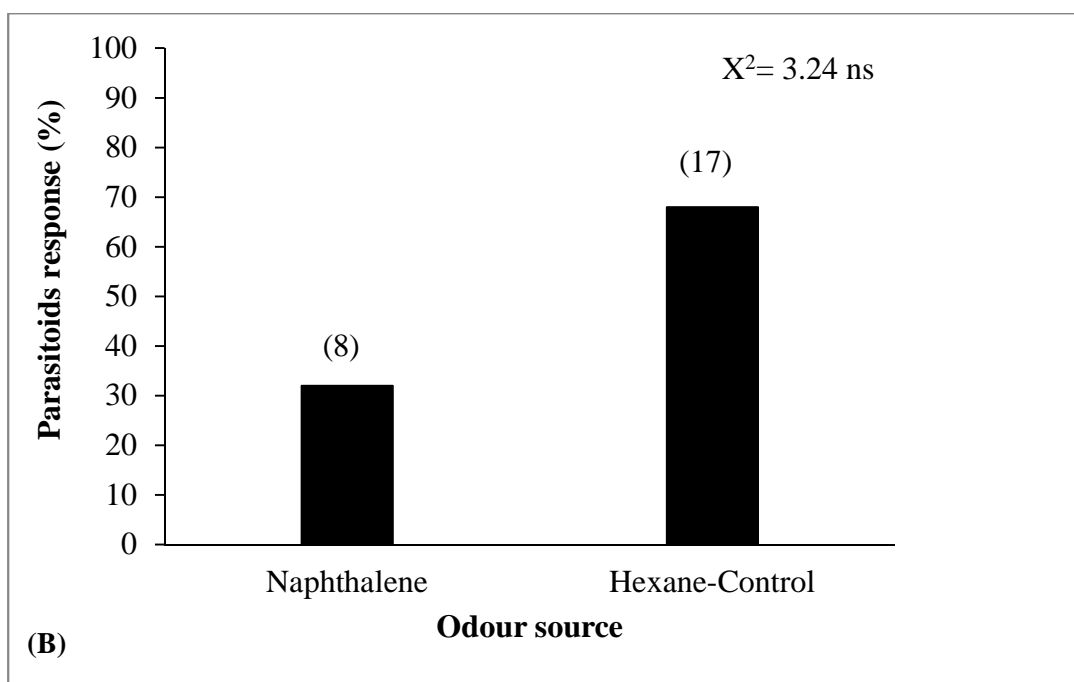
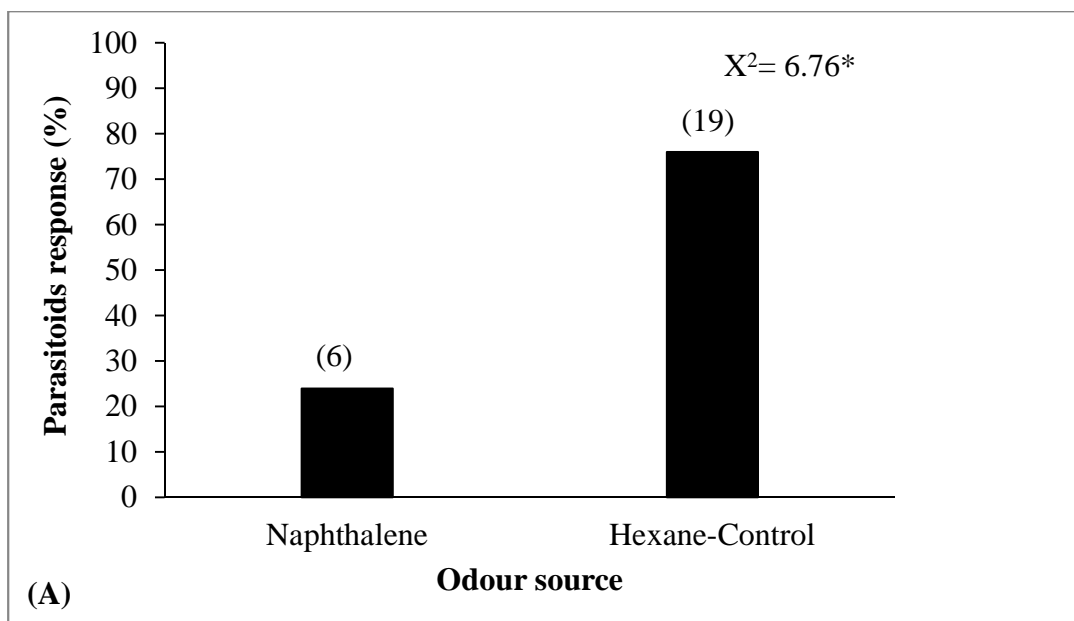


Figure 3.7 Preference of mated naïve (A) and experienced (B) female *A. bishopi* to naphthalene versus control in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to the treatment (naphthalene versus control) was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *T. leucotreta*.)

3.2.2.5 Four compound blend

Although more parasitoids were attracted to the four-compound blend than the control, there were no significant differences noted in the preference of mated naïve parasitoids between the options offered ($X^2 = 0.36$, d.f. =1, $P > 0.05$) (Fig. 3.8 A). Percentage response for the four-compound blend and control were 56% and 44% respectively. Conversely, highly significant differences were observed in the response of mated experienced parasitoids between the two options ($X^2 = 11.56$, d.f. =1, $P < 0.001$) (Fig. 3.8 B). More female parasitoids were attracted to the 4-compound blend (84%) than the control (16%).

3.2.3 Attractiveness of *Thaumatotibia leucotreta* infested fruit to mated naïve and experienced female *Agathis bishopi* in a flight tunnel

When all cues were offered simultaneously, mated naïve parasitoids were more attracted to infested fruit (52%) than healthy fruit (48%) (Fig. 3.9 A). This difference was however not statistically significant ($X^2 = 0.04$, d.f. = 1, $P > 0.842$). For mated experienced parasitoids (Fig. 3.9 B), a highly significant difference in preferences between the two options was measured ($X^2 = 21.16$, d.f. = 1, $P < 0.001$). Only 4% of parasitoids were attracted to the healthy fruit while 96% preferred infested fruit. Further all mated experienced parasitoids that alighted on infested fruit located *T. leucotreta* frass filled holes and attempted to oviposit.

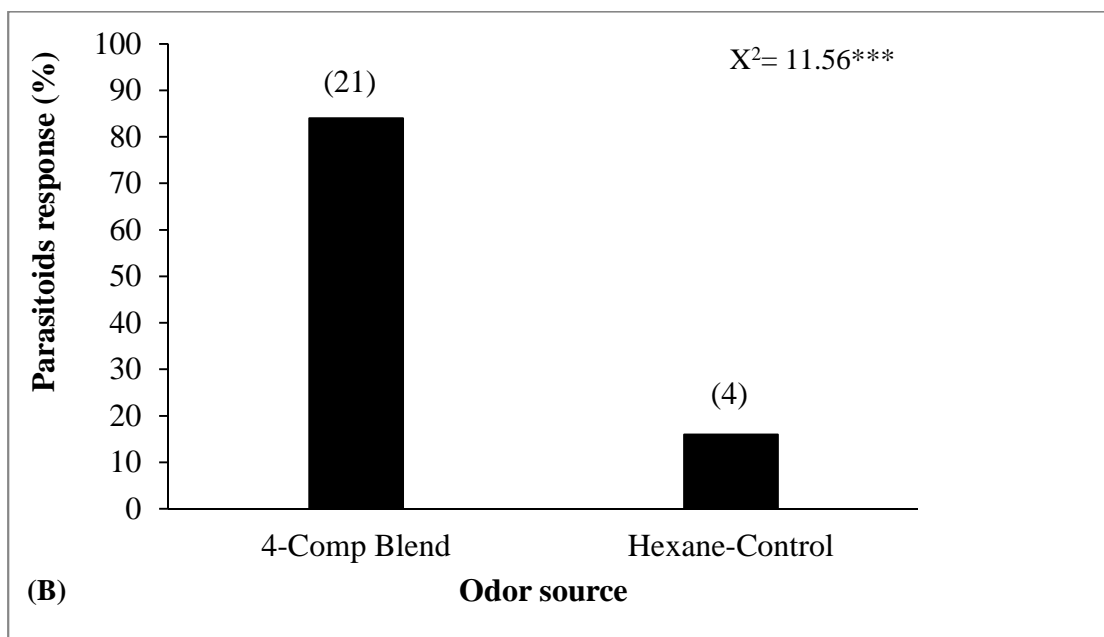
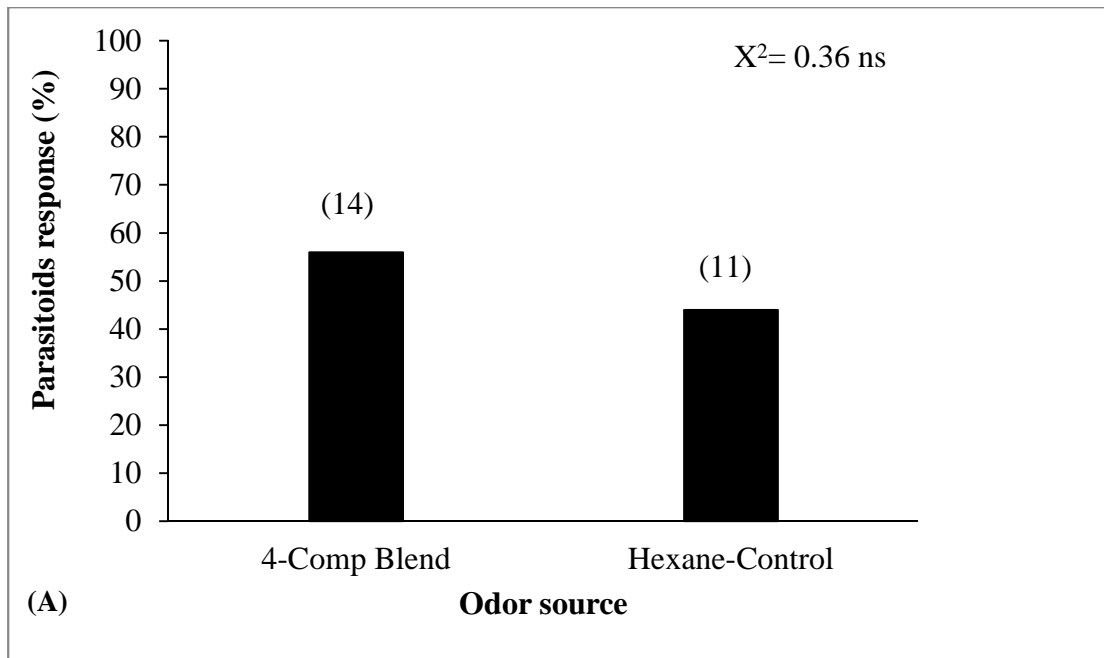


Figure 3.8 Preference of mated naïve (A) and experienced (B) female *Agathis bishopi* to 4-compound blend versus control in a Y-tube olfactometer. Percentage response of parasitoids (25 females) to the treatment (four-compound blend versus control) was recorded over a bioassay duration of 10 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *Thaumatotibia leucotreta*.)

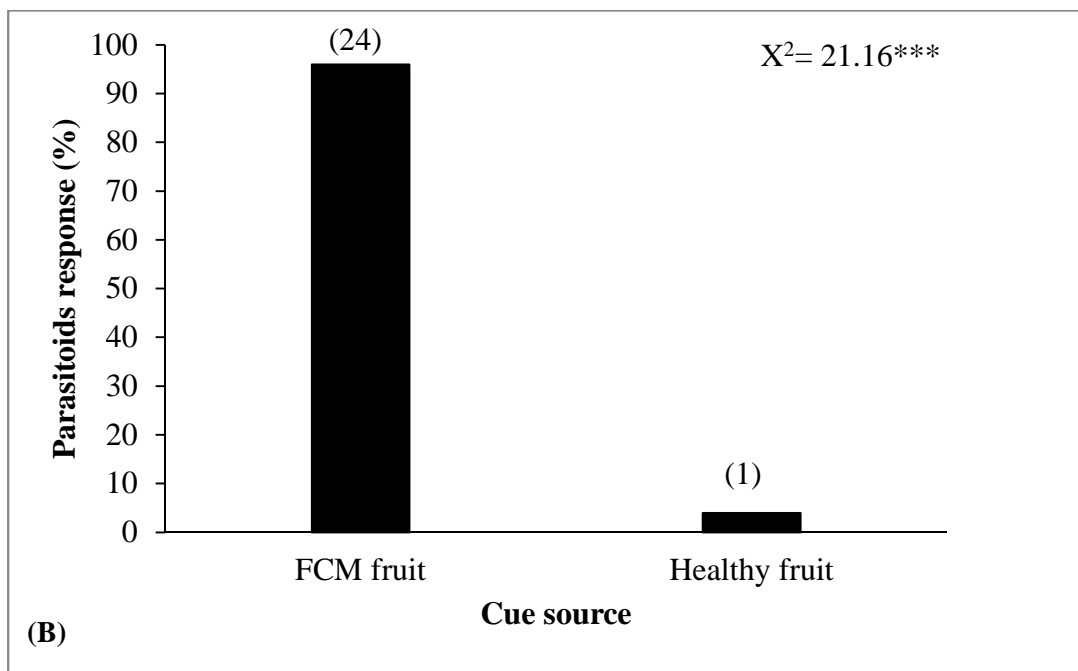
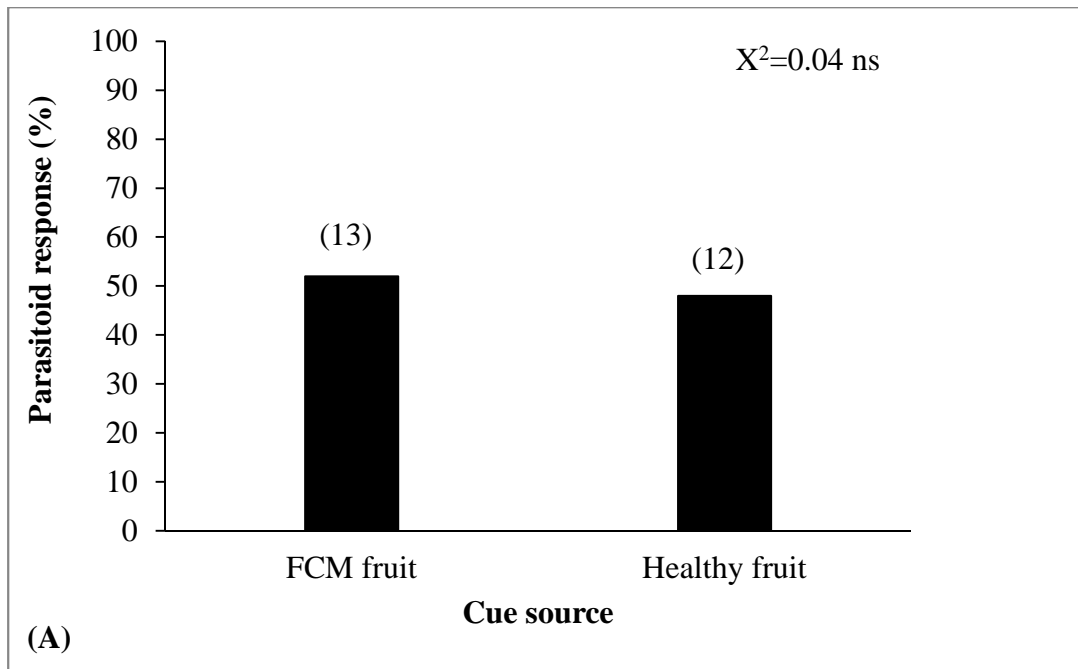


Figure 3.9 Preference of naïve (A) and experienced (B) mated female *Agathis bishopi* to *Thaumatotibia leucotreta* infested fruit versus healthy fruit in a flight tunnel. Percentage response of parasitoids (25 females) to the treatment (FCM fruit versus healthy fruit) was recorded over a bioassay duration of 5 min. Differences in the parasitoids' preferences for the options offered were analysed using X^2 statistical test. (* denotes $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and n.s non-significant $P > 0.05$; FCM fruit- fruit infested by *Thaumatotibia leucotreta*).

3.3 Discussion

Previous evidence that experienced as opposed to naïve parasitoids show a stronger and more specific response to their hosts' cues supports the findings in this study (Mattiacci *et al.* 1999; Gandolfi *et al.* 2003; Canale & Benelli 2011). Between the two groups (mated naïve and mated experienced) of parasitoids tested in all bioassays, experienced *A. bishopi* females showed a relatively much stronger attraction to *T. leucotreta* volatiles when presented either from infested fruit, individual synthetic compounds (except in the case of caryophyllene and naphthalene) or compound blend. This stronger response in parasitoids was consistent in both bioassays. This different response measured suggests that associative learning plays a key role in the host location process of *A. bishopi* females. It is possible that after a successful oviposition, *A. bishopi* females learn to associate cues from *T. leucotreta* larvae (frass and regurgitate) with fruit volatiles, which then improves their search efficiency in subsequent encounters. This result further suggests that naïve *A. bishopi* require a pre-host cue learning period before they can successfully locate their host. This behaviour in *A. bishopi* conforms to previous studies with braconid wasps. For example, a mated experienced female *M. croceipes* parasitoid exhibited a stronger attraction to its lepidopteran host larva, *H. zea*, compared to naïve wasps (Tomberlin *et al.* 2008), and *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) a parasitic wasp for a lepidopteran caterpillar, *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae), showed an improved response to cues from its host after a successful oviposition experience (Costa *et al.* 2010).

The Y-tube bioassays indicated that olfactory cues from *T. leucotreta* infested fruit elicited a stronger attraction to *A. bishopi* females than healthy fruit. In this bioassay, the female wasps were only exposed to olfactory cues. It is therefore presumed that the presence of the susceptible larval instars (1st or 2nd) of *T. leucotreta* in fruit (infested fruit), including their products (frass, silk, regurgitate) on or within the fruit play a crucial role in the host location process for *A. bishopi* females. This observation is consistent with results from several previous studies that provide evidence on the role of host olfactory cues in host location for most larval parasitoids of concealed lepidopteran larvae (Mattiacci *et al.* 1999; Ero *et al.* 2011; Kaplan 2012; McCormick *et al.* 2012; Graziosi & Rieseke 2013). For example, *Hyssopus pallidus* (Askew) (Hymenoptera: Eulophidae), a larval parasitoid of codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), locates its host using olfactory cues from host infested apple fruit (Mattiacci *et al.* 1999). *Psytalia concolor*, an endoparasitoid of *Ceratitis capitata*, locates its host by responding to its host induced volatiles (Beneli *et al.* 2013).

Further, a braconid wasp, *M. croceipes*, uses odour stimuli to locate its host, *Heliothis virescens* F. (Lepidoptera: Noctuidae) (Ngumbi *et al.* 2010).

In the flight tunnel bioassays, *A. bishopi* females were simultaneously exposed to a combination of host and fruit cues (visual, olfactory, contact). As observed in the Y-tube bioassays, *A. bishopi* females showed a strong attraction to *T. leucotreta* infested fruit over healthy fruit. This result suggests that *A. bishopi* females mainly rely on olfactory stimuli from the host-fruit complex as a long range host location cue as noted in other similar studies (Sullivan *et al.* 2000; Belda & Riudavets 2010; Gols *et al.* 2011). It was further noted that all the female parasitoids that flew and alighted on infested fruit in the flight tunnel probed their ovipositor into the frass holes on fruit. Conversely, *A. bishopi* did not show any oviposition attempts on healthy fruit in the flight tunnel. Thus in addition to long range cues (olfactory cue), *A. bishopi* females possibly use an extra set of short range cues from their host (i.e. odour from host frass, regurgitate, silk or vibrotaxis) for host location, acceptance and oviposition. This behavioural response in *A. bishopi* is consistent with results from several authors who confirmed and reported the complementary effects of both long range (HIPV) and short range (host odour; frass, silk, regurgitate, vibrotaxis) cues in the host location process of parasitoids (Rogers & Potter 2002; Rojas *et al.* 2006; Obonyo *et al.* 2010; Wang *et al.* 2010).

One of the four synthetic compounds that were previously found to be consistently abundant in *T. leucotreta* infested orange fruit (van der Walt 2012) elicited the strongest attraction to *A. bishopi* females in the Y-tube bioassays. D-limonene was strongly attractive to *A. bishopi* females. D-limonene and ocimene are the main terpene compounds found in citrus fruit peel (Fisher & Phillips 2008; Hosni *et al.* 2010; Chamberlain *et al.* 2012). The two compounds are both found in healthy fruit but are quantitatively elevated by insect herbivory or mechanical injury of the fruit peel (Mulas *et al.* 1996; Kendra *et al.* 2011; Chamberlain *et al.* 2012). The elevated concentrations of the above two compounds in *T. leucotreta* infested fruit (van der Walt, 2012) could possibly be due to the feeding injury on the fruit rind caused by *T. leucotreta* larvae, which consequently attracts *A. bishopi* females to their host. Therefore, it could further be suggested that production of D-limonene and ocimene in citrus is part of the indirect defence strategy against *T. leucotreta* infestation by attracting its natural enemies. Thus d-limonene and ocimene play a key role as long range attractants of *A. bishopi* females to *T. leucotreta* infested fruit. This tritrophic interaction (orange fruit, *T. leucotreta* larvae and

A. bishopi females) compares well with several other tritrophic systems (Mattiacci *et al.* 1999; Beneli *et al.* 2013).

Conversely, caryophyllene and naphthalene generally elicited a negative response to *A. bishopi* females indicating they were repellent. Caryophyllene is a sesquiterpene (Huang 2012) produced in most plant including maize (Robert *et al.* 2013), and citrus (Kendra *et al.* 2011). A previous study showed that caryophyllene has a repellent effect to several lepidopteran pests (Degenhardt *et al.* 2009) and is attractive to certain parasitoids of lepidopteran pests (Sasso *et al.* 2007) and some entomopathogenic nematodes (Robert *et al.* 2013). Surprisingly, caryophyllene did not elicit any attraction to *A. bishopi* females. It is possible that *T. leucotreta* elevates the production of caryophyllene through its feeding action as an ecological defence strategy against its parasitoid. The repellent effect elicited by naphthalene conforms well to previous reports confirming its repellence and toxicity to several arthropods (Daisy *et al.* 2002; Galera *et al.* 2004). Besides van der Walt (2012), no studies have reported naphthalene as a component of orange fruit either healthy or insect damaged fruit. It is unclear whether this could have been due to the presence of pesticide residues on fruit. Naphthalene is known to be a component of some pesticide formulations (Barr *et al.* 2002).

The four compound blend, a simulation of the volatile profile produced by *T. leucotreta* infested orange fruit strongly attracted *A. bishopi* females. Even though d-limonene and ocimene individually strongly attracted *A. bishopi* females, their combined effect in the blend elicited the strongest attraction, overcoming the repellent effects of caryophyllene and naphthalene. It could therefore be assumed that the two terpene compounds within the volatile plume from *T. leucotreta* infested fruit are responsible for long range attraction of *A. bishopi* females. This result further suggests that a combination of major *T. leucotreta* infested fruit compounds, including their specific blending ratios provide *A. bishopi* females with a more specific signal for their host as previously noted in similar studies (Pare & Tumlinson 1999; Fatouros *et al.* 2005).

This study demonstrated that *A. bishopi* females mainly rely on olfaction as long range cue in their host location process, with d-limonene as the major attractant compound in volatiles from *T. leucotreta* infested fruit. Therefore *A. bishopi*'s olfactory response to *T. leucotreta* induced fruit volatiles could potentially be harnessed in the development of a detection system for *T. leucotreta* infested fruit. Application of such an olfactory behavioural response

in odour detection systems using braconid wasps and other arthropods has previously been tested successfully (Rains *et al.* 2004; Tomberlin *et al.* 2008; Wackers *et al.* 2011; Chamberlain *et al.* 2012). It was clear that mated experienced *A. bishopi* female parasitoids showed a relatively stronger and more specific response to *T. leucotreta* infested fruit volatiles. Therefore prior oviposition experience in *A. bishopi* female parasitoids should be considered before their use in a host detection system. However, the average duration for odour learning in *A. bishopi* females should be ascertained in future studies. Future research should also consider characterisation of odour from *T. leucotreta* larvae silk and frass and assessing their specific influence as short range host location cues for *A. bishopi* females.

CHAPTER 4: Host location behaviour of *Agathis bishopi*, a larval endoparasitoid of false codling moth *Thaumatotibia leucotreta*

4.1 Introduction

The behavioural response of insects to resource (food, host, mate) cues has been widely studied due to their value for various applications including; biological control of pests (Foster & Harris 1997), integrated pest management (push and pull approach) (Khan *et al.* 2011), pest monitoring systems (Campbell *et al.* 2002; Suckling & Brockerhoff 2010), mating disruptions in pests (Witzgall *et al.* 2010) and chemical detection systems for host related or non-host chemical compounds such as drugs and explosives (Tomberlin *et al.* 2008; Chamberlain *et al.* 2012).

Hymenopteran parasitoids in particular exhibit several behavioural modifications during their host location process (Colazza *et al.* 2014). Generally, behaviour modification results in the arrestment of the parasitoid within the patch which is reflected through flight suppression, progressive reduction in mobility and increased antennal drumming (Colazza *et al.* 2014). Host stimuli are sensed by chemoreceptors that transmit the signal to the central nervous system, after which specific behavioural modifications are initiated (Colazza *et al.* 2014). According to Waage (1978), twofold behavioural patterns result, (1) random non-directed searching pattern, and (2) directed trail searching pattern. Random non-directional searching pattern is a general increase in parasitoid locomotion with reduced turning frequency at low concentration of stimuli (Waage 1978). Conversely, reduced movement and increased turning frequency results when the concentration of host stimuli increases. Random non-directed searching behaviour is known to be elicited by indirect host cues (frass, honey dew, lepidopteran scales) which attract the parasitoids to their host vicinity (González *et al.* 2011). On the other hand, directed trail searching behaviour is characterised by a series of movements in straight lines with relatively less turning frequency (Colazza *et al.* 2014). This behavioural pattern is known to be elicited by direct host stimuli (larval mandibular secretions, larval trails, chemical foot prints) which aid the parasitoid to locate hosts (González *et al.* 2011). In cases where the host is not encountered during directed trail searching, some species of parasitoids have been reported to engage in area restricted searching in which they exhibit increased mobility and turning frequency in a localised area at relatively high concentration of host cues (Rogers & Potter 2002).

In this study, a tritrophic system involving orange fruit, its major pest false codling moth *T. leucotreta* and the braconid larval endoparasitoid *A. bishopi* was examined. As described in the previous chapters, *T. leucotreta* is a pest of economic importance to South Africa's citrus industry (Moore 2002). Its phytosanitary status and zero tolerance by major export markets pose a challenge to the citrus industry. Consequently, a reliable and more sensitive technique is needed for detecting infested fruit before it is packaged for export.

Insects have been reported to detect chemical compounds almost instantaneously and with greater sensitivity and therefore offer great potential for developing systems for detecting infested fruit due to volatile chemical changes that occur in fruit following infestation by pests (Wäckers *et al.* 2011; Chamberlain *et al.* 2012). Furthermore, behavioural modifications such as area restricted searching exhibited by *M. croceipes* parasitoids in presence of host related or conditioned cues has been harnessed to develop a biosensor called a “wasp hound[®]” which has been successfully tested (Tomberlin *et al.* 2008; Wäckers *et al.* 2011). As described in the previous chapters, *A. bishopi* is a larval endoparasitoid of *T. leucotreta*. Its behavioural modifications in the presence of host cues could provide valuable information for developing a biosensor for *T. leucotreta* infested fruit. Although the olfactory and visual responses of *A. bishopi* to *T. leucotreta* infested fruit cues have been evaluated (Chapter 3), behavioural modifications exhibited by female parasitoids during the host searching process are unknown. Therefore, evaluation of parasitoid behavioural response to *T. leucotreta* infested fruit and characterisation of host searching and location behaviour is needed.

The aim of this chapter was to examine in detail, the host searching behavioural modification of *A. bishopi* females in presence of *T. leucotreta* infested fruit. In particular, the variation in the duration of female parasitoid behaviours (walking, resting, and oviposition) was evaluated and the host searching and oviposition behaviours on *T. leucotreta* infested fruit were characterised.

4.2 Materials and Methods

4.2.1 Insects

A culture of *A. bishopi* parasitoids was established and maintained at Rhodes University (Waainek Research Laboratory). The parasitoids were reared on their natural host *T. leucotreta* using a method previously described by Gendall (2007) with the improvements and changes outlined in chapter two. After eclosion and before the bioassays, male and female parasitoids were kept in different glass jars (250 ml). To produce mated females, male and female parasitoids (sex ratio 1:2 male: female) were kept in the same jar for at least 24 hours before the bioassay. A small piece of cotton wool soaked in water was placed in the jar as a water source for the parasitoids while food was provided by soaking a piece of cotton wool in dilute honey and placing it through a hole on the metal lid of the jar. The water and dilute honey was replenished daily. The glass jars containing parasitoids were kept in the rearing room (25°C ±2, 50% RH ±10 and 12:12 (Light: Dark) photoperiod. All bioassays were carried out using 2-5 day old female parasitoids.

Thaumatotibia leucotreta larvae used for fruit infestation were obtained from an established colony maintained at Rhodes University (Waainek Research Laboratory). The larvae were reared on artificial diet using a method described by Moore et al. (2014), as explained in chapter two. Until the larvae (1st instar) were used for fruit infestation, the moths were kept under the following conditions; 25°C ±2, 30% RH ±10 and 12:12 (Light: Dark) photoperiod.

4.2.2 Cue sources tested

Orange fruit of uniform size and similar cultivar (cv Navel) were used in the bioassays. Fruit infestation by *T. leucotreta* was carried out as described previously (Chapter 3). Orange fruit of fairly uniform size were collected from farms in Sundays River Valley (South Africa, Eastern Cape Province) and stored in a cold room at 4°C until they were used in the trials. Before infestation with *T. leucotreta* larvae, fruits were removed from the cold room and kept at room temperature for 24 hours. In order to obtain uniformly infested fruit, ten newly emerged 1st instar *T. leucotreta* larvae were placed individually on the fruit rind. Preliminary observations showed that most larvae died before they burrowed into the fruit, thus a relatively higher number of larvae were inoculated per fruit to ensure successful infestation. Both infested and healthy fruit were kept in the rearing room (25°C ±5, 30% RH ±10 and 12:12 (Light: Dark) photoperiod for 3-4 days before their use in bioassays. Based on

preliminary observations under the above conditions, 1st instar *T. leucotreta* larvae placed on fruit reached 2nd instar between 3-4 days after inoculation.

4.2.3 Bioassay setup

The testing arena consisted of a 50 × 40 × 30 cm plexiglass box with an open side and top. Within the arena, an individual orange fruit (healthy or infested) was placed on a plastic petridish, sitting on a platform (Fig 4.1).

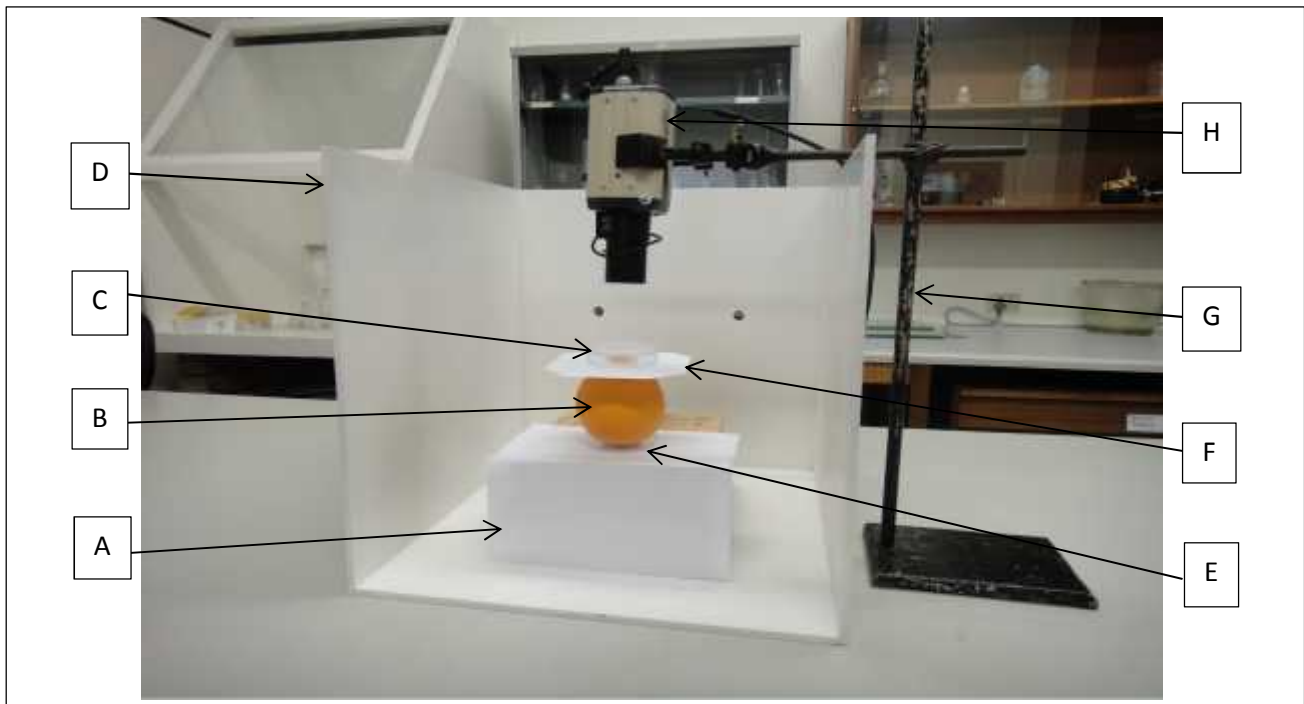


Figure 4.1 Bioassay design. A=fruit platform; B=Orange fruit; C=inverted petridish; D=Perspex box; E=Petridish; F=piece of white plain paper; G=Clamp stand; H=video camera

A 100 cm² square piece of white plain paper with a central circular hole (7 cm²) was placed on top of the fruit, such that only the fruit area through the circular hole was exposed above the paper. The exposed area of the fruit was covered by inverting a petri dish (6 cm diameter) over the plain paper, with the fruit area positioned centrally. The petridish was inverted in order to later house and prevent the parasitoid from escaping. A digital video camera was mounted on a clamp stand and positioned centrally and directly above (15 cm) the petridish. An individual *A. bishopi* mated female (naïve or experienced) was released into the inverted petri dish such that the parasitoid was exposed to the fruit area through the paper. All the bioassays were video recorded and saved for later analyses.

4.2.4 Behavioural observation and recording

Behavioural activities of *A. bishopi* were observed on healthy and *T. leucotreta* infested fruit. Essentially, the duration of searching behavioural activities involved in host searching were observed and video recorded using a digital video camera, and later their respective duration was analysed using a software ‘The observer[®] XT version 11.5’ (Noldus 2013) (Mattiacci et al. 1999). After the parasitoid was introduced into the arena, it was allowed 1 min to acclimatize to the petri dish environment before commencement of the bioassay. The behavioural activities of *A. bishopi* females that were recorded included; ‘Walking’ which was defined as the time spent by the parasitoid walking within the petridish, ‘Resting’ defined as the time spent by the parasitoid standing still or while preening, ‘Oviposition attempt’ defined as time spent by the parasitoid pushing its ovipositor into the frass hole on fruit to lay eggs. For each treatment (healthy or infested fruit), twenty female parasitoids from each category (Naïve or Experienced) were tested. A new fruit was used in each replicate and each parasitoid was used only once. The bioassay ran for 5 min and 10 min for naïve and experienced parasitoids respectively. Naïve parasitoids were exposed 5 min bioassay in order to prevent parasitoids from gaining experience due to prolonged exposure to fruit. In order to avoid circadian variation in parasitoids behaviour, all experiments were carried out between 08:00 – 17:00 hrs. The bioassays were conducted in a laboratory (22°C±3, 40-50% RH) and the laboratory was illuminated by two fluorescent tubes with a light intensity of 5 lux.

4.2.5 Statistical design and analyses

The experiment consisted of four factors, fruit state (*T. leucotreta* infested fruit and healthy fruit) and three behaviours (resting, walking and oviposition). Resting, walking and oviposition were chosen as dependent variables while fruit state (*T. leucotreta* infested fruit and healthy fruit) served as independent variable. For each behaviour, mean duration, total duration, frequency of behavioural event and standard errors were calculated using, The observer[®] XT, version 11.5; Noldus Information Technology, Wageningen, The Netherlands, 2013). Further, all statistical analyses were done using R-statistical package version 3.0.2. Given the non-normality distribution of the parasitoid behavioural data even after transformations, a non- parametric statistical test (Wilcoxon rank-sum test ($P < 0.05$)) was used to identify whether fruit condition had a significant effect on the duration of the three behaviours (resting, walking and oviposition) tested.

4.3 Results

4.3.1 Mean duration of behaviours (resting, walking and oviposition) for mated naïve female *Agathis bishopi* in presence of healthy and *Thaumatotibia leucotreta* infested fruit

The mean duration per behaviour (sum of durations of behaviour events/number of behaviour events) and total mean time of behaviours (sum of durations of behaviour events) for mated naïve female parasitoids when exposed to fruit treatments (healthy and FCM fruit) are shown below (Table 4.1). Significant differences were observed in the mean duration of resting behaviour for mated naïve *A. bishopi* female parasitoids (Wilcoxon rank sum test, $W = 287$, $P = 0.01929$) indicating that they rested longer on healthy fruit than on *T. leucotreta* infested fruit (Table 4.1). However, there was no statistical difference in mean duration for walking (Wilcoxon rank sum test, $W = 128$, $P = 0.05306$) and oviposition (Wilcoxon rank sum test, $W = 220$, $P = 0.1626$) behaviours between the fruit treatments (Table 4.1).

4.3.2 Mean duration of behaviours (resting, walking and oviposition) for mated experienced female *Agathis bishopi* in presence of healthy and *Thaumatotibia leucotreta* infested fruit

The mean duration per behaviour (sum of durations of behaviour events/number of behaviour events) and total mean time of behaviours (sum of durations of behaviour events) for mated experienced female parasitoids when exposed to fruit treatments (healthy and FCM fruit) are shown below (Table 4.2). Highly Significant differences were observed in the mean duration of oviposition behaviour for mated experienced *A. bishopi* female parasitoids when exposed to fruit treatments (Wilcoxon rank sum test, $W = 350$, $P = 3.308e-06$) (Table 4.2) showing that they oviposited longer on *T. leucotreta* infested fruit than on healthy fruit (Table 4.2). Furthermore, it was observed that 75% of parasitoids tested attempted to oviposit and this behaviour was expressed exclusively on *T. leucotreta* infested fruit. Conversely, there was no statistical difference in mean duration for resting (Wilcoxon rank sum test, $W = 161$, $P = 0.3013$) and walking (Wilcoxon rank sum test, $W = 165$, $P = 0.3507$) behaviours between the fruit treatments (Table 4.2).

Table 4.1 Mean duration of each behaviour event and total mean time per behaviour (\pm SE) of 20 mated naïve female parasitoids observed over a 5 min bioassay for three behaviours (resting, walking and resting) when exposed to healthy and FCM fruit.

Fruit state	Parameter	Behaviour		
		Resting	Walking	Ovipositing
Healthy Fruit	Total duration (s)	114.20 \pm 21.34	185.93 \pm 21.33	0.00 \pm 0.00
	Mean time (s)	20.21 \pm 5.18	41.43 \pm 8.22	0.00 \pm 0.00
FCM fruit	Total duration (s)	182.05 \pm 23.90	98.41 \pm 21.29	19.64 \pm 13.52
	Mean time (s)	80.59 \pm 23.14	29.77 \pm 14.41	3.93 \pm 2.71

Table 4.2: Mean duration of each behaviour event and total mean time per behaviour (\pm SE) of 20 mated experienced female parasitoids observed over a 10 min bioassay for three behaviours (resting, walking and resting) when exposed to healthy and FCM fruit.

Fruit state	Parameter	Behaviour		
		Resting	Walking	Ovipositing
Healthy Fruit	Total duration (s)	413.68 \pm 44.97	186.73 \pm 44.90	0.00 \pm 0.00
	Mean time (s)	141.26 \pm 40.23	24.71 \pm 6.43	0.00 \pm 0.00
FCM fruit	Total duration (s)	285.89 \pm 49.74	105.11 \pm 18.88	236.65 \pm 43.17
	Mean time (s)	98.82 \pm 34.36	13.55 \pm 2.08	61.16 \pm 16.78

4.3.3 Host searching and oviposition behavioural sequence of *Agathis bishopi* on *Thaumatotibia leucotreta* infested fruit.

The host searching and oviposition behavioural sequence for mated experienced *A. bishopi* female parasitoids on *T. leucotreta* infested fruit was observed during the 10 min bioassays. After being introduced into the petridish arena with infested fruit, the parasitoid either walked around the arena immediately or stood quietly or while grooming before it started walking on the fruit. Grooming behaviour involved the cleaning of sensory parts and always started with the antennae followed by the tarsi and ovipositor respectively. This was followed by searching behaviour in which the parasitoid walked around the arena while antennating on

the petridish or fruit surface. When the parasitoid got closer to a pile of *T. leucotreta* frass on the fruit surface, it stopped walking and examined the frass thoroughly by rubbing the tip segments of antennae against the frass pile. If the frass did not indicate the presence of the host, the parasitoid discontinued the examination and continued searching. When the quality of frass was acceptable, (indicating presence of host) the parasitoid positioned itself to oviposit by slowly moving forward, raising its abdomen, bending its ovipositor downwards and probing in to the frass pile. The parasitoid probed the frass pile several times until the ovipositor contacted the host larvae. This was preceded by a quiet inactive state of the parasitoid with the antennae resting on the surface of the fruit indicating egg deposition into its host. However, it was not ascertained whether oviposition was successful by checking for parasitoid eggs in the host larvae. At the end of the assumed oviposition event, the parasitoid withdrew the ovipositor from the frass pile, cleaned it by rubbing with its hind legs (tarsals) and continued searching for the next host.

4.4 Discussion

In this study, the host searching behavioural response of *A. bishopi* females to *T. leucotreta* infested fruit (orange, cv Navel) was examined. In particular, changes in the duration of three main parasitoid behaviours (resting, walking and oviposition), in the presence of healthy and *T. leucotreta* infested fruit was highlighted, including the characterisation of host searching and oviposition behaviour of experienced female parasitoids on infested fruit.

During the bioassay with healthy and *T. leucotreta* infested fruit, mated naïve female *A. bishopi* parasitoids spent more time resting on infested fruit than on healthy fruit (Table 4.2). The behavioural alteration observed in mated naïve parasitoids when exposed to infested fruit suggests that although the fruit and host stimuli are innately recognised cues, the female parasitoid requires an initial priming or exposure in order to gain optimum responsiveness to host cues. Prolonged resting durations in several species of parasitoids have been reported as a common behaviour expressed by naïve female parasitoids (Mattiacci *et al.* 1999; Canale & Benelli 2012). It is possible that the parasitoids initiated the learning of host related cues by spending more time resting (which was almost always associated with grooming behaviour) when exposed to *T. leucotreta* infested fruit than healthy fruit. Odour learning and experienced based behavioural response to host cues as exhibited by mated naïve *A. bishopi* females has been reported previously in parasitoids (Mattiacci *et al.* 1999; Tomberlin *et al.* 2008; Canale & Benelli 2012).

Furthermore, two out of the twenty mated naïve female parasitoids tested exhibited oviposition behaviour only when exposed to *T. leucotreta* infested fruit. It is presumed that host cue learning was still in its infancy stage resulting in only a small proportion of parasitoids ovipositing on infested fruit. This response conforms to similar previous studies that showed that the success of host location increased with level of host cue learning (Meiners *et al.* 2003; Tomberlin *et al.* 2008). Even though grooming behaviour was not examined in this study, preliminary observations showed that the resting behaviour of naïve female parasitoids was coupled with high occurrence of grooming behaviour when exposed to *T. leucotreta* infested fruit than on healthy fruit. This observation suggests that as the parasitoid begins to perceive and learn host cues, it modifies its behaviour by spending more time resting and grooming during which the antennae, tarsals and ovipositor are cleaned repeatedly to increase their sensitivity to host cues (Robinson 1996; Orchard *et al.* 2012; Böröczky *et al.* 2013). The poor behavioural response of mated naïve female parasitoids to *T. leucotreta* infested fruit is consistent with results from the previous study (Chapter 3) in which naïve female parasitoids did not show preference between healthy and *T. leucotreta* infested fruit cues in both Y-tube and flight tunnel bioassays. Although mated naïve *A. bishopi* female parasitoids modified the duration of their resting behaviour, including initiation of oviposition behaviour when exposed to *T. leucotreta* infested fruit, the magnitude of this behavioural change was not distinct enough to show clear discrimination between healthy and *T. leucotreta* infested fruit.

The bioassay for mated experienced *A. bishopi* females on healthy and *T. leucotreta* infested fruit showed that parasitoids spent significantly more time ovipositing on infested fruit than healthy fruit (Table 4.3). In particular, 75% of parasitoids attempted to oviposit only on infested fruit. The expression of oviposition behaviour exclusively on *T. leucotreta* infested fruit by mated experienced female parasitoids suggests that prior oviposition experience strongly enhances the responsiveness of *A. bishopi* female parasitoids to *T. leucotreta* infested fruit. Furthermore, it is suggested that oviposition behaviour is only elicited by direct host related cues (frass, larvae silk). Voluminous literature highlights the importance of host related cues for successful host location (Rogers & Potter 2002; Rojas *et al.* 2006; Hare 2011; Kaplan 2012; Benelli *et al.* 2013; Kessler *et al.* 2001). This behavioural response is similar to the findings in the previous study (Chapter 3) in which experienced parasitoids showed a stronger attraction to *T. leucotreta* infested than healthy fruit. This finding suggests that in the presence of both fruit and host cues, host searching is elicited in which resting and walking

behaviours are reduced while probing and oviposition behaviours are initiated. The observed prolonged duration of oviposition behaviour for mated experienced *A. bishopi* parasitoids in the presence of *T. leucotreta* infested fruit, including the expression of oviposition behaviour exclusively on infested fruit could provide a more distinct indication of fruit infestation by *T. leucotreta*.

The host searching and location behaviour of *A. bishopi* compares well with other similar species in the genus *Agathis* such as *Agathis laticintus* (Cress) (Hymenoptera: Braconidae) (Dondale 1954), *Agathis gibbosa* (Say) (Hymenoptera: Braconidae) (Odebiyi & Oatman 1972) and other braconid parasitoids (Canale & Benelli 2012). On average, it took 2.57 ± 0.64 min (\pm SE) for mated experienced *A. bishopi* female parasitoids to locate their host after being introduced in to the testing arena while oviposition behaviour (including probing) lasted for 61.16 ± 16.78 sec (\pm SE) (Table 4.3). Furthermore, the mated experienced female parasitoids showed a more random searching movement on the infested fruit surface and did not exhibit an oriented or directed movement towards frass filled holes (host site) on fruit. Parasitoids only turned to the direction of frass on fruit only when they were in very close proximity. It is presumed that while the direct host cues in frass are key for successful host location, their abundance and volatility is relatively much lower as compared to fruit compounds making frass to be detectable only at very close range. This observation confirms the findings in the previous study (Chapter 3) in which parasitoids did not attempt to oviposit when exposed to *T. leucotreta* infested fruit odour in a Y-tube bioassay. Similar studies have also confirmed the low abundance and volatility of compounds in larval frass (Wajnberg *et al.* 2008). Therefore, while oviposition behaviour provides a more consistent and accurate indication of fruit infestation by *T. leucotreta*, the behaviour is only elicited when the parasitoid is in contact with the fruit and in close proximity with frass.

Results in this study demonstrated that *A. bishopi* female parasitoids modify the duration of resting and oviposition behaviours when exposed to *T. leucotreta* infested fruit. Oviposition was especially a very distinct behaviour which was only elicited when experienced female parasitoids were exposed to *T. leucotreta* infested fruit and therefore provides a reliable indication of fruit infestation. Mated experienced *A. bishopi* females showed a stronger expression of oviposition behaviour as compared to mated naïve parasitoids. It seemed that compounds in frass were responsible for eliciting oviposition behaviour in parasitoids. Future studies should therefore consider characterisation of compounds in frass and conditioning female parasitoids to these compounds in order to improve the directed or oriented response

of parasitoids to frass. This would improve the potential of applying *A. bishopi* in the development of a detection system for *T. leucotreta* infested fruit.

CHAPTER 5: General discussion

5.1 Introduction

Thaumatotibia leucotreta is one of the major pests of citrus in South Africa (Moore 2002). It is a phytosanitary pest with zero tolerance by most markets for its presence in fruit (Moore 2002; Moore *et al.* 2004; Hattingh 1996). Due to its cryptic nature, accurate detection of fruit newly infested with *T. leucotreta* at the packhouse is difficult and this poses a market threat to the South African citrus industry. The ultimate aim of this study was to investigate the potential for using *A. bishopi*, a larval parasitoid of *T. leucotreta* for early post-harvest detection of *T. leucotreta* infested fruit. In order to achieve this aim, several objectives were set out: establishment of an *A. bishopi* culture to facilitate bioassay trials and improvement of the rearing protocol; identification of which volatile compounds produced by *T. leucotreta* infested fruit are attractive to *A. bishopi* female parasitoids; determination of whether there are distinct observable and interpretable behavioural responses from *A. bishopi* that can be associated with *T. leucotreta* infested fruit; conditioning *A. bishopi* females to respond behaviourally to volatiles from *T. leucotreta* infested fruit; and finally, developing a system where *A. bishopi* could be practically applied for post-harvest identification of *T. leucotreta* infested fruit in the packhouse. The first three objectives were to a large extent achieved while the last two objectives were not carried out due to challenges with the establishment of a parasitoid culture, which was more difficult to establish than expected. This chapter aims to place the findings of this thesis into a broader context and to discuss their implications for development of a detection system for *T. leucotreta* infested fruit.

Parasitoids have been used widely for biological control of pests in agricultural systems (Obonyo *et al.* 2010; Ode & Crompton 2013; Balmer *et al.* 2014; Niu *et al.* 2014; Wang *et al.* 2014), but in the past decade, there has been growing interest in using parasitoids in chemical detection systems (Rains *et al.* 2004; Tomberlin *et al.* 2008; Wäckers *et al.* 2011). In order to establish a reliable supply of sufficient numbers of insects for biological studies for applications mentioned above, parasitoids are usually reared in the laboratory on their host insects, which are fed on artificial diet (Sishuba 2003; Gendall 2007). A rearing protocol described by Gendall (2007) provided the basis to facilitate the establishment of an *A. bishopi* culture, aimed at producing parasitoids for bioassays (chapter 3 and chapter 4) and studies for improvement of the existing rearing protocol (chapter 2). Adult parasitoid feeding is an important factor that affects fecundity of female parasitoids in rearing programmes (Azzouz *et al.* 2004). While the effect of adult feeding on the fecundity of several parasitoids is quite

well known (Azzouz *et al.* 2004; Eliopoulos 2007; Balmer *et al.* 2014), nothing is known about this with *A. bishopi* parasitoids. Evaluation of the influence of food quality and accessibility to parasitism success in *A. bishopi* was therefore essential. As described in chapter two, changes in the feeding method for *A. bishopi* parasitoids in the rearing jars, resulted in improved parasitism success. This finding provides some insights for augmentation of the existing rearing protocol for possible mass rearing. Since *T. leucotreta* larvae leave infested fruit to pupate on the soil underneath the citrus tree (Kirkman & Moore 2007), it is possible that parasitoids naturally fly up the tree to search for food immediately after emergence from the soil. Therefore the positioning of the honey (with optimal concentration) under the lid in the rearing jar, increases the accessibility of honey to *A. bishopi* female parasitoids, resulting in increased fecundity and longevity, consequently optimising offspring yield and sex ratio (Jacob & Evans 1998; Géneau *et al.* 2013). In a broader context, this finding has significant implications for the orchard situation as increasing the availability of food resources (extra floral nectar and pollen) through integration of flowering plant in the orchards could improve the effectiveness of *A. bishopi* as a biological control agent for *T. leucotreta* (Eliopoulos 2007; Amaral *et al.* 2013; Géneau *et al.* 2013).

Resource foraging by parasitoids involves the use of various stimuli (visual taxis, chemical taxis and vibratory taxis) in order to successfully locate food or hosts (Beneli *et al.* 2013). Many studies have reported that parasitoids discern infested from healthy plant by exploiting the novel profile of volatile compounds produced by their host's herbivorous action (Hare 2011; Kaplan 2012; Beneli *et al.* 2013). This alteration of volatile profiles due to herbivory has also been reported on citrus fruit (Kendra *et al.* 2011; Chamberlain *et al.* 2012; van der Walt 2012). Furthermore, it is well known that parasitoids are capable of learning both host and food related stimuli (Rains *et al.* 2004; Tomberlin *et al.* 2008). This ability of parasitoids to learn and respond to food, host or non-host cues has been used to develop odour detection systems (Tomberlin *et al.* 2008; Wäckers *et al.* 2011). Identification of compounds in *T. leucotreta* infested fruit that are attractive to *A. bishopi* female parasitoids was a key preliminary step in evaluating the potential for using *A. bishopi* parasitoids in a detection system for infested fruit. Evidence from chapter three of this thesis indicated that from the volatile compounds produced from *T. leucotreta* infested fruit (van der Walt, 2012), D-limonene is the key compound responsible for attracting *A. bishopi* female parasitoids to infested fruit. Several studies have demonstrated that D-limonene production in citrus is

elevated by insect herbivory on fruit (Kendra *et al.* 2011; Chamberlain *et al.* 2012; van der Walt 2012), including its role as an attractant for parasitoids (Wei *et al.* 2013; Dias *et al.* 2014). This result showed that D-limonene production is elevated due to the feeding injury on citrus fruit by *T. leucotreta* larvae, which consequently attracts *A. bishopi* parasitoids to their host on fruit. The natural ability of *A. bishopi* female parasitoids to discriminate infested from healthy fruit, based on the concentration of D-limonene in fruit, presents an important preliminary step in the evaluation of *A. bishopi* parasitoids for development of a detection system for *T. leucotreta* infested fruit. However, D-limonene did not elicit oviposition behaviour in the bioassay trials (chapter 3), showing that this compound is only a long-range attractant for parasitoids and that female parasitoids require an extra cue(s) to finally locate *T. leucotreta* larvae on fruit. In addition, it was also observed that when female *A. bishopi* parasitoids were allowed contact with *T. leucotreta* infested fruit, particularly frass, oviposition behaviour was elicited. Similar to what other studies have reported (Kessler *et al.* 2001; Hare 2011; Goubert *et al.* 2013), the compounds in frass account for the final short range location of *T. leucotreta* larvae on fruit by *A. bishopi* female parasitoids. The fact that D-limonene is not only elevated by *T. leucotreta* infestation on fruit (van der Walt, 2012) but also by several other pests (Kendra *et al.* 2011; Chamberlain *et al.* 2012; Dias *et al.* 2014) makes it a less reliable cue for specific detection of *T. leucotreta* fruit by *A. bishopi* parasitoids.

Further conditioning *A. bishopi* parasitoids to respond to compounds in *T. leucotreta* frass would provide a more specific and accurate discrimination of *T. leucotreta* infested fruit in a detection system. The stronger attraction to D-limonene and *T. leucotreta* infested fruit observed in mated and oviposition experienced female parasitoids confirms the ability of *A. bishopi* females to associatively learn their host cues. This learning ability in *A. bishopi* females suggests the possibility of conditioning parasitoids to respond behaviourally to compounds in *T. leucotreta* frass and consequently be used to detect infested fruit. In addition, prioritisation of food to host foraging as described in chapter two, particularly when *A. bishopi* female parasitoids are starved, showed that conditioning females to associate frass compounds to food as opposed to oviposition would result in a stronger response of parasitoids to *T. leucotreta* infested fruit which would be a desired quality for developing a detection system.

During the resource searching process, parasitoids are known to modify their behaviour which usually culminates in the arrestment of parasitoids within the resource patch (Colazza

et al. 2014). Attraction and consequent arrestment of behavioural response observed in *M. croceipes* when exposed to conditioned stimuli has been harnessed to develop an odour detection system called “Wasp hound[®]” (Tomberlin *et al.* 2008). Therefore, evaluations of behavioural modifications in *A. bishopi* females that are associated with the parasitoid response to *T. leucotreta* infested fruit are essential for consideration as positive or typical signals of detection in a detection system. Chapter four provided evidence that *A. bishopi* female parasitoids modify their behaviour in the presence of *T. leucotreta* infested fruit, in which the resting and walking behaviour are considerably reduced while probing and oviposition behaviours are initiated. The noticeable elicitation of probing and oviposition behaviour when *A. bishopi* females are exposed to *T. leucotreta* frass on fruit could be used as typical detection signals in a detection system. Similar to observations in chapter three, probing and oviposition behaviours are elicited exclusively on *T. leucotreta* infested fruit, consolidating the assumption that only compounds in frass or larval silk are responsible for their activation.

Agathis bishopi female parasitoids did not show an oriented movement towards a frass pile on fruit but only turned towards the location of frass on fruit when in very close proximity to it. A possible explanation to this could be that the compounds in frass have lower abundance and volatility relative to fruit compounds. This influence of *T. leucotreta* frass on host location behaviour has implications for the application of *A. bishopi* parasitoids for detecting *T. leucotreta* infested fruit. Therefore, determination of which compounds in *T. leucotreta* frass are attractive to *A. bishopi* female parasitoids is essential.

5.2 Conclusion

Through this study a significant stride has been made towards the development of a detection system for *T. leucotreta* infested fruit using *A. bishopi* parasitoids. In light of the observations made in this study, it is proposed that optimal feeding of *A. bishopi* parasitoids in rearing programmes should be considered in order to optimise offspring yields and sex ratio. It was evident in this study that discrimination of *T. leucotreta* infested from healthy fruit by *A. bishopi* female parasitoids is mainly based on the compounds in frass. Therefore, it is suggested that chemical analysis of *T. leucotreta* frass, particularly produced by younger larvae (1st - 2nd instar), on fruit be examined in order to ascertain the chemical compound/s that are responsible for eliciting oviposition behaviour in *A. bishopi* females. Given the poor innate oriented movement of *A. bishopi* female parasitoids towards frass spots on fruit, it is advisable to consider conditioning of *A. bishopi* female parasitoids to compounds in frass in

order to realise a stronger oriented movement of parasitoids towards frass which is a desired quality for developing a detection system for *T. leucotreta* infested fruit.

In order to actualise the proposed detection technology for *T. leucotreta* infested fruit using *A. bishopi* parasitoids as proposed in this thesis, conditioning of parasitoids to respond behaviourally to compounds in *T. leucotreta* frass and construction of a “Wasp hound[®]” to harness the conditioned behaviour should be considered in taking this study further.

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