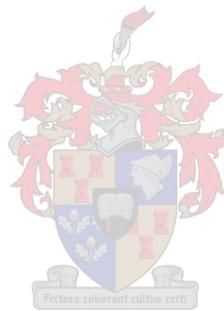


**Biological control of two sporadic grapevine pests,
Plangia graminea and *Lobesia vanillana*,
using entomopathogenic nematodes**

by
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Thesis presented in partial fulfilment of the requirements for the degree of
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at

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2019

SUMMARY

Plangia graminea (Serville) (Orthoptera: Tettigoniidae) and *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae) are two sporadic, minor pests of wine grape vineyards in the Western Cape province of South Africa. Recent years have seen an increase in their abundance and damage, which necessitates their control. Little is known about the biology, ecology and distribution of these insects. The aim of this study was to collect their basic ecological data and to evaluate entomopathogenic nematodes (EPN) as potential biocontrol agents. Nymphs of *P. graminea* were evaluated against 12 *in vivo*-cultured EPN species, of which *Heterorhabditis zealandica* (SF41), *H. indica* (SGS), *Steinernema jeffreyense* (J192), *S. yirgalemense* (157-C) and *H. baujardi* (MT19), resulted in > 82 % mortality after 48 h. Larvae of *L. vanillana* were evaluated against *S. jeffreyense* (J192) and *S. yirgalemense* (157-C), sourced from both *in vivo* and *in vitro*-cultures of the same isolates. Results show that they were susceptible to all treatments, resulting in > 72% mortality, and that there was no significant difference in mortality between *in vivo* and *in vitro*-cultured nematodes of the same EPN species, but that in the *in vitro*-culture, *S. yirgalemense* (98%) performed significantly better than *S. jeffreyense* (73%). Cadavers from screening bioassays were dissected to evaluate the presence of infective juveniles (IJ), which in turn confirmed insect mortality by EPN infection. The ability of IJs to complete their lifecycle *in vivo*, and their ability to produce a new cohort of IJs, suggests that they may be able to provide persistent control in favourable environments. Observations on the biology and ecology of *P. graminea* in grapevine, suggests that they do not have a soil stage and only a single generation per year. *Plangia graminea* were mainly reported from the Cape Winelands region in the Western Cape province of South Africa, from where they were collected in an attempt to establish a laboratory colony. They did not perform well in captivity, therefore field collected individuals had to be used in laboratory bioassays. Observations on *L. vanillana* suggests that this species also does not have a soil stage. They seem to have a generational life cycle of 4-5 weeks and to overwinter as pupae. *Lobesia vanillana* was successfully reared from field-collected larvae using an agar-based modified codling moth diet. The present study contributes to new knowledge of *P. graminea* and *L. vanillana*, and indicates that EPNs are promising as biological control agents when considered as part of an integrated pest management program.

OPSOMMING

Plangia graminea (Serville) (Orthoptera: Tettigoniidae) en *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae) is twee, minder belangrike, sporadiese peste van wyndruiwe in die Wes-Kaap provinsie van Suid Afrika. In die laaste paar jaar was daar 'n toename in hul teenwoordigheid en skade opgemerk, wat beheermaatreëls noodsaak vir hulle beheer. Min is bekend oor hierdie insekte se biologie, ekologie en verspreiding. Die doel van hierdie studie was om hul basiese ekologiese inligting te versamel en om entomopatogeniese nematodes (EPN) as potensiële biologiese beheer agent te evalueer. Nimfe van *P. graminea* was geëvalueer teen 12 *in vivo* gekweekte EPN spesies, waarvan *Heterorhabditis zealandica* (SF41), *H. indica* (SGS), *Steinernema jeffreyense* (J192), *S. yirgalemense* (157-C) en *H. baujardi* (MT19), meer as 82% mortaliteit na 48 uur veroorsaak het. Larwes van *L. vanillana* was geëvalueer teen *S. jeffreyense* (J192) en *S. yirgalemense* (157-C), afkomstig vanaf beide *in vivo* en *in vitro* kulture van dieselfde isolate. Resultate toon dat hulle vatbaar was vir alle behandelinge, wat meer as 72% mortaliteit veroorsaak het, en dat daar geen beduidende verskil tussen *in vivo* en *in vitro* gekweekte kulture was nie, maar dat binne die *in vitro* kultuur, *S. yirgalemense* (98%) beduidend beter gevaar het as *S. jeffreyense* (73%). Kadawers van laboratoriumtoetse was gedissekteer om die teenwoordigheid van invektiewe onvolwassenes te evalueer, wat dus insekmortaliteit deur EPN infeksie bevestig het. Die vermoë van hierdie invektiewe onvolwassenes om hul lewenssiklus *in vivo* te voltooi, en hul vermoë om 'n nuwe gros invektiewe onvolwassenes te vorm, dui daarop dat hulle moontlik die vermoë het om voortgesette beheer te kan uitoefen in gunstige omstandighede. Waarnemings ten opsigte van die biologie en ekologie van *P. graminea* op wyndruiwe, dui daarop dat hulle nie 'n grondgebonde fase het nie en slegs 'n enkele generasie per jaar. *Plangia graminea* was hoofsaaklik gerapporteer vanuit die Kaapse Wynland distrik in die Wes-Kaap provinsie van Suid Afrika, en van daar versamel is met die doel om 'n laboratoriumkolonie te stig. *Plangia graminea* het nie goed presteer in gevangenskap nie, wat dit genoodsaak het om individue uit die veld te versamel en te gebruik vir laboratoriumtoetse. Waarnemings van *L. vanillana* in die veld dui daarop dat hierdie insek ook nie 'n grondgebonde lewensstadium het nie. Dit wil voorkom dat hulle 'n lewenssiklus van 4-5 weke het en oorwinter as 'n papie. Veld versamelde *L. vanillana* was suksesvol geteel op 'n gewysigde agar-gebaseerde kodling mot dieet. Die huidige studie dra by tot nuwe kennis van *P. graminea* en *L. vanillana*, en dui daarop dat EPNs as belowende agente vir biologiese beheer oorweeg kan word, indien dit deel vorm van 'n geïntegreerde plaagbeheer program.

BIOGRAPHICAL SKETCH

Francois du Preez earned his Bachelor of Science degree in Conservation Ecology and Entomology at Stellenbosch University in 2014. His final year's project evaluated the state of the Eerste River in Stellenbosch, using a modified version of the South African Scoring System (miniSASS). From 2014-2016 he was employed in the industry as a consultant for agriculture-related software and hardware solutions. In the latter half of 2016 he decided to pursue his Master of Science degree in Entomology, with a focus on the biological control of insect pests. This interest stems from his awareness of current challenges in the production and marketing of agricultural produce, and the many dialogs with growers, consultants and marketers in this regard. His eagerness to learn and to contribute to the industry is his primary motivation.

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PREFACE

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Entology and Viticulture*.

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CHAPTER 1: LITERATURE REVIEW

Plangia graminea (Orthoptera: Tettigoniidae) and *Lobesia vanillana* (Lepidoptera: Tortricidae) as sporadic pests in South African grapevine

INTRODUCTION

Jan van Riebeeck established the first vineyard in South Africa in 1655 using *Vitis vinifera* L. (Vitales: Vitaceae) cuttings originating from France and Germany (Van Zyl, 1987). After apartheid ended in 1994, the resulting influx of foreign investment and the relaxation of international sanctions led to the exponential growth of the industry (Estreicher, 2014). Nowadays, South Africa produces about 4% of the world's wine and is the 8th largest producer internationally (Floris-Samuels, 2016), and the industry and its associated tourism sector is an integral part of local economies in the Western Cape (Bruwer, 2003).

In 2013, the wine industry contributed R36 145 million (1.2%) to the South African gross domestic product and employed almost 300 000 people in unskilled (55.6%) and semi-skilled (29.3%) positions (Conningarth Economists, 2015). As a percentage of local production, exports have increased from 38.3% in 2003 to 57.4% in 2013 (Conningarth Economists, 2015), and to 48.8% in 2017 (WOSA, 2018). The 2016/2017 season produced 1.4 million tons of grapes and 919 million litres of wine, from approximately 95 000 ha of vineyard planted with 280 million vines (Vinpro, 2017). The 2017/2018 harvest was 15% smaller, however, mostly due to extended drought conditions and severe water restrictions (Vinpro, 2018).

Since 2005, the number of primary grape producers reduced from 4360 (2005) to 3029 (2017), indicating some consolidation (WOSA, 2018), although wine grape surface area also decreased, from 102 146 ha in 2006 to 94 545 ha in 2017 (Floris-Samuels & Uren, 2017). Statistics show that about a third of wine farms operate at a loss and that the remaining majority have low profitability (Van Zyl & Van Niekerk, 2017). On average, as a percentage of total on-farm expenses, pest and disease control absorbed 41.36% of direct costs and 7.65% of total production costs during the 2016/2017 season (Van Zyl & Van Niekerk, 2017).

Traditional agrochemicals are still major components of pest and weed control programmes, but preferences are slowly shifting towards more environmentally-friendly and sustainable solutions. Certification schemes such as GlobalGAP, Fairtrade, the Integrated Production of Wine (IPW) scheme and the increase of organically farmed produce, signify this demand from consumers, whereas increasingly strict regulations on the use of agrochemicals during

production and export processes, indicate their discouragement from marketplaces (Blignaut *et al.*, 2014). Wine research in South Africa is largely funded by Winetech (Wine Industry Network of Expertise and Technology) through applied research projects (Giuliani *et al.*, 2010).

All South African wine cultivars belong to *V. vinifera* (WOSA, 2016). South African grapevine is host to 35 pests, the most important of which include species in the families of Cicadellidae (Hemiptera), Curculionidae (Coleoptera), Margarodidae (Hemiptera), Noctuidae (Lepidoptera), Pseudococcidae (Hemiptera) and Phylloxeridae (Hemiptera) (Table 1.1) (Allsopp *et al.*, 2015).

TABLE 1.1

Common grapevine pests of South African grapevine. Adapted from Allsopp *et al.* (2015).

Order	Family	Common name	Species	
Hemiptera	Cicadellidae	Grapevine leafhoppers	<i>Acia lineatifrons</i> (Naudé)	
			<i>Mgenia fuscovaria</i> (Stål)	
	Phylloxeridae	Grapevine phylloxera	<i>Daktulosphaira vitifoliae</i> (Fitch)	
Coleoptera	Margarodidae	Ground pearls	<i>Margarodes capensis</i> Giard	
			<i>M. greeni</i> Brian	
			<i>M. prieskaensis</i> (Jakubski)	
			<i>M. trimeni</i> Giard	
			<i>M. vredendalensis</i> De Klerk	
	Pseudococcidae	Grapevine mealybug	<i>Planococcus ficus</i> (Signoret)	
	Curculionidae	Black snout beetle	<i>Eremnus atratus</i> (Sparman)	
			Speckled beetle	<i>E. cerealis</i> Marshall
			Vine weevil	<i>E. chevrolati</i> Oberprieler
			Grey weevil	<i>E. setulosus</i> Boheman
Banded fruit weevil			<i>Phlyctinus callosus</i> (Schönherr)	
Bud nibbler			<i>Tanyrhynchus carinatus</i> Boheman	
Lepidoptera	Cossidae	Apple trunk borer	<i>Coryphodema tristis</i> (Drury)	
	Tortricidae	Pear leaf roller	<i>Epichoristodes acerbella</i> (Walker)	
	Agaristidae	Trimen's false tiger	<i>Agoma trimenii</i> (Felder)	
	Noctuidae	African bollworm	<i>Helicoverpa armigera</i> (Hübner)	
Thysanoptera	Thripidae	Western flower thrips	<i>Frankliniella occidentalis</i> (Pergande)	
		Guava thrips	<i>Heliothrips sylvanus</i> Faure	

This review aims to consolidate all available information regarding the two sporadic grapevine insect pests, *Plangia graminea* (Serville) (Orthoptera: Tettigoniidae) and *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae). Information will be evaluated with regards to the potential biological control of *P. graminea* and *L. vanillana* in an integrated pest management system (IPM) for grapevine.

PLANGIA GRAMINEA

Plangia graminea is considered a minor and sporadic pest of vineyards in South Africa. They are locally known as "krompokkels" (Afrikaans), which translates to "hunched-over fatty", a reference to their hunched-back appearance (Fig. 1.1). In English they are called katydids, although this term generally refers to all species of the Tettigoniidae family.



FIGURE 1.1

Adult (male) of *Plangia graminea*.

A recent study by Doubell *et al.* (2017) referred to *P. graminea* as "chirping katydids", a reference to the sound males make during sexual signalling (stridulation). Together with crickets, katydids belong to the Ensifera suborder (long horned grasshoppers), "ensifer" (Latin) translating to "sword bearing", referring to the blade-like ovipositor of females, while grasshoppers and locusts belong to the Caelifera suborder (short-horned grasshoppers). *Plangia graminea* was previously known as *Plangia compressa* (Walker), but a taxonomic review by Hemp *et al.* (2015) synonymised the two species. *Plangia graminea* is now considered a species complex (Hemp *et al.*, 2015). The holotype for both *P. graminea* and *P. compressa* is lost (Hemp *et al.*, 2015), though a neotype for *P. graminea* was proposed by the same author in a recent study (Hemp, 2017). Despite the number of species described, the *Plangia* Stål genus is still taxonomically unclear and little is known about the biology and ecology of its members (Hemp, 2017).

The *P. graminea* complex is considered widespread in tropical and sub-Saharan Africa (Hemp *et al.*, 2015). Outside of the Cape Winelands region, they are rarely considered pestiferous and thus receive minimal attention. *Searsia angustifolia* (L.) Barkley (Sapindales: Anacardiaceae), previously known as *Rhus angustifolia* L. and commonly known as the Willow Karee or

smalblaar, is believed to be a natural host (Doubell, 2017). Males are slightly smaller than females (Castner & Nickle, 1995) and all *Plangia* species are fully winged (Hemp *et al.*, 2015). Observations since 2012 indicate an increase in katydid abundance and damage intensity, possibly due to changes in agrochemical use or weather conditions (Allsopp, 2012), affecting either katydid persistence or that of its natural enemies, although the exact causes are unknown. Under normal conditions, katydids are unobtrusive and not of much agricultural importance (Annecke & Moran, 1982). In grapevine, nymphs and adults feed on foliage within the canopy (Fig. 1.2).



FIGURE 1.2

Nymphs of *Plangia graminea* on a grapevine leaf, with visible feeding damage.

The entomopathogenic fungi (EPF) *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) was identified from a katydid cadaver (PPRI 12353) by Doubell (2017), and *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae) is available commercially as Bio-Insek[®], for the control of “krompokkel”, mealybug and snoutbeetle (Agro-Organics, 2010). In South Africa, the commercial product, Green Muscle[®] (L6198), with the active ingredient *Metarhizium anisopliae* var. *acridum*, is registered against locust and grasshoppers (Hatting *et al.*, 2018). Generalist predators may include birds, lizards, spiders (F. Le Roux & M. Steyn, Plaisir De Merle, pers. comm., 2016; R. Maree & K. Du Toit, Kanonkop, pers. comm., 2016), and parasitoid wasps (Doubell, 2017).

Entomopathogenic nematodes (EPN), as biocontrol agent, have not yet been evaluated for use in katydid biocontrol. A study by Shim *et al.* (2013) found that early nymphs of the katydid *Paratlanticus ussuriensis* (Uvarov) (Orthoptera: Tettigoniidae) were highly susceptible to *Photorhabdus temperate* Fischer-Le Saux, Viillard, Brunel, Normand & Boemare (Enterobacteriales: Enterobacteriaceae), a symbiotic bacterium of EPNs that belong to the genera of *Heterorhabditis* (Rhabditida: Heterorhabditidae). They found that susceptibility to the bacterium diminished as the nymphs aged, from 93.3% mortality of the first instar to 36.6% of the third instar, while no significant mortality was observed for fourth and fifth instar nymphs, versus the control. No mortalities were observed for adult females, though their fecundity was significantly inhibited, following the ingestion of the bacterium (Shim *et al.*, 2013).

LOBESIA VANILLANA

Lobesia vanillana (Fig. 1.3) is a sporadic pest of wine grapes in the Western Cape of South Africa and has been reported from the Breede River Valley. It does not have a common name and is locally referred to as "*Lobesia* moths".



FIGURE 1.3

(A) Hatching eggs, (B) larva, (C) pupa, (D) adult, and (E) larva hidden within a grape bunch, of *Lobesia vanillana*.

Few records of *L. vanillana* exist in literature, except for some taxonomic and historical locality information. Joseph de Joannis first described the species in 1900 from individuals collected from Réunion Island (De Joannis, 1990), originally named *Conchylis vanillana*, which was later renamed to *Lobesia vanillana* (Krüger, 2007). Two synonyms for this species exist, namely *Lobesia oluducha* Razowski, collected from Nigeria (Razowski & Wojtusiak, 2012), and *Lobesia triancanthis* Diakonoff, collected from Madagascar (Diakonoff, 1992). *Lobesia vanillana* has also been reported from Cosmoledo Island, Aldabra Island (Diakonoff, 1969) and Cerf Island (Gerlach & Matyot, 2006) in the Seychelles, Nigeria (Razowski & Wojtusiak, 2012), Príncipe Island and Tanzania (Razowski & Wojtusiak, 2014), Kenya and Mauritius (Razowski & Brown, 2009), and is otherwise considered widespread in the Afrotropical region (Razowski & Brown, 2009; Brown *et al.*, 2014). Brown *et al.* (2014) reported that although *L. vanillana* was initially described from vanilla plantations (Asparagales: Orchidaceae: *Vanilla planifolia* Jacks. ex Andrews) (De Joannis, 1990) and cashew (Sapindales: Anacardiaceae) (Diakonoff, 1977), they seem to be fairly polyphagous, as they were able to rear it from Rutaceae, Anacardiaceae, Solanaceae and Icacinaceae, among six other plant families (Brown *et al.*, 2014). *Lobesia vanillana* seem to share some biological and ecological features with *Lobesia botrana* (Denis & Schiffermüller), commonly known as the European grapevine moth (EGVM), a similar but much more serious pest native to Europe (Thiéry & Moreau, 2005).

In South Africa, *L. vanillana* has been reported from the Bonnievale, Ashton and McGregor region ($\pm 300 \text{ km}^2$), located in the Breede River Valley of the Western Cape province in South Africa. During the 2013/2014 season, Morland (2015) reported their presence from damaged citrus in the Bonnievale area, where it was attracted to the pheromone lure of carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), and captured using yellow delta sticky traps. During the 2016/2017 season, viticulturists and consultants from the Robertson/McGregor area reported multiple incidences of larval damage in wine grapes, and captured *L. vanillana* in grapevine using similar traps. Morphological analysis (J. Brown, USDA, Smithsonian, Washington, USA) identified the insect as *Lobesia vanillana*, which was confirmed by DNA barcoding (C. Bazelet, Stellenbosch University).

Growers in the Robertson/McGregor region seemed to be the most affected, as feeding damage quickly reached economic thresholds if not properly controlled (J. Lerm, Bemchem Marketing, pers. comm., 2017; K. Van Zyl, AgriRos, pers. comm., 2017). Signs of larval infestation were observed to be similar to that of *L. botrana* (Varela *et al.*, 2010), such as frass, the black beady excrement of larvae as they feed, webbing, the spinning together of plant parts to form a nest,

and bunch rot, the progressive decay of an entire bunch due to damage and secondary infections. Some growers successfully suppressed the *L. vanillana* population using chemical insecticides (R. Wilsdorf, Viking, pers. comm., 2017).

Biological control agents for *L. vanillana* are unknown. Green lacewings and spiders may be generalist predators, and parasitoid wasps may target eggs. For *L. botrana*, biological control agents, such as *Bacillus thuringiensis* (Bt) (Bacillales: Bacillaceae) and products for mating disruption have been registered, and natural enemies, including *Trichogramma* parasitoid wasps, have been identified in Europe (Scaramozzino *et al.*, 2017).

BIOLOGICAL CONTROL

In agricultural ecosystems, the structure and function of natural enemy complexes are typically restricted, resulting in sub-optimal natural pest suppression (Landis *et al.*, 2000). Biological control can be considered as the deliberate intervention to restore or enhance these interactions in the favour of natural enemies (Gullan & Cranston, 2014), for example in IPM programs. The goal of such programs is often not aimed at pest eradication, but rather towards the suppression of insect damage to below economic damage thresholds (Kogan, 1998).

EPN and EPF are small organisms that infect their insect hosts through natural openings or through their cuticle. EPNs occur in soil across the world and are considered useful for the biological control of insect pests. Important species include those of Heterorhabditidae and Steinernematidae (Rhabditida), which are associated with symbiotic gut bacteria of the genera *Photorhabdus* and *Xenorhabdus* (Enterobacteriales: Enterobacteriaceae), respectively (Stock & Goodrich-Blair, 2012), which together are pathogenic to insects (Kaya & Gaugler, 1993).

Their infective juveniles (IJs) (special third instar, also known as the dauer stage) are free-living and survive outside their host in moist environments, typically in the soil, where they are natural enemies to many belowground insect life stages (Shapiro-Ilan *et al.*, 2016). After penetration, they release their symbiotic bacteria in haemocoel of the host, where it multiplies rapidly and kills the host within 48 h (Fig. 1.4). Nematodes feed off the bio-converted cadaver and reproduce *in vivo* for multiple generations, depending on the size of the insect (Dillman & Sternberg, 2012). These non-IJ stages cannot survive outside their host. When food resources become scarce, third stage juveniles turn into a new cohort of IJs, with the ability to seek out and infect new hosts (Shapiro-Ilan *et al.*, 2016).

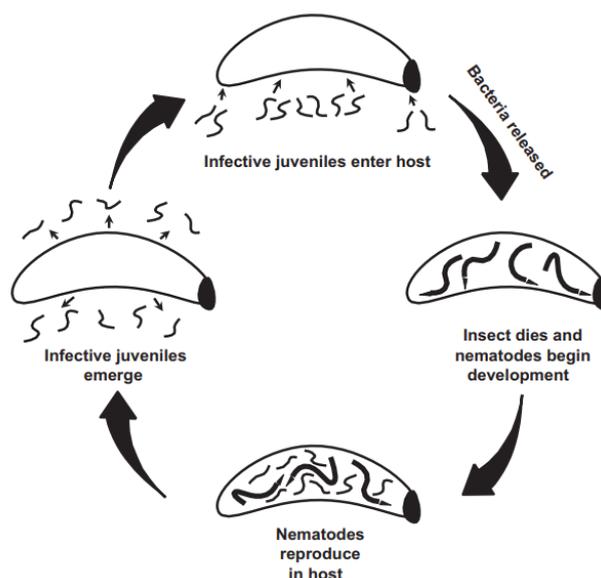


FIGURE 1.4

The life cycle of entomopathogenic nematodes. Diagram taken from Shapiro-Ilan *et al.* (2016).

The virulence of these pathogens is usually correlated with host abundance and their associated microclimate (Shapiro-Ilan *et al.*, 2006). In agricultural applications, they are used as inundative control (similar to chemical insecticides), but in favourable conditions they can successfully establish to provide persistence (i.e. inoculative control) (Dillman & Sternberg, 2012). Nematodes can be mass-cultured using *in vivo* (Van Zyl & Malan, 2015) or *in vitro* (Ferreira *et al.*, 2014; 2016, Dunn & Malan, 2019) techniques and IJs can be applied using conventional spray equipment (Shapiro-Ilan *et al.*, 2006).

Insects without a soil stage may be more susceptible to EPNs, as they may not have had the opportunity to evolve the resistance necessary to protect them from nematode infections. This weakness of above-ground pest defence mechanisms against microbiological pathogens can thus be exploited to provide biological control opportunities, for example as previous research on mealybugs (Van Niekerk & Malan, 2012; Le Vieux & Malan, 2013; Platt *et al.*, 2018) has shown. Above-ground applications, however, do not provide the moisture and temperature buffer that soil provides, influencing nematode survival, efficacy and reliability (Arthurs *et al.*, 2004; Lacey & Georgis, 2012). EPNs have also been evaluated for use against lepidopteran pests of South Africa, such as the false codling moth, *Thaumatotibia leucotreta* L. (Meyrick) (Malan *et al.*, 2011; Malan & Moore, 2016) and the codling moth, *Cydia pomonella* L.

(Lepidoptera: Tortricidae) (De Waal *et al.*, 2011; Odendaal *et al.*, 2016a), with promising results for field applications (Odendaal *et al.*, 2016b; Steyn, 2019).

The possibility of applying biologicals, such as EPNs and EPF, to hot-spot areas and during sporadic pest outbreaks of *P. graminea* and *L. vanillana*, would be of great advantage. These agents use a 'softer' approach to suppress pest populations, relative to the harsher (and often non-registered) chemicals currently used, and they do not disturb natural enemies or interfere with IPM practices in the vineyard.

AIMS AND OBJECTIVES

The aim of the study was to collect basic ecological data for *P. graminea* in grapevine in the Western Cape province and to evaluate EPNs as potential biocontrol agents of *P. graminea* and *L. vanillana*. These two sporadic pests were highlighted as priority pests by the wine grape industry, from which funding for this study was obtained.

The objectives of the study were:

1. To evaluate the biocontrol potential of various entomopathogenic nematodes against *Plangia graminea*; and
2. To evaluate the biocontrol potential of *in vivo* and *in vitro*-cultured *Steinernema yirgalemense* and *S. jeffreyense* against *Lobesia vanillana*

Each objective was written as a separate chapter, therefore some repetition was unavoidable. The format of the *South African Journal of Enology and Viticulture* was followed. Two appendices are included which feature key qualitative observations on these pests in the laboratory and in the field, for which very little information is currently available.

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CHAPTER 2:

Entomopathogenic nematodes for the control of *Plangia graminea* (Orthoptera: Tettigoniidae) under laboratory conditions

ABSTRACT

Plangia graminea, known as katydids or “krompokkels”, is a minor pest of vineyards in the Western Cape province of South Africa, where they feed primarily on grapevine foliage. In natural conditions, katydids are not of much agricultural importance, but pest outbreaks during favourable conditions can result in significant foliar damage. Observations indicate an increase in katydid abundance and damage intensity in recent years. There are at present no agrochemicals registered for the control of this species and current natural enemies are unlikely to provide sufficient control without augmentation. In this study, 12 entomopathogenic nematode (EPN) species have been evaluated against the nymphs of *P. graminea* in laboratory bioassays. Mortality by infection, and the reproductive potential of nematodes in the host were investigated. Seven locally occurring nematode species achieved significant mortality, of which *H. zealandica*, *H. indica*, *S. jeffreyense* and *S. yirgalemense* performed the best (> 90% mortality). High infectivity of EPN species tested against *P. graminea* nymphs were found, with the ability of nematodes to complete their life-cycle within the host confirmed.

Keywords: grapevine, *Heterorhabditis*, katydids, *Steinernema*

INTRODUCTION

Plangia graminea (Serville) (Orthoptera: Tettigoniidae) is considered a minor and sporadic pest of vineyards in South Africa, locally known as "krompokkels" (Afrikaans) or generally as katydids, and primarily feed on the foliage of grapevine. Nymphs seem to mimic black beetles (barring their long antennae) to evade predation, while adults camouflage well within the leafy canopy, which makes their monitoring, especially that of the adults, difficult.

Like most katydids of the Phaneropterinae subfamily, *P. graminea* is believed to have only one generation per year (Bailey & Rentz, 1990) and that the soil is not utilised for any of its life stages (Appendix 1). Observations since 2012 indicate an increase in katydid abundance and damage intensity, possibly due to changes in agrochemical use or weather conditions (Allsopp, 2012). Katydids seem to occur throughout most of the Western Cape province, especially in the Cape Winelands region (Appendix 1), but due to their inconspicuous nature, their distribution is likely underestimated.

In grapevine, the eggs of *P. graminea* were observed to start hatching in early spring (September), during the onset of bud break. Nymphs underwent 3-4 moults over the course of three months and the population peaked between late-October and November. Adults emerged starting late-November, and by December, at least half of the katydid population matured into adults (Appendix 1; Doubell, 2017). Adults were observed to be highly mobile, with the ability to disperse to and lay eggs in adjacent blocks or vegetation. Females of *P. graminea* lay their eggs under the bark of vine, in contrast to other Phaneropterinae that lay their eggs in-between the epidermal layer of leaves (Picker *et al.*, 2004). Their eggs overwinter until spring of the following season (Appendix 1; Doubell, 2017). Pest outbreaks in vineyards can cause significant foliar destruction, which degrades the vigour and growth of vines, in turn also affecting grape berry health and quality. These outbreaks seem concentrated around certain hotspots within the Cape Winelands region, but the causative factors are still unknown (Appendix 1).

Generalist predators may include birds, lizards, spiders (F. Le Roux & M. Steyn, Plaisir De Merle, pers. comm., 2016; R. Maree & K. Du Toit, Kanonkop, pers. comm., 2016), and parasitoid wasps (Doubell, 2017). The entomopathogenic fungi (EPF) *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) was identified from a katydid cadaver (PPRI 12353) by Doubell (2017), and *Beauveria bassiana* (Bals.-Criv) Vuill. (Hypocreales: Cordycipitaceae) is available commercially as Bio-Insek[®] for the control of "krompokkel",

mealybug and snoutbeetle (Agro-Organics, 2010). In South Africa, the commercial product, Green Muscle® (L6198), with *Metarhizium anisopliae* var. *acridum* as the active ingredient, is registered against locust and grasshoppers (Hatting *et al.*, 2018)

Entomopathogenic nematodes (EPN) are insect parasitic roundworms that occur naturally in soil across the world. Nematodes of Heterorhabditidae and Steinernematidae (Rhabditida) are associated with the symbiotic bacteria *Photorhabdus* and *Xenorhabdus* (Enterobacteriales: Enterobacteriaceae) respectively (Stock & Goodrich-Blair, 2012), which together are pathogenic to insects (Kaya & Gaugler, 1993). Through inundative releases, these nematodes can be utilised as biological control agents against a wide range of insect species, as they achieve significant mortality within 48 h, find insects in cryptic habitats and have the ability to persist in the environment after their application (Dillman & Sternberg, 2012).

Local research evaluated above-ground applications against adults of banded fruit weevil, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) (Ferreira & Malan, 2014; Dlamini *et al.*, 2019), vine mealybug, *Planococcus ficus* (Signoret) (Le Vieux & Malan, 2013, 2015; Platt *et al.*, 2018, 2019a, b) citrus mealybug, *Planococcus citri* (Risso) (Van Niekerk & Malan, 2012) and codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) (De Waal *et al.*, 2011, 2013; Odendaal *et al.*, 2016a, b). The diapausing larval population of codling moth overwinters in cryptic habitats, such as in old pruning wounds and cracks in the bark of apple trees, which offers an opportunity for using nematodes as a biological control agent before their emergence the next growing season. EPNs were also evaluated in laboratory and field bioassays against false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Malan *et al.*, 2011; Malan & Moore, 2016; Steyn, 2019), and Mediterranean fruit fly, *Ceratitidis capitata* (Wiedemann) (Malan & Manrakhan, 2009; James *et al.*, 2018).

EPNs have not yet been evaluated as a biocontrol agent for katydids. A study by MacVean & Capinera (1992) evaluated *Steinernema carpocapsae* (Weiser) Wouts, Mráček, Gerdin & Bedding against the Mormon Cricket *Anabrus simplex* Hald. (Orthoptera: Tettigoniidae), but the nematode did not successfully infect or reduce survival of the crickets.

The aim of this study was to evaluate the pathogenicity of *in vivo* cultured South African species (and one exotic species) of EPNs against the nymphs of *P. graminea*. Screening was conducted under optimum laboratory conditions and mortality by infection confirmed. Reproduction of the nematode in the insect cadaver was investigated.

MATERIALS AND METHODS

Source of insects

Nymphs of *P. graminea* were obtained from multiple wine grape vineyards in the Western Cape province. Three sites with a persistent katydid presence were prioritised for the collection of katydids during the summer months of 2016 and 2017 (Fig. 2.1).

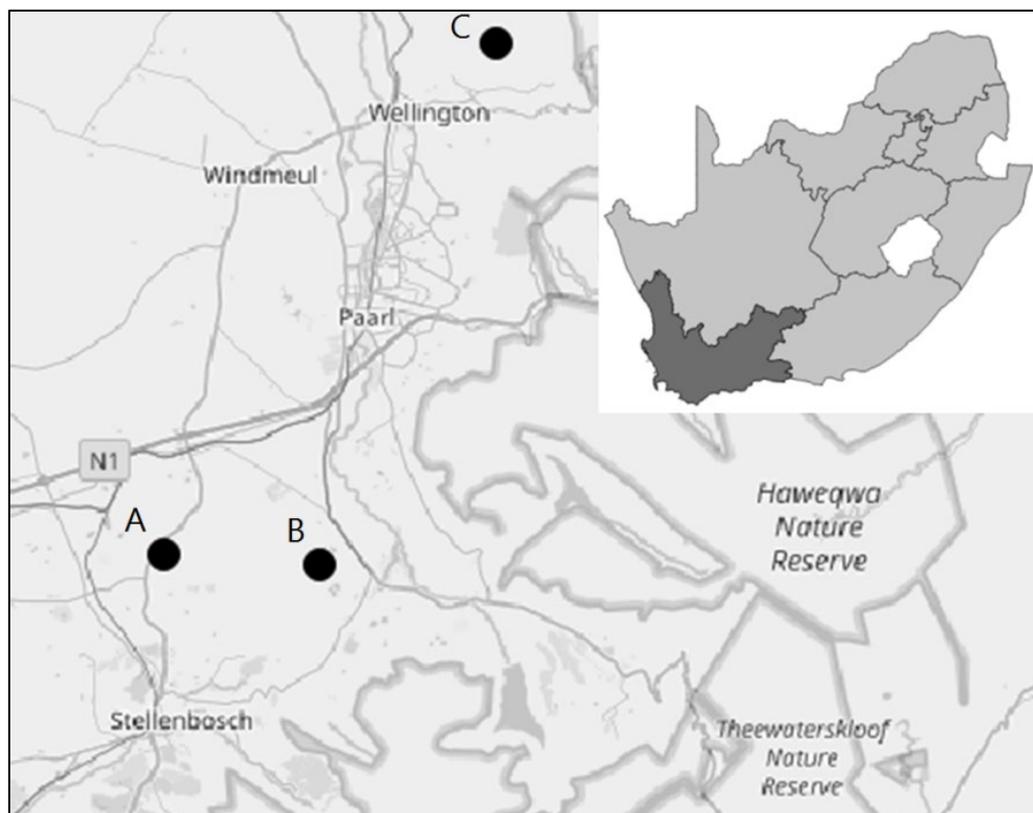


FIGURE 2.1

Map of katydid nymph collection sites in the Western Cape province, South Africa. Locations are approximate: (A) 10 km north of Stellenbosch, (B) 2 km west of Simondium, (C) 4 km north-east of Wellington.

Nymphs were collected using rigid cylindrical plastic containers with perforated lids, and taken to the laboratory of the Department of Conservation Ecology and Entomology at Stellenbosch University, with the aim of establishing a laboratory colony. This colony never successfully stabilised, and field collected individuals were prioritised for laboratory bioassays (Appendix 1). Individuals were kept for a minimum of one and maximum of three days prior to bioassays, first to assess their health and to discard unsuitable individuals, and second, to limit the deterioration of their health over time due to causes currently unknown.

Source of nematodes

The EPNs used in the present study were obtained from the nematode collection stored at the Department of Conservation Ecology and Entomology, Stellenbosch University (Table 2.1). All species were locally isolated, except for *Steinernema feltiae* (Filipjev) Wouts, Mráček, Gerdin & Bedding, which is an exotic species. Infective juveniles (IJ) were cultured *in vivo* using last instar larvae of either the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) or of the common mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), at room temperature using the methods described by Stock & Goodrich-Blair (2012) and Van Zyl & Malan (2015).

Infective juveniles were harvested over the course of 2 weeks, transferred to vented culture flasks (50 ml rec. max, NUNC) and stored horizontally at 14°C. Culture flasks were shaken biweekly to mitigate nematode clumping and to aerate the mixture. Nematodes were used within 3 weeks of their culture and inspected for fitness (motility, mortality) prior to bioassays.

TABLE 2.1

Heterorhabditis and *Steinernema* nematode species used against *Plangia graminea* nymphs, including Genbank accession number and nematode size.

Nematode species	Isolate	Genbank ID	Origin	GPS location (DMS)	Associated host plant	Mean length of IJ (µm)	Reference
<i>H. bacteriophora</i>	SF351	FJ455843	Wellington, Western Cape	33°36'24"S 18°59'48"E	Grapevine	588 (512-671)	Malan <i>et al.</i> , 2006
<i>H. baujardi</i>	MT19	MF535520	KwaZulu-Natal	n/a	Natural vegetation	551 (497-595)	Abate <i>et al.</i> , 2018
<i>H. indica</i>	SGS	KU945293	Bonnievale, Western Cape	33°55'38"S 20°00'35"E	Grapevine	528 (497-573)	n/a
<i>H. noenieputensis</i>	SF669	JN620538	Noenieput, Northern Cape	27°16'15"S 20°03'05"E	Fig	528 (484-563)	Malan <i>et al.</i> , 2014
<i>H. zealandica</i>	SF41	EU699436	Brenton-on-Sea, Western Cape	33°41'28"S 24°35'23"E	Natural vegetation	685 (570-740)	Malan <i>et al.</i> , 2006
<i>S. feltiae</i> *	S. fel	-	Germany	n/a	n/a	876 (766-928)	n/a
<i>S. innovationi</i>	SGI-60	KJ578793	Free State	n/a	Grain	1053 (1000-1103)	Hatting <i>et al.</i> , 2009
<i>S. jeffreyense</i>	J192	KC897093	Jeffrey's Bay, Eastern Cape	34°02'43"S 24°55'35"E	Guava	924 (784-1043)	Malan <i>et al.</i> , 2011
<i>S. khoisanae</i>	SF87	DQ314287	Villiersdorp, Western Cape	33°12'33"S 19°06'57"E	Apple	1062 (994-1159)	Nguyen <i>et al.</i> , 2006
<i>S. litchii</i>	WS9	KP325086	Mbombela, Mpumalanga	25°30'56"S 30°58'41"E	Litchi	1054 (953-1146)	Steyn <i>et al.</i> , 2017a
<i>S. sacchari</i>	SB10	KC633095	Gingindlovu, KwaZulu-Natal	29°01'37"S 31°35'37"E	Sugarcane	680 (630-722)	Nthenga <i>et al.</i> , 2014
<i>S. yirgalemense</i>	157-C	EU625295	Friedenheim, Mpumalanga	25°27'50"S 30°59'16"E	Citrus	635 (548-693)	Malan <i>et al.</i> , 2011

*Imported species from e-nema, Germany; n/a = not available

Screening

Bioassays were prepared using six-well bioassay plates (BioLite 6-Well Multidish, Thermo Scientific), lined with one circular filter paper disk (30 mm, Grade 1 Whatman, GE Healthcare Life Sciences) per well. The concentration of nematodes was determined using the technique of Glazer & Lewis (2000). A nematode concentration of 200 IJs per 100 μ l of water was inoculated onto each circular disk, while the control received 100 μ l of distilled water only. The number of trays used was scaled to utilise the maximum number of insects available at the time of bioassays, and to prevent the health deterioration often observed in "older" laboratory katydids, which may influence their susceptibility to infection.

Katydid nymphs were added to the wells using soft metal forceps, and a glass rectangle was placed over the tray as each well was filled. Using sleight of hand, the original tray cover was slid in place of the glass cover once all wells were filled. Rubber bands secured the tray lids and trays were transferred into 2-L plastic ice cream containers, each lined with paper towels moistened with distilled water, and closed with their lid to maintain high humidity. The containers were then incubated in a growth chamber at 25°C, in the dark, for 48 h. The mortality of katydids was determined by gently poking the insect with forceps. Dissection kit equipment, glassware and other potential sources of contaminants, were submerged in boiling water and dried prior to the handling of each treatment and batch.

All treatments had repetitions on different days, except for *H. baujardi*, *H. zealandica*, *S. innovationi* and *S. khoisanae*, which had none, due to a shortage of katydids. The number of katydids used for each treatment, per repetition, is listed in Table 2.2. Treatment repetitions were carried out on different days and each repetition had a control group present.

TABLE 2.2

Number of insects used for each treatment with *Heterorhabditis* and *Steinernema* species, per repetition, in the screening bioassay.

Treatment	Isolate	R1	R2	R3	R4	R5	Total
Control	-	24	18	8	36	42	128
<i>H. bacteriophora</i>	SF351	12	18		12	18	60
<i>H. baujardi</i>	MT19					12	12
<i>H. indica</i>	SGS				12	30	42
<i>H. noenieputensis</i>	SF669	12	18		12	30	72
<i>H. zealandica</i>	SF41					12	12
<i>S. feltiae</i>	S. fel				12	18	30
<i>S. innovationi</i>	SGI-60				12		12
<i>S. jeffreyense</i>	J192			12	18	30	60
<i>S. khoisanae</i>	SF87				18		18
<i>S. litchii</i>	WS9				12	18	30
<i>S. sacchari</i>	SB10			12	18		30
<i>S. yirgalemense</i>	157-C		18	6	18	42	84

Penetration and reproduction

Following screening bioassays, cadavers of the different treatments were placed on a sieve, gently rinsed with a handheld water jet and patted dry on hand towel paper to remove surface nematodes. Cadavers were then placed in 90 mm diameter petri dishes lined with one circular filter paper disk (85 mm, Grade 1 Whatman, GE Healthcare Life Sciences), inoculated with 800 µl of distilled water and incubated at 25°C and > 95% RH in the dark for 24-36 h, to allow for IJ growth and development. The infectivity of nematodes was determined by dissecting cadavers and evaluating the presence of nematodes.

Large sample sizes necessitated the storage of cadavers at 14°C to slow the growth and development of nematodes to evaluate IJ penetration on a later day. Second generation nematodes did develop within cadavers, despite their cooling, and were noted as it confirms the ability of nematodes to complete their life cycle *in vivo*.

Data analysis

Data were analysed in Microsoft Excel 2010 for descriptive statistics and processed in Statistica 13.3 (TIBCO Software Inc., 2017) for comparative analysis. For the screening bioassay, residuals of the mortality response were considered normally distributed (Shapiro-Wilk's $W = 0.984$, $p = 0.267$), permitting the use of a one-way ANOVA, however Levene's test for

homogeneity of variances failed, necessitating the use of a Games-Howell post hoc analysis to evaluate the response between nematode species. Results are given as the mean response of all repetitions \pm SEM, unless otherwise specified.

RESULTS

Screening

The highest percentage mortality was obtained by *Heterorhabditis zealandica* Poinar (n = 12; 100%), *Heterorhabditis indica* Poinar, Karunakar & David (n = 42; 95.24% \pm 3.07%), *Steinernema jeffreyense* Malan, Knoetze & Tiedt (n = 60; 93.33% \pm 3.69%) and *Steinernema yirgalemense* Kguyen, Tesfamariam, Gozel, Gaugler & Adams (n = 84; 91.67% \pm 3.81%), with no significant differences between each other, but significantly different from the control (p < 0.01). *Heterorhabditis noenieputensis* (n = 72; 70.83% \pm 9.65%, p = 0.034) and *Heterorhabditis bacteriophora* Poinar (n = 60; 65% \pm 8.03%, p = 0.041) also achieved significant mortality relative to the control treatment (n = 128; 25% \pm 3.7%). *Heterorhabditis zealandica* was significantly different from *H. bacteriophora* (p = 0.047), but no other treatments were significantly different from each other (Fig. 2.2).

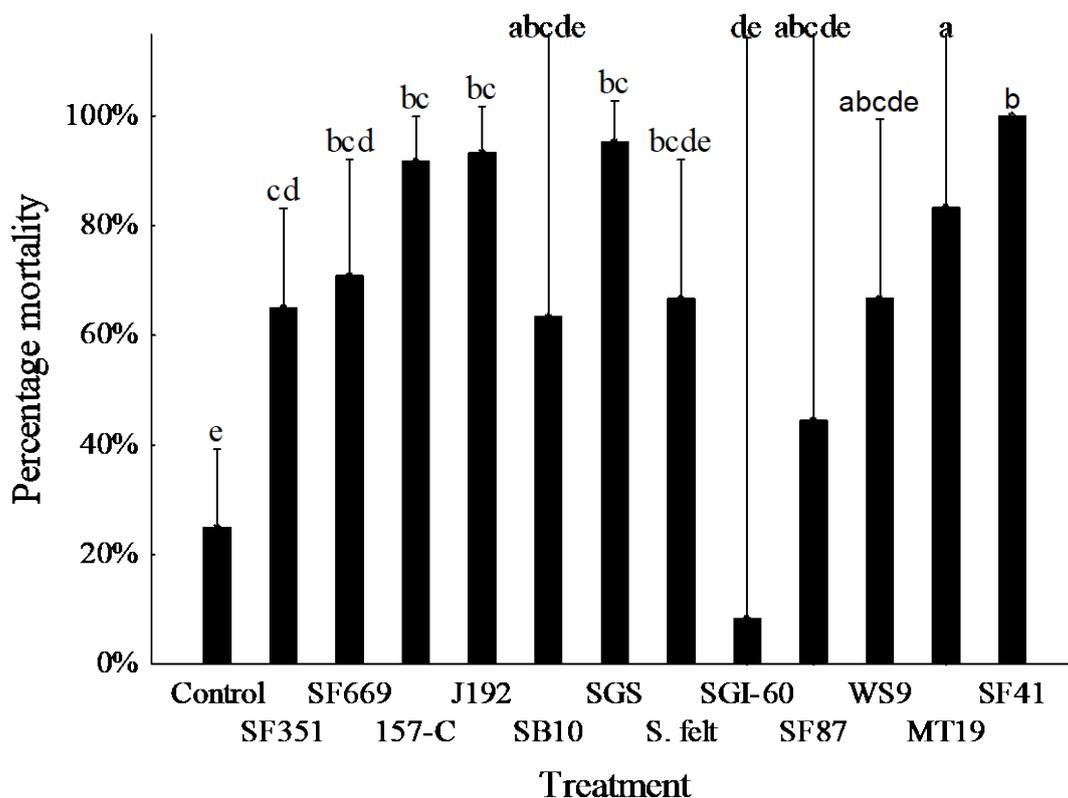


FIGURE 2.2

Percentage mortality (95% confidence intervals) of *Plangia graminea* nymphs, 48 h after inoculation with 200 IJ/100 μ l of *Heterorhabditis bacteriophora* (SF351), *H. noenieputensis* (SF669), *Steinernema yirgalemense* (157-C), *S. jeffreyense* (J192), *S. sacchari* (SB10), *H. indica* (SGS), *S. feltiae* (S. fel), *S. innovationi* (SGI-60), *S. khoisanae* (SF87), *S. litchii* (WS9), *H. baujardi* (MT19) and *H. zealandica* (SF41). Vertical bars were calculated using weighted means, while differing letters denote significance, calculated using a Games-Howell post hoc analysis (Error between MS = 0.717; df = 86; $p < 0.05$).

Steinernema feltiae (Filipjev) Wouts, Mráček, Gerdin & Bedding (n = 30; 66.67% \pm 9.13%), *Steinernema litchii* Steyn, Knoetze, Tiedt & Malan (n = 30; 66.67% \pm 11.79%), *Steinernema sacchari* Nthenga, Knoetze, Berry, Tiedt & Malan (n = 30; 63.33% \pm 19.29%), *Steinernema khoisanae* Nguyen, Malan & Gozel (n = 18; 44.44% \pm 29.4%) and *Steinernema innovationi* Çimen, Lee, Hatting, Hazir & Stock (n = 12; 8.34% \pm 8.34%) did not differ significantly from the control. *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (n = 12; 83.34% \pm 16.67%) achieved high average mortality, but did not compute in the Games-Howell post-hoc

analysis due to high variance within the treatment, and thus comparative analysis for this species was not possible.

Penetration

The cadavers of *P. graminea* nymphs inoculated with *S. innovationi*, *S. khoisanae* and *S. sacchari* were lost following screening bioassays and neither penetration nor reproduction could be confirmed for these species. In all other treatments, the presence of nematodes was confirmed for at least 70% of cadavers (Fig 2.3).

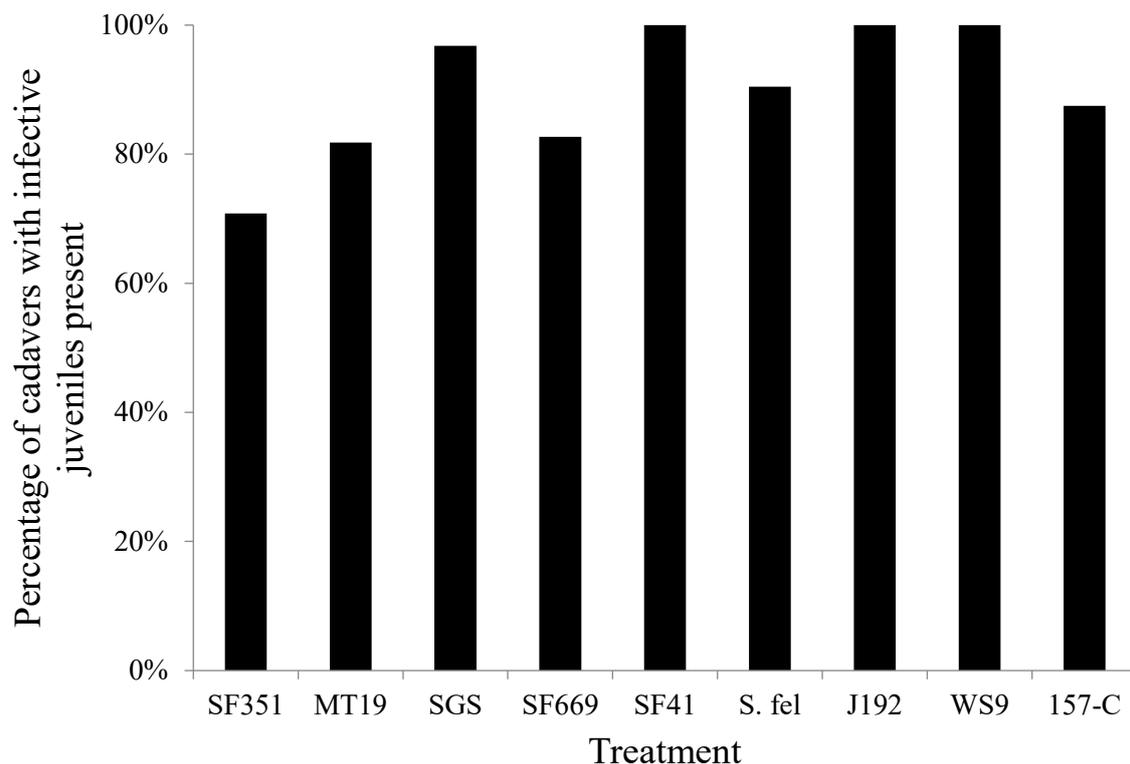


FIGURE 2.3

Percentage of *Plangia graminea* nymph cadavers following the screening bioassay with infective juveniles present, for *Heterorhabditis bacteriophora* (SF351), *H. baujardi* (MT19), *H. indica* (SGS), *H. noenieputensis* (SF669), *H. zealandica* (SF41), *Steinernema feltiae* (S. fel), *S. jeffreyense* (J192), *S. litchii* (WS9) and *S. yirgalemense* (157-C).

Heterorhabditis zealandica (n = 12), *S. jeffreyense* (n = 20) and *S. litchii* (n = 18), had nematodes present in 100% of cadavers, followed by *H. indica* (n = 31; 97%), *S. feltiae* (n = 21; 90%), *S. yirgalemense* (n = 56; 88%), *H. noenieputensis* (n = 52; 83%), *H. baujardi* (n = 11; 82%) and *H. bacteriophora* (n = 48; 71%).

Reproduction

Nematodes within the host cadaver were able to complete their lifecycle *in vivo* and produce second generation offspring with varying success (Fig. 2.4).

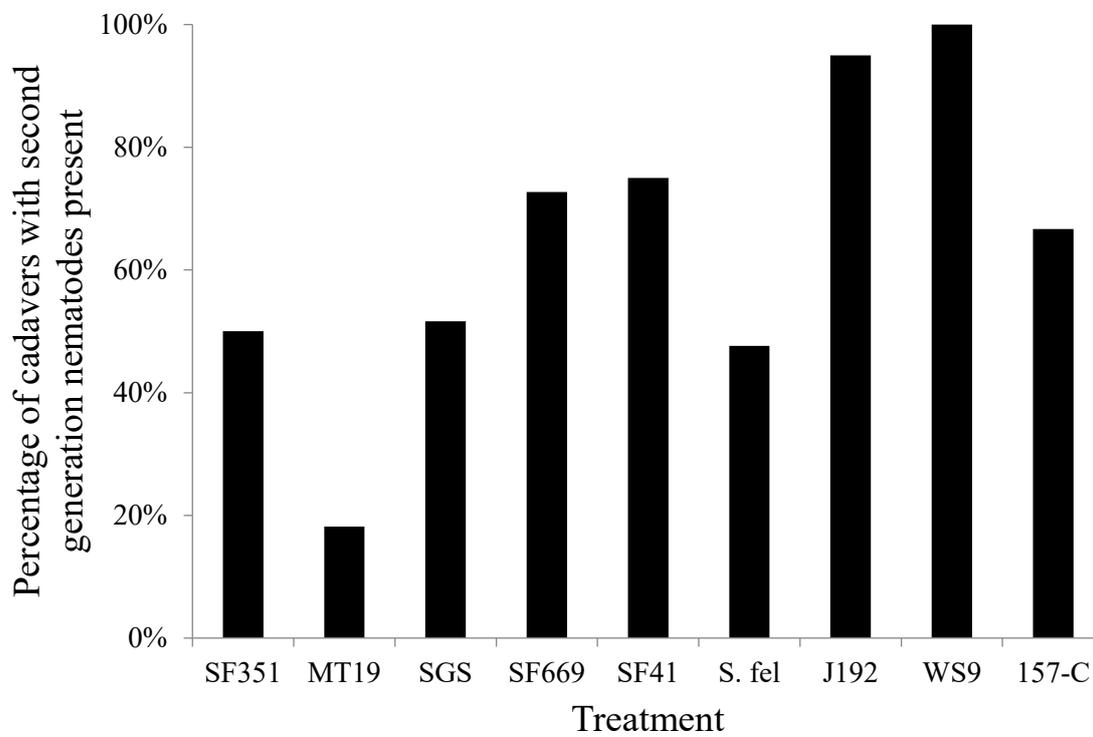


FIGURE 2.4

Percentage of *Plangia graminea* nymph cadavers, following the screening bioassay, with second generation nematodes present for *Heterorhabditis bacteriophora* (SF351), *H. baujardi* (MT19), *H. indica* (SGS), *H. noenieputensis* (SF669), *H. zealandica* (SF41), *Steinernema feltiae* (S. fel), *S. jeffreyense* (J192), *S. litchii* (WS9) and *S. yirgalemense* (157-C).

Second generation nematodes were confirmed in *P. graminea* cadavers inoculated with *S. litchii* (n = 18; 100%); *S. jeffreyense* (n = 20; 95%), *H. zealandica* (n = 12; 75%), *H. noenieputensis* (n = 22; 73%), *S. yirgalemense* (n = 39; 67%), *H. indica* (n = 31; 52%), *H. bacteriophora* (n = 20; 50%), *S. feltiae* (n = 21; 48%) and *H. baujardi* (n = 11; 18%).

DISCUSSION

This is the first study to evaluate the biocontrol potential of EPNs for the control of *Plangia graminea*. In the present study, 12 *in vivo* cultured EPN species were evaluated using a total of 590 katydid nymphs. Five EPN species, namely *H. indica*, *H. zealandica*, *S. jeffreyense*, *S.*

yirgalemense and *H. baujardi*, achieved > 80% control in the laboratory environment, while *H. noenieputensis* (71%) and *H. bacteriophora* (65%) performed significantly better than the control (25%). *Heterorhabditis zealandica* performed significantly better than *H. bacteriophora*, but there were no other statistically significant differences between other treatments. This lack of significance is likely due to relatively high control group mortality and variation within treatments, which can be resolved in future bioassays by increasing the sample size and number of repetitions, in addition to limiting control group mortality.

The most effective five EPN species, namely *H. indica*, *H. zealandica*, *S. jeffreyense*, *S. yirgalemense* and *H. baujardi*, had nematodes present in $\geq 82\%$ of cadavers, supporting mortality caused by EPN infection, and suggests high pathogenicity of these EPN species against nymphs of *P. graminea*. In addition, second generation nematodes were discovered in cadavers of *H. indica* (52%), *H. zealandica* (75%), *S. jeffreyense* (95%) and *S. yirgalemense* (67%) treatments, but with low presence in the *H. baujardi* (18%) treatment. This indicates that nematodes have the ability to complete their life cycle and produce new generations *in vivo*, theoretically able to produce a new cohort of IJs capable of finding and infecting new insect hosts. Cadavers were not suitable for evaluating IJ production by means of a modified White trap (White, 1927), due to the destructive nature of dissection. Mortality of *P. graminea* ascribed to *H. baujardi* could not be evaluated statistically, due to high variability and low sample sizes within the treatment results, while the treatments of *S. feltiae*, *S. litchii*, *S. sacchari*, *S. khoisanae* and *S. innovationi* did not result in significant mortality relative to the control.

Of the five EPN species with high mortality, three belong to *Heterorhabditis* and two to *Steinernema*. Two of these species, *H. baujardi* and *H. indica*, have not yet been tested against pests associated with grapevine. During South African surveys for the occurrence of EPNs, *H. bacteriophora* was found to be the most common species (Malan *et al.*, 2006, 2011; Hatting *et al.*, 2009) and is the only EPN used in the current study available commercially (Hatting *et al.*, 2018). The methods of mass culturing this EPN have been well documented (Shapiro-Ilan & Gaugler, 2002; Inman *et al.*, 2012) and it has been evaluated against multiple species since its discovery in 1976 (Smart, 1995; Van Lenteren, 2012). However, in the present study, it was found that mortality against katydids using *H. bacteriophora* was relatively low (65%), compared to the other local isolates tested.

Heterorhabditis baujardi was recently reported from South Africa from two independent surveys (Steyn *et al.*, 2017b; Abate *et al.*, 2018), both claiming it to be the first record of this species. It was also reported from Cameroon in Africa (Kanga *et al.*, 2012). The potential of mass culturing *H. baujardi* as a biocontrol agent is unknown.

Heterorhabditis indica was reported from Bonnievale, located in the Western Cape province of South Africa, during 2016 (KU945293). Ehlers *et al.* (2000) successfully mass cultured *H. indica* from isolates in India, and the success of this nematode to infect *P. graminea* in the laboratory encourages future research on this heterorhabditid for local mass culture. It would be beneficial to evaluate this EPN against other insect pests of grapevine to determine its biocontrol potential.

Heterorhabditis zealandica was the best performing EPN in the present study, and success in the mass culture of this nematode has been demonstrated by Ferreira *et al.* (2014). This species was previously evaluated against banded fruit weevil (Ferreira & Malan, 2014; Dlamini *et al.*, 2019) and false codling moth (Malan *et al.*, 2011; Malan & Moore, 2016), both of which are key pests of grapevine. Research on the mass culture and application of *H. zealandica* in integrated pest management programmes will also likely benefit its control potential against katydids.

Steinernema jeffreyense was previously evaluated against codling moth and false codling moth in both laboratory and field environments (De Waal *et al.*, 2011, 2013; Odendaal *et al.*, 2016a; Steyn, 2019), as well as against vine mealybug (Platt *et al.*, 2018, 2019a, b). Methods of mass culturing this nematode species was demonstrated by Dunn and Malan (2019), and its application in grapevine is likely to provide simultaneous control of several insect pests.

Steinernema yirgalemense was previously evaluated against false codling moth in laboratory and field environments (Malan *et al.*, 2011; Steyn, 2019), codling moth (De Waal *et al.*, 2011) and mealybugs (Van Niekerk & Malan, 2012; Le Vieux & Malan, 2013, 2015; Platt *et al.*, 2018). It has also been evaluated as an above-ground application for the control of codling moth (Odendaal *et al.*, 2016b). This nematode is currently regarded as the best candidate for a biological control agent in South Africa, due to its demonstrated pathogenicity against multiple insect pests. It has also been successfully mass cultured, and research on its production (Ferreira *et al.*, 2016) and formulation (Kagimu, 2018) is currently underway.

A significant constraint in the present study was the number of katydids available for bioassays. High mortality in the laboratory colony necessitated the use of field collected individuals, as

strategies to increase katydid fitness and survival did not result in satisfactory results. Treatments and repetitions were scaled to use the maximum number of katydids available, which resulted in unequal sample sizes between treatments and repetitions. This sensitivity to suboptimal environments likely resulted in the higher control group mortality and variance within treatments observed in the present study. As a result, efficacy may have been underestimated, resulting in suppressed statistical significance. It poses the question, however, whether and to what extent the susceptibility of katydid nymphs to EPN infection is influenced by environmental conditions in the laboratory, and how this would translate to field applications, which may better resemble their natural habitat.

It is recommended that future research evaluate the field efficacies of the best performing EPN species of the present study, and to also evaluate their *in vitro* produced counterparts where available. This may help establish an EPN control profile for *P. graminea* and motivate further research, development and investment into biological control agents for insect pest control and its adoption into integrated pest management programmes.

In conclusion, results of the present study show high susceptibility of *P. graminea* to *H. indica*, *H. zealandica*, *S. jeffreyense*, *S. yirgalemense* and *H. baujardi*, which in turn confirms their pathogenicity. It also demonstrated that nematodes of the aforementioned species are able to penetrate and complete their life cycle within the host, as well as to produce second generation nematodes, suggesting their ability to produce a new cohort of IJs able to find and infect new hosts.

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CHAPTER 3:

Evaluating *in vivo* and *in vitro* cultured entomopathogenic nematodes to control *Lobesia vanillana* (Lepidoptera: Tortricidae) under laboratory conditions

ABSTRACT

Lobesia vanillana is a sporadic pest of grapevine in the Western Cape province of South Africa. Growers experienced an increasing number of pest outbreaks in recent years, necessitating the use of chemical control. Markets are increasingly sensitive towards traditional agrochemicals, which creates a demand for affordable and sustainable biological control options. The *in vivo* and *in vitro* cultured South African entomopathogenic nematodes (EPNs), *Steinernema yirgalemense* and *S. jeffreyense* (Rhabditida: Steinernematidae), were evaluated against larvae of *L. vanillana* in laboratory bioassays. High mortality was observed for all treatments. *In vitro*-cultured *Steinernema yirgalemense* (98%) performed significantly better than *S. jeffreyense* (73%), while within *in vivo* cultures, there was no significant difference between nematode species (both 83%). No significant difference was detected between *in vivo* and *in vitro* cultures of the same nematode species. The LD₅₀ of the *in vitro*-cultured *S. yirgalemense*, the best performing species of the present study, was calculated by probit analysis and estimated as 7.33 nematodes per larva. Mortality by nematode infection was confirmed by the dissection of cadavers and confirming the presence of nematodes, which was > 90% for all treatments. Within *in vitro* cultures, both *S. yirgalemense* and *S. jeffreyense* were able to produce a new cohort of infective juveniles from host cadavers. The relative success of local *in vivo* and *in vitro*-cultured EPN species against South African tortricid species in laboratory and field assays, is encouraging for further research and development of this technology

Keywords: grapevine, *in vivo*-cultured, *in vitro*-cultured, *Lobesia*, *Steinernema jeffreyense*, *Steinernema yirgalemense*

INTRODUCTION

Lobesia vanillana (De Joannis) (Lepidoptera: Tortricidae), is a recent, sporadic pest of grapevine in the Western Cape province of South Africa. Viticulturists and farm consultants witnessed an increase of damage in recent years, necessitating the use of insecticides for its control. Apart from some taxonomy and locality information, very few records of *L. vanillana* exist in literature (Chapter 1). They seem to occur sporadically throughout the Afrotropical region (Razowski & Brown, 2009) and have been reported to be polyphagous (Brown *et al.*, 2014).

In South Africa, Morland (2015) reported the sporadic presence of *L. vanillana* in damaged fruit and pheromone traps of carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), in the Bonnievale/Robertson area, located in the Breede River Valley. During the 2016/2017 season, growers of the Roberson/McGregor area reported multiple incidences of larval damage in grapevine berries and adult moth catches in carob moth baited traps. Pest outbreaks resulted in economic losses, which necessitated the use of chemical insecticides for its control (K. Van Zyl, AgriRos, pers. comm., 2017).

Natural enemies are unknown. For *Lobesia botrana* (Denis & Schiffermüller), a visually similar moth pest of grapevine in Europe, biological control agents, including *Bacillus thuringiensis* (Bt) (Bacillales: Bacillaceae) and mating disruption products, have been registered and used (Altindisli, 2014). Natural enemies such as *Trichogramma* parasitoid wasps (Hymenoptera: Trichogrammatidae) have been identified in Europe (Scaramozzino *et al.*, 2017), but they may be unlikely to provide sufficient suppression in an orchard ecosystem without augmentation.

Diapausing pupae of *L. botrana* overwinter in the bark of vine and emerge the following spring (Gutierrez *et al.*, 2018), and *L. vanillana* is expected to have a similar life cycle (Appendix 2). During the summer, adult females oviposit their eggs on berries, where larvae feed on and later pupate within the bunch. Similar to *L. botrana*, the life stages of *L. vanillana* is not expected to depend upon the soil for its development.

Insect parasitic nematodes, or entomopathogenic nematodes (EPN), occur in soil across the world and are natural enemies of many insect species. Entomopathogenic nematodes of the family Steinernematidae (Rhabditida) are associated with the symbiotic bacteria of *Xenorhabdus* (Enterobacteriales: Enterobacteriaceae) (Stock & Goodrich-Blair, 2012). Infective juveniles (IJs) of these species release their symbiotic bacteria shortly after

penetrating the haemocoel of their target insect, causing mortality within 48 h (Kaya & Gaugler, 1993), depending on the number of nematodes that penetrated, and the size of the insect host (Dillman & Sternberg, 2012).

Steinernema has been previously evaluated against lepidopteran pests of South Africa under laboratory and field conditions. Relevant EPN biocontrol research in South Africa includes that of the above-ground diapausing larval population of the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) (De Waal *et al.*, 2011, 2013; Odendaal *et al.*, 2016a, b) and the soil stages of false codling moth *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Malan *et al.*, 2011; Malan & Moore, 2016; Steyn, 2019).

Steinernema yirgalemense Kguyen, Tesfamariam, Gozel, Gaugler & Adams and *S. jeffreyense* Malan, Knoetze & Tiedt have been successfully mass produced using *in vitro* liquid culture methods (Ferreira *et al.*, 2016; Dunn & Malan, 2019). Promising results were obtained against false codling moth, using both *in vivo* and *in vitro*-cultured nematodes, with no significant difference between culture type in laboratory and field trials (Steyn, 2019). The *in vitro* liquid mass production of EPNs is much more cost and labour effective than *in vivo* production, the latter of which is better suited for small-scale experiments and insecticidal applications (Shapiro-Ilan & Gaugler, 2002).

The aim of this study was to evaluate the pathogenicity of *in vivo* and *in vitro*-cultured *S. yirgalemense* and *S. jeffreyense* against the larvae of *L. vanillana*. Screening was conducted under optimum laboratory conditions and mortality by infection confirmed. The LD₅₀ of the most efficient species was determined. Additionally, reproduction of the nematode in the insect cadaver was investigated.

MATERIALS AND METHODS

Source of insects

Lobesia vanillana were collected from the Breede River Valley in the Western Cape province, from two farms approximately three kilometres south to the town of Robertson (33°48'7"S, 19°53'15"E). A commercial pest monitoring company (AgriRos, Robertson), used yellow delta sticky traps baited with carob moth lure to monitor the distribution and abundance of adult moths, and from this information, blocks with a persistent presence of *L. vanillana* were prioritised for collection. Grape bunches were collected and taken to the insectary of the Department of Conservation Ecology and Entomology, Stellenbosch University on the same day, and stored at 25°C for approximately two weeks to allow egg hatch and larval growth.

Over the course of three days, bunches were broken apart by hand and inspected for eggs, larvae, pupae and damage. Larvae, placed in separate containers, were taken to a mass rearing facility in Stellenbosch to establish a colony (Appendix 2). Larvae were fed an agar-based modified codling moth diet (Stenekamp, 2011) and kept at a constant 25°C with a 18:6 h light-dark cycle. Adults were placed next to a window to receive natural indirect sunlight, and were given a cotton ball, dipped in a 2% sugar-water solution, for nourishment, without which oviposition was poor.

Source of nematodes

In vitro-cultured *S. yirgalemense* (157-C) (EU625295) (Malan *et al.*, 2011; Ferreira *et al.*, 2016) and *S. jeffreyense* (J194) (KC897093) (Malan *et al.*, 2016) were produced according to the technique of Dunn & Malan (2019). *In vivo* cultured nematodes, of the same two species, were sourced from the departmental collection of cultures according to the technique of Van Zyl & Malan (2015). Approximately 20 ml of each nematode suspension was prepared, counted and placed in culture flasks. Flasks were shaken biweekly to discourage nematode clumping and to aerate the suspension. Cultures were used within two months of their preparation. The viability of nematodes was evaluated by inspecting the culture for motility and mortality.

Bioassay protocol

The test arena consisted of 24-well bioassay trays (flat-bottom, Nunce, Cat. No.144530, Thermo Fisher Scientific (Pty) Ltd, Johannesburg, Gauteng, South Africa). Filter paper discs (Grade FN 30, 12.7 mm diam., Ahlstrom-Munksjö, Lot 15-187, Germany) were added to each alternate well, on which IJs were inoculated at a predetermined concentration (Glazer & Lewis, 2000) by pipetting 50 µl of the nematode suspension onto the filter paper. Glass rectangles were placed in-between the lid and the tray to prevent larval escape during the incubation period. The trays were placed in closed 2-L plastic ice cream containers, each with wet paper towels at the bottom to provide an environment of high humidity, then incubated at 25°C for 48 h. Mortality was assessed by prodding larvae with a dissection needle and inspecting their body integrity.

Following bioassays, cadavers were carefully rinsed with a handheld water jet to rid the cadaver body of surface nematodes. Cadavers were then either placed on a modified White Trap (White, 1927) to evaluate *in vivo* nematode production, or dissected to evaluate the penetrability of nematodes.

Susceptibility

Bioassays consisted of two treatment groups (*in vivo* and *in vitro*) inoculated with *S. yirgalemense* and *S. jeffreyense*, and one control group treated with water only. A total of four 24-well bioassay plates were used for each treatment, as described in the bioassay protocol, with six final instar *L. vanillana* larvae evenly distributed per tray, using 24 larvae per treatment. The insect larvae were inoculated with ± 100 IJ/50 μ l for all treatment groups and with 50 μ l distilled water only for control groups. The experiment was repeated on a different test date with a new batch of nematodes.

Concentration

Following the results of the EPN screening, the most effective species was selected for calculating the estimated LD₅₀. The same procedure as described in the bioassay protocol was followed. Dosages were calculated logarithmically from the most effective concentration downwards, which in this case were 100, 50, 25, 12, 6 and 0 IJs/larvae, respectively. Dilutions were made individually from the culture flask and mixed with distilled water to provide the required concentration at an inoculation volume of 50 μ l. Cadavers were discarded afterwards.

Evaluation

To assess the penetration success of nematodes, cadavers from the screening bioassay were incubated at 25°C at high humidity and dissected 18-36 h after mortality. The extra incubation time allowed nematodes to grow within the cadaver, which improved their visibility during counting. Cadavers were placed singly on a petri dish, with a droplet of distilled water to suspend the cadaver contents, and dissected with the aid of a Leica MZ75 stereo microscope. The presence and number of nematodes within the cadaver was recorded.

To assess the reproductive ability of nematodes within the host, cadavers of the screening bioassay were placed in 90 mm diam. plastic petri dishes with one filter paper circle (85 mm, Grade 1 Whatman, GE Healthcare Life Sciences) inoculated with 800 μ l of distilled water. Approximately 10-12 cadavers were placed per petri dish. With the top removed, plastic petri dishes were transferred to 150 mm glass petri dishes, the bottom of which contained just enough distilled water to not float the plastic petri dishes, and covered with the glass lid. Subsequent IJs suspended in distilled water of the glass petri dish were counted to confirm the reproductive ability of nematodes, and not necessarily the performance of nematode production. After counting, nematodes were removed, and fresh distilled water was added to the glass petri dish.

Data analysis

Data were analysed in Microsoft Excel 2010 for descriptive statistics and processed in Statistica 13.3 (TIBCO Software Inc., 2017) for comparative analysis. Probit analysis and LD₅₀ estimates were calculated using NCSS (Hintze, 2007). For the screening bioassays, residuals of the mortality response were considered normally distributed (Shapiro-Wilk's $W = 0.967$, $p = 0.187$), permitting the use of a factorial ANOVA and Fisher's LSD post-hoc test to evaluate responses between nematode production types and between nematode species. All analyses were evaluated for, and passed, Levene's test for homogeneity of variances ($p > 0.05$). Results are given as the mean response for all repetitions \pm SEM, unless otherwise specified.

RESULTS

Susceptibility

There were significant differences between the control and treatment groups for both the *in vitro* and *in vivo* nematode culture types ($p < 0.01$), while there was no significant difference between nematode culture types, and no significant effect between nematode culture type and nematode species.

Within *in vitro* cultures, *S. yirgalemense* ($97.88\% \pm 2.13\%$) was significantly ($p < 0.01$) more effective than *S. jeffreyense* ($72.88\% \pm 6.21\%$), while both were significantly ($p < 0.01$) more effective than the control ($8.38\% \pm 6.31\%$). Within *in vivo* cultures, *S. yirgalemense* ($83.25\% \pm 6.28\%$) and *S. jeffreyense* ($83.38\% \pm 5.4\%$) were significantly different from the control ($16.63\% \pm 7.02\%$), but not to each other (Fig. 3.1).

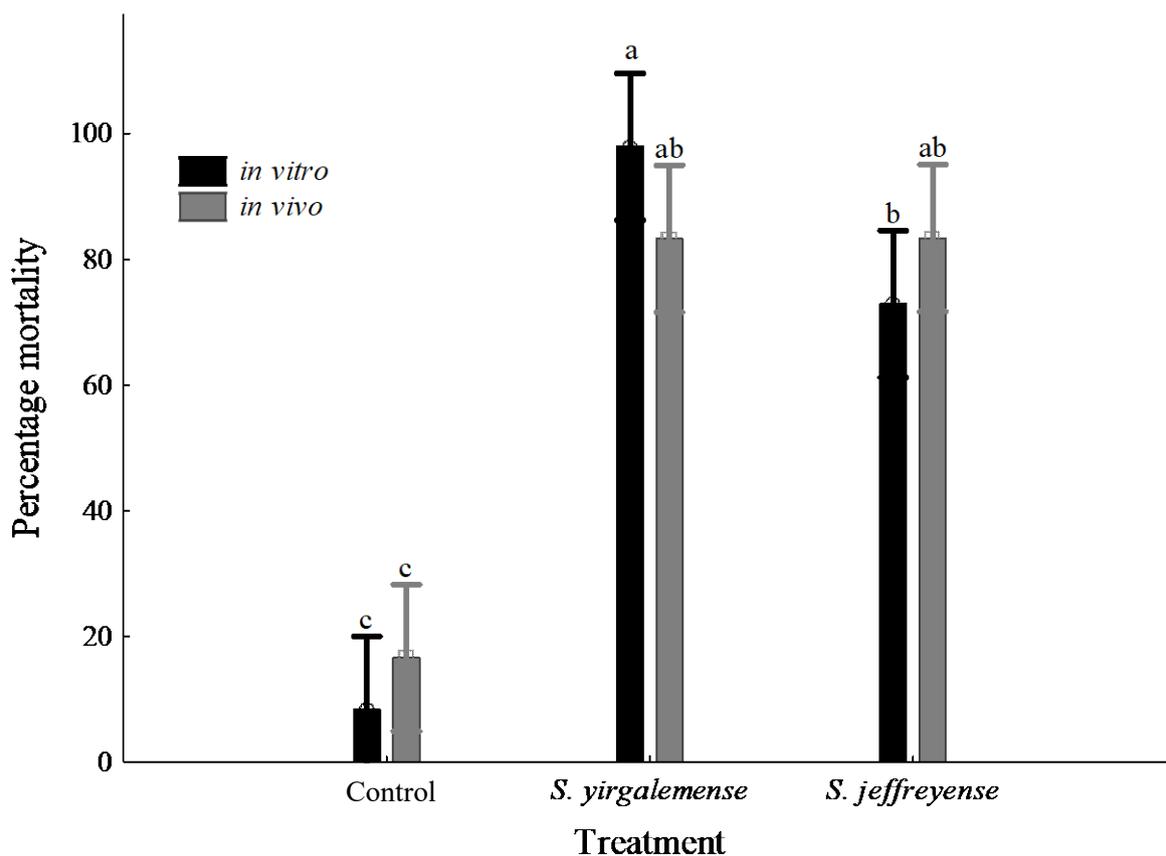


FIGURE 3.1

Percentage mortality (95% confidence intervals) of *Lobesia vanillana* larvae, 48 h after inoculation with *in vitro* and *in vivo* produced infective juveniles (IJ) of *Steinernema yirgalemense* (157-C) and *S. jeffreyense* (J194), at a concentration of 100 IJ/50 µl. Vertical bars were calculated using least square means. Different letters between treatments denote a difference of statistical significance, calculated using Fisher's LSD (Error between MSE = 267.64; df = 42; $p < 0.05$).

Concentration

The data fits the probit model well (Fig. 3.2) (Chi-Square = 1.29; DF = 3; Prob. level = 0.73). Lethal dosage estimates were calculated as follows: $LD_{25} = 2.37 \pm 1.37$; $LD_{50} = 7.335 \pm 2.485$; $LD_{90} = 62.761 \pm 23.224$ and $LD_{95} = 115.339 \pm 57.426$ nematodes per host. The probit model may be expressed by the linear function $P = 3.81 \pm 0.466 + 1.375x \pm 0.344$ where P is Probit-Mortality and x is \log_{10} -Dose.

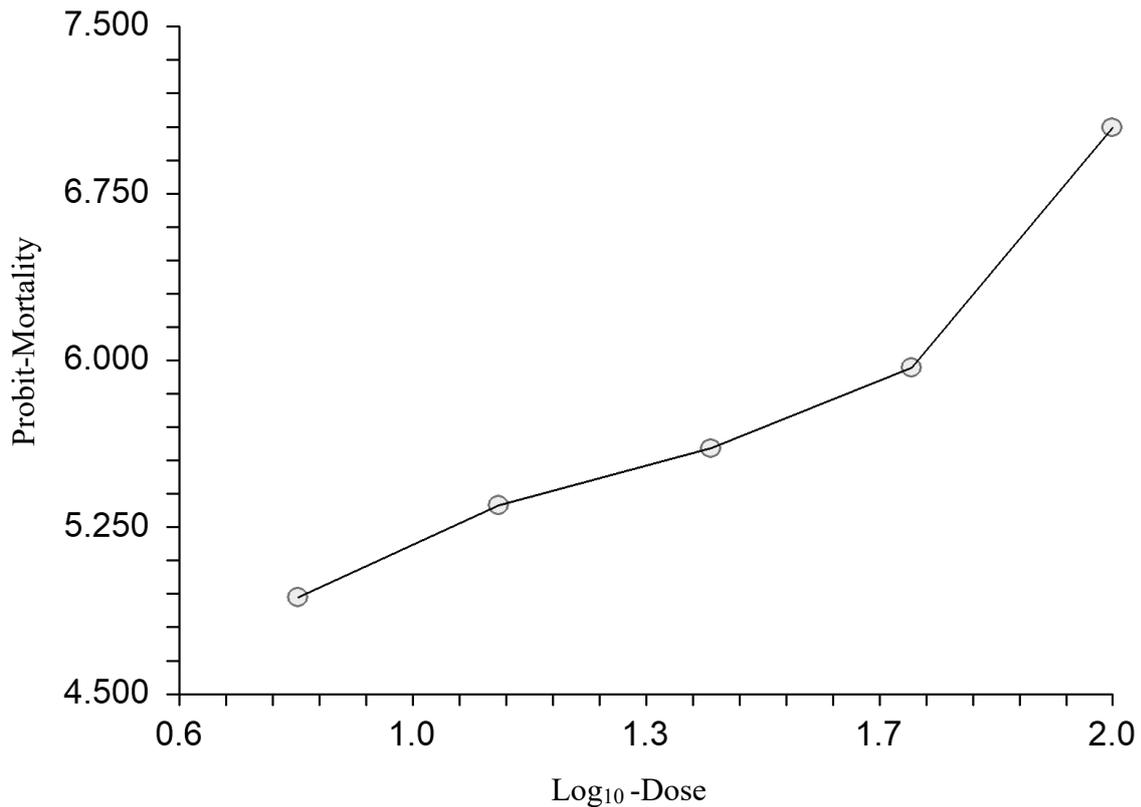


FIGURE 3.2

Probit mortality of *Lobesia vanillana* larvae 48 h after inoculation at different dosages (100, 50, 25, 12, 6 and 0 IJs/larvae) of *in vitro* cultured *Steinernema yirgalemense* (157-C). The LD_{50} was estimated as 7.335 ± 2.485 nematodes.

Penetration

In all treatments, > 90 % of the cadavers had nematodes present (Fig. 3.3). A higher percentage of nematodes penetrated *L. vanillana* in the *in vivo*-cultured treatments, versus their *in vitro* counterparts, however this difference was not significant.

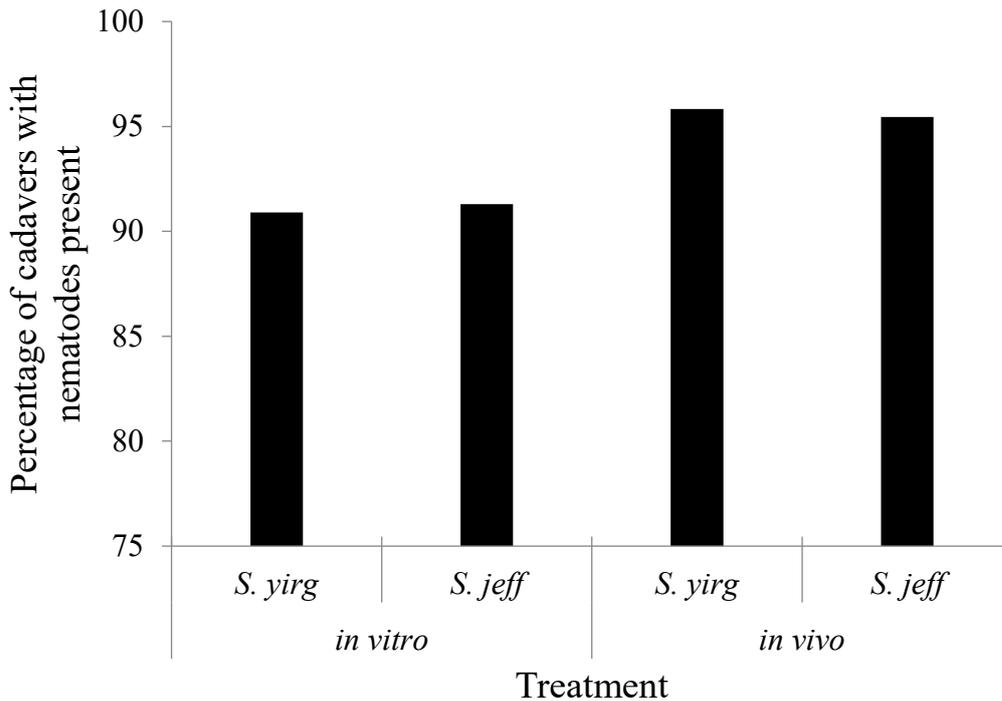


FIGURE 3.2

Percentage of *Lobesia vanillana* cadavers with nematodes present following screening bioassays of *in vitro* and *in vivo* produced *Steinernema yirgalemense* (157-C) and *S. jeffreyense* (J194). No significant differences were found between treatments.

Chi-square revealed no significant differences between nematode presence within cadavers and nematode culture type or nematode species. Residuals of nematode counts failed the normality assumption (Shapiro-Wilk's $W = 0.771$, $p < 0.01$), but relatively large sample sizes per group ($n \geq 22$) allowed for an ANOVA bootstrap analysis. There was a significant difference in the average number of nematodes per cadaver within the *in vivo* culture type, between *S. jeffreyense* (17.091 ± 0.187) and *S. yirgalemense* (4.917 ± 0.334) (Bootstrap $p = 0.045$), while within the *in vitro* culture type, there was no significant difference between them (Fig. 3.4).

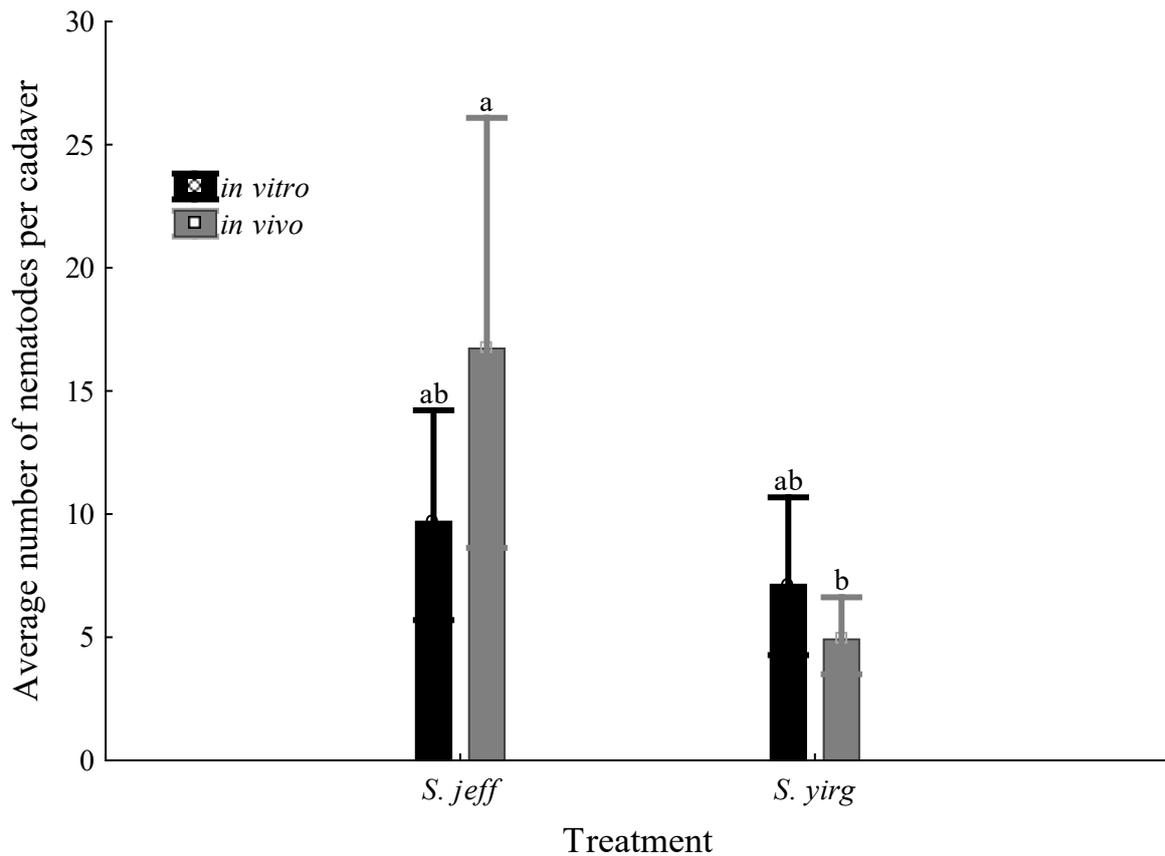


FIGURE 3.3

Average number of nematodes per *Lobesia vanillana* cadaver following screening bioassays, using *in vitro* and *in vivo* produced *Steinernema yirgalemense* (157-C) and *S. jeffreyense* (J194). Different letters between treatments denote a difference of statistical significance ($p < 0.05$), calculated using Bootstrap.

Reproduction

Both *in vitro*-cultured *S. yirgalemense* and *S. jeffreyense* nematodes were able to produce IJs from cadavers following the screening bioassay, using a modified White trap (White, 1927). Cumulative production after 45 days totalled 2130 IJs for *S. yirgalemense* and 2356 IJs for *S. jeffreyense*, per insect cadaver (Fig. 3.5).

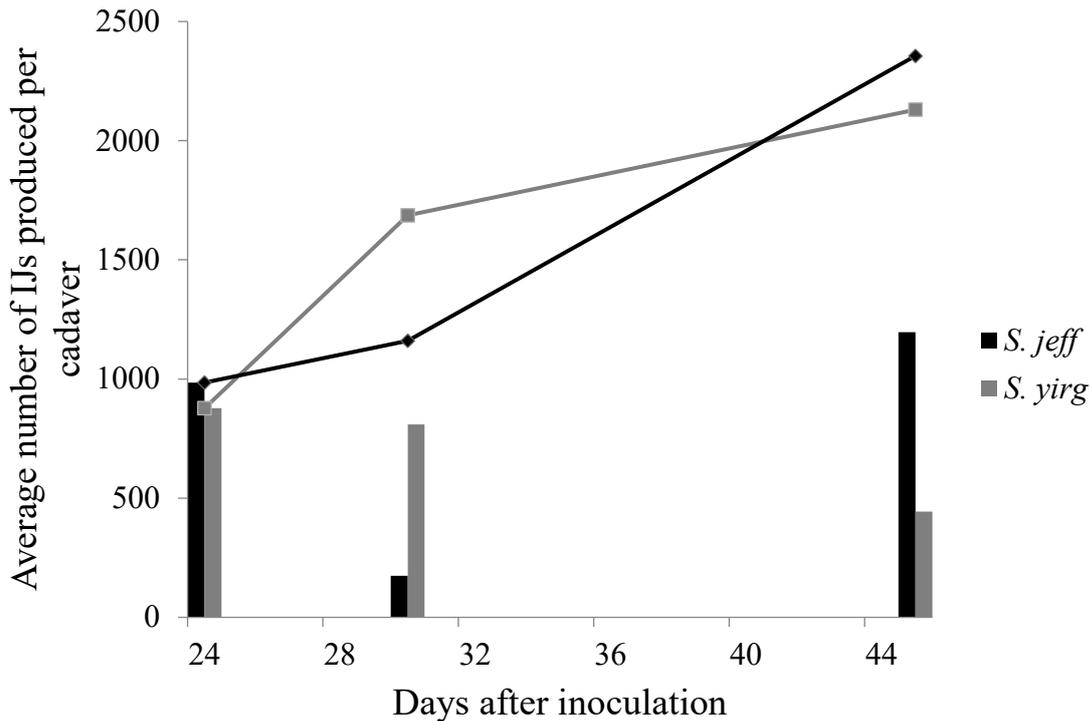


FIGURE 3.4

Average number of infective juveniles produced per *Lobesia vanillana* cadaver, following screening bioassays, using *in vitro*-cultured *Steinernema yirgalemense* (157-C) and *S. jeffreyense* (J194). Lines indicate cumulative production.

DISCUSSION

This was the first study evaluating EPNs for the control of *L. vanillana*. All treatments resulted in significant mortality of *L. vanillana* larvae. Specifically, within *in vitro* cultures, *S. yirgalemense* (98%) performed significantly better than *S. jeffreyense* (73%), while both performed significantly better than the control (8%). Within *in vivo* cultures, there were no significant difference in mortality between *S. yirgalemense* (83%) and *S. jeffreyense* (83%), while both performed significantly better than the control (17%).

There was no significant difference found between *in vitro* and *in vivo* cultures of the same species. In a study by De Waal *et al.* (2011), both *in vivo*-cultured *S. yirgalemense* and *S. jeffreyense* were evaluated against the above-ground diapausing codling moth larvae, at half the concentration used for *L. vanillana* in the present study. Both nematode species resulted in a mortality of close to 100%. However, Odendaal *et al.* (2016b) found that in a semi-field spray trial, *S. jeffreyense* performed better than *S. yirgalemense*, which is contrary to what was expected.

Mortality, caused by nematode infection, was confirmed by dissecting *L. vanillana* cadavers and evaluating the presence of nematodes, which was > 90% for all treatments. The average number of nematodes per *L. vanillana* cadaver was also investigated. Within the *in vivo* culture there was a significant difference between *S. jeffreyense* (17 nematodes per cadaver) and *S. yirgalemense* (five nematodes per cadaver), but no significant difference between the species of *in vitro* cultures. It was expected that the number of IJ penetrated would be higher in the case of *S. yirgalemense*, as it is a smaller nematode (635 µm) when compared to the body length of *S. jeffreyense* (> 900 µm). Examination of cadavers directly after screening bioassays revealed that few of the final instar insect larvae treated with *S. yirgalemense* managed to pupate in their trays, with little webbing present, compared to those treated with *S. jeffreyense*, where pupae and webbing were more common, suggesting that *S. yirgalemense* is faster-acting than *S. jeffreyense*, in laboratory bioassays at least, but more research is needed to support this theory.

While investigating the reproductive ability of nematodes, both *S. yirgalemense* and *S. jeffreyense* were able to produce new IJs, and after 45 days produced an estimated 2130 IJ and 2356 IJs per cadaver, respectively. Generally, the larval stages of lepidopterans were found to support nematode infection and reproduction (Malan & Hatting, 2015), and more so when insects are believed to not have a soil stage. In rare cases, especially where insects have soil stages, resistance against nematodes can develop, such as the case with woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Stokwe & Malan, 2017).

Local research established *in vitro* liquid culture methods for *H. zealandica* (Ferreira *et al.*, 2014), *S. jeffreyense* (Dunn & Malan, 2019) and *S. yirgalemense* (Ferreira *et al.*, 2016), while research on the formulation, packaging and storage of these species is still ongoing (Kagimu *et al.*, 2017; Kagimu, 2018). *In vivo* produced IJs can provide affordable, high quality nematodes that are easy to culture, but only on small scales (Shapiro-Ilan & Gaugler, 2002). Increased complexity, risk, labour and running costs are prohibitive when scaling towards mass production (Ehlers, 2001). The start-up capital and complexity of *in vitro* production methods are excessive for small-scale use, but for mass production and augmentative releases where a large number of nematodes is required, it is arguably the most cost-effective solution (Shapiro-Ilan & Gaugler, 2002). Results from this study indicate that the quality of *in vitro*-cultured nematodes (Dunn & Malan, 2019) are comparable to those cultured *in vivo*, the latter of which is considered the more natural method. Previous studies by Ferreira *et al.* (2014, 2016) evaluated the efficacy of *in vitro* and *in vivo*-cultured *S. yirgalemense* and *H. zealandica* against

the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), and found *in vivo*-cultured nematodes to cause significantly higher mortality than their *in vitro*-cultured equivalents. Wax moth larvae are useful in EPN studies as they are highly susceptible to nematode infection and relatively easy to rear (Van Zyl & Malan, 2015).

Steyn (2019) evaluated *S. jeffreyense* and *S. yirgalemense*, both *in vivo* and *in vitro*, produced under the same conditions as the present study, and assessed their mortality against false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), both in the laboratory and in the field. Using 25 IJs/larva against false codling moth, half the concentration of the current study, > 85% mortality was found in the laboratory, compared to semi-field applications which resulted in a mortality of \approx 70%. Similar to the present study, the author did not find any significant difference between *in vivo* and *in vitro*-cultured nematodes of the two species (Steyn, 2019).

Internationally, EPNs have been evaluated against a wide range of pests (Georgis *et al.*, 2006). Important South African moths of the Tortricidae family that attack grapevine include false codling moth and pear leafroller, *Epichoristodes acerbella* (Walker) (Rentel, 2013). As there is no information on the efficacy of EPNs against *E. acerbella*, a study investigating alternate control methods would be of value. The isolate of *S. yirgalemense* (157-C) demonstrated high efficacy against other key insect pests of grapevine, including banded fruit weevil (Dlamini *et al.*, 2019), fruit fly (James *et al.*, 2018) and mealybugs (Platt *et al.*, 2018), and has been prioritised for commercialisation (Kagimu, 2018). For this reason, it would be beneficial to also evaluate this particular isolate against *E. acerbella* in future research.

False codling moth is a notorious phytosanitary pest of South Africa. Local research evaluated false codling moth against a range of locally isolated EPNs (Malan *et al.*, 2011, 2018; Manrakhan *et al.*, 2014; Steyn *et al.*, 2017). In laboratory assays, *S. yirgalemense* (157-C) resulted in 100% mortality against final instar larvae at a concentration of 50 IJs/ml at 25°C after 48 h, and 74% mortality against pupae at a concentration of 200 IJs/ml at 25°C after 48 h (Malan *et al.*, 2011). Similarly, a study by Steyn *et al.* (2017) found this isolate to result in 100% mortality against final instar larvae at a concentration of 50 IJs/ml at 25°C after 48 h, under laboratory conditions.

Environmental parameters and fluctuations (temperature, relative humidity, wind, UV, etc.) may affect the interactions between the nematode and its insect host, as well as their survival, dispersal and fecundity. Lower efficacies can be expected in field trials due to harsher

conditions and environmental incompatibilities relative to the laboratory bioassay protocol. For example, in codling moth trials, lower temperature resulted in slower and lower mortalities compared with warmer temperatures (Odendaal *et al.*, 2016b). The origin of nematode cultures may also influence their performance, due to their ability (or inability) to adapt to different climates. For example, following results from field trials, Odendaal *et al.* (2016a) proposed that because *S. yirgalemense* originated from Mpumalanga (Malan *et al.*, 2011), it may not be well adapted to Mediterranean climates.

Larvae of *L. vanillana* in the present study were sourced from a laboratory colony established in February 2018 from field-collected individuals, and the susceptibility of these individuals to EPNs may differ slightly to that of the natural field population. More nematode species, especially *H. zealandica* and other native species that show effective control against lepidopteran pests of grapevine, can be evaluated in future research to establish a nematode susceptibility profile for *L. vanillana*. Additional life stages of *L. vanillana* should also be evaluated for susceptibility, for example, targeting the overwintering pupae may result in significant pest suppression and reduced damage in the following season. The application of nematode formulations to the canopy and soil of wine grapes may have the ability to control multiple grapevine pests simultaneously, especially when used in an integrated pest management programme.

Results of the present study thus indicate that *in vitro* and *in vivo* produced *S. yirgalemense* and *S. jeffreyense* nematodes are able to infect and kill the larvae of *L. vanillana*, and that the *in vitro* cultures are able reproduce within this host and produce a new cohort of IJs, capable of finding and infecting new hosts. The relative success of *S. yirgalemense*, *S. jeffreyense* and other local EPN species against South African tortricid species in laboratory and field assays, the ability to produce nematodes using *in vitro* liquid culture techniques, and the industry demand for such products, encourages further research and development of this technology.

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CHAPTER 4:

General discussion

Plangia graminea (Serville) (Orthoptera: Tettigoniidae) and *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae) are two sporadic, minor pests of vineyards in the Western Cape province of South Africa. As insecticides evolved to better target pest species and be less toxic to non-target organisms, minor pests that were usually suppressed, now have the ability to exploit agricultural resources and become secondary pests (Gullan & Cranston, 2014). Indeed, both *P. graminea* and *L. vanillana* were reported by growers to be susceptible to broad-spectrum agrochemicals, for example, organophosphates and pyrethroids, which were not explicitly registered for their control.

Growers reported an increased number of localised pest outbreaks in recent years. *Plangia graminea* is considered widespread in the Western Cape, whereas the distribution of *L. vanillana* is reported to be much more limited. In Robertson (Breede River Valley), *P. graminea* adults were observed in a *L. vanillana* infested block, albeit at low abundance, suggesting that in favourable conditions, a dual outbreak of *P. graminea* and *L. vanillana* may result in significant economic damage.

Macrobial and microbial control agents have been successfully used for the control of many insect pests worldwide, which includes entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF). Local research has shown that the main insect pests of grapevine are susceptible to EPN infection, including false codling moth, vine mealybug, fruit fly and banded fruit weevil (Malan & Hatting, 2015). It is believed that neither *P. graminea* nor *L. vanillana* depend upon the soil for their development (Appendix 1, 2). This may increase their susceptibility to EPN infection, as these nematodes occur naturally in the soil, and above-ground insects are likely not to have had the opportunity to develop resistance.

It has been demonstrated that nymphs of *P. graminea* were highly susceptible to EPN infection, in particular to the species of *Heterorhabditis zealandica* Poinar (SF41), *Heterorhabditis indica* Poinar, Karunakar & David (SGS), *Steinernema jeffreyense* Malan, Knoetze & Tiedt (J192), *Steinernema yirgalemense* Kgyuen, Tesfamariam, Gozel, Gaugler & Adams (157-C) and *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens (MT19) (Malan & Hatting, 2015), all of which are local South African isolates. Results from these bioassays are regarded as novel, as it was the first study to indicate the susceptibility of *P. graminea* to EPN biocontrol

agents. The collection of sufficient field individuals for laboratory bioassays was challenging due to the cryptic nature of *P. graminea*. They also did not perform well in captivity and had to be used directly after their collection from the field. For future research in the use of biocontrol agents, it is highly recommended to develop a way in which to rear *P. graminea* in artificial conditions.

Larvae of *L. vanillana* proved to be susceptible to infection by both *in vivo* and *in vitro*-cultured *S. jeffreyense* (J192) and *S. yirgalemense* (157-C), with no significant difference in mortality between the culture types. The dissection of cadavers after screening bioassays confirmed mortality by EPN infection, while further incubation thereof indicated the ability of nematodes to survive and complete their life cycle *in vivo*. This suggests that *L. vanillana*, as a host, can sustain nematode reproduction and should a nematode-infected insect drop to the soil, recycling of the nematode can continue. The bioassay results from this study are novel, as the susceptibility of *L. vanillana* against EPNs has not been tested before. This study has shown exceptionally high pathogenicity of *S. yirgalemense* (>80%) against *P. graminea* and *L. vanillana*, which is a nematode species found to be effective against a range of key grapevine insect pests (Malan & Hatting, 2015).

The performance of *in vivo* and *in vitro*-cultures may differ, but is not always the case, as Steyn (2019) and the present study have found. Exotic species have been successfully cultured in Europe and the USA, while Ehlers *et al.* (2000) successfully mass cultured *H. indica* from isolates in India. Local research has established *in vitro* liquid culturing methods for *H. zealandica* (Ferreira *et al.*, 2014), *S. jeffreyense* (Dunn & Malan, 2019) and *S. yirgalemense* (Ferreira *et al.*, 2016). The ability to mass culture nematodes *in vitro* can be considered a prerequisite for its commercialisation, as the production of *in vivo* nematodes is not viable at large scales (Ehlers, 2001). Local research on the formulation and storage of EPNs is promising (Malan & Hatting, 2015; Kagimu, 2018). Results from this study support future *in vitro* liquid mass culture and nematode formulation research, aimed towards foliar and soil application techniques of EPNs against grapevine pests. Informal correspondence with various farmers during the course of this study, indicates an overall positive and optimistic attitude regarding the integration of this technology in practice.

Research on the katydid, *Paratlanticus ussuriensis* (Uvarov) (Orthoptera: Tettigoniidae), by Shim *et al.* (2013), found that early nymphs were significantly more susceptible to the symbiotic bacterium of a heterorhabditid than older nymphs. This suggests the timing of field

applications when nymphs are still small, but after eggs have hatched, which seems to coincide around late-September to mid-October. Doubell (2017) described a method by which the number of katydid eggs, deposited on wine grapes, can be used as an estimate for katydid abundance, with a lag period of approximately ten weeks. The author demonstrated that this method may be more reliable than monitoring for leaf damage or for katydids in the grapevine canopy. Since *P. graminea* is believed to have only one generation per year, achieving sufficient mortality early in the season may significantly suppress the katydid population, reducing damage, but also reduce the number of eggs laid and thus the size of future generations.

Field applications targeting *L. vanillana* in grapevine should occur prior to bunch closure and before the canopy grows too dense for proper cover and infiltration of the nematode formulation. Nematodes have the ability to actively seek their host in cryptic habitats where chemicals may not be able reach (Malan & Hatting, 2015). Monitoring flights can be useful for forecasting damage and for timing insecticidal applications to target certain life stages. Developing pheromone lures specific to *L. vanillana* may also increase the capturing performance of traps. Monitoring of *L. vanillana* in the Breede River Valley should also include alternative crops, especially citrus and olives, as it was reported to be polyphagous and its distribution in unmonitored regions is unknown.

Establishing susceptibility profiles, both for insect pests and for EPNs, are vital for their consideration and implementation in IPM programmes. For example, from previous research we know that *S. yirgalemense* is effective against the main insect pests of grapevine (Malan & Hatting, 2015). In turn, we know that certain grapevine pests are susceptible to, for example, *H. zealandica*, *S. jeffreyense*, *S. yirgalemense* and *H. bacteriophora* (Malan & Hatting, 2015). Using such a susceptibility matrix, an EPN with high efficacy against multiple insect pests can be selected for *in vitro* mass culture and commercial application. Alternatively, during the outbreak of a single pest species, the most effective EPN species can be identified for targeted, augmentative release. In addition, a full field application would not only target above-ground grapevine insect pests, but also will have an effect on pests with soil connected stages such as false codling moth, mealybugs and fruit flies. At present, however, there is not enough data available to construct such a matrix, as field performance trials for many of the equivalent laboratory assays have not yet been performed.

The effects of adjuvants, including wetters, surfactants and emulsifiers, should also be evaluated in order to develop an understanding of the nematode and host response, with the aim of increasing the efficacy of EPN species. The purpose of these adjuvants is primarily to mitigate the desiccation of nematodes in above-ground applications. In addition, it may also aid in the mobility of nematodes and improve its infection potential under suboptimal, above-ground field conditions. Previous South African research have demonstrated the benefits of using adjuvants, such as the case of vine mealybug and codling moth, for which control is mainly targeted in the canopy of the vine (Malan & Hatting, 2015). Such applications can also be considered against *P. graminea* and *L. vanillana* in hot-spots and during pest outbreaks, without compromising current IPM programs.

This study emphasised that the rearing of *P. graminea* is essential for future research in the use of biological control agents against it. In the case of *L. vanillana*, rearing the insect on an agar-based modified codling moth diet produced sufficient larvae for laboratory bioassays. Although rearing conditions for *L. vanillana* was not optimised, results from this study are useful for future research on biological control agents in laboratory bioassays.

The objectives of this research thesis were to evaluate the biocontrol potential of EPNs against the katydid, *P. graminea*, and the moth, *L. vanillana*, two sporadic pests of vineyard in South Africa. Results identified multiple local South African EPN species that were highly pathogenic to these insects, which motivates for further research to advance their biocontrol potential. This study also contributes to new knowledge regarding the biology, ecology, behaviour, distribution and rearing of these two insects, for which little to no information was available.

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APPENDIX 1:

Observations on *Plangia graminea* (Serville) (Orthoptera: Tettigoniidae) in grapevine of the Western Cape

INTRODUCTION

Plangia graminea (Serville) (Orthoptera: Tettigoniidae), is a minor and sporadic pest of vineyards in the Western Cape province of South Africa (Allsopp, 2012). It is considered widespread in tropical and sub-Saharan Africa (Hemp *et al.*, 2015), but their distribution in South Africa is largely unknown. In nature they are inconspicuous (Annecke & Moran, 1982). The Willow Karee or smalblaar, *Searsia angustifolia* (L.) Barkley (Sapindales: Anacardiaceae), is believed to be a local natural host (Doubell, 2017).

In agricultural ecosystems, katydids feed on grapevine foliage, but during pest outbreaks, their feeding activity, especially on young vines, can result in significant economic loss (Allsopp, 2012). Little is known about the biology, ecology and distribution of this species. Allsopp (2012) reported that their abundance and distribution have increased in recent years, possibly due to changes in agrochemical use and/or weather conditions, but that the exact drivers of change are unknown.

Previous attempts to establish a laboratory colony and to rear this insect was reported to be challenging (M. Doubell, pers. comm., 2016). Researchers of the San Diego Zoo (California, USA) attempted to rear the dragon-headed katydid, *Lesina intermedia* (Karny) (Orthoptera: Tettigoniidae). These katydids were housed in large, glass containers, with a variety of substrates and habitat features. Their diet consisted of various seeds and grains, supplemented by fish food and sea salt. After initial difficulties to produce offspring, katydids seemed to thrive on live banana plants and females oviposited their eggs under several layers of stem. The authors noted, however, that the survival rate of nymphs to adults was approximately 25%, irrespective of whether they were reared communally or separately after hatching. They also noted missing nymphs or leftover bodyparts, suggesting that cannibalism contributed to the high mortality (Chang, 2014).

Researchers from the Singapore Zoo (Singapore, Singapore) attempted to rear another species of dragon-headed katydid, *Lesina blanchardi* (Brongniart) (Orthoptera: Tettigoniidae), which they mentioned is a difficult insect to breed, due to low egg hatch rates. The authors developed a method by which > 96% of eggs hatch, however nymph survivability was still \pm 45%. Other

methods resulted in a nymph survivability between 4.8% and 15.2%, with nymph mortality being the highest within the first eight days after hatching (Kaur *et al.*, 2018).

The aim of this supplementary chapter was to investigate the basic biology, ecology and impact of *P. graminea*, as observed in vineyards, and to determine their geographical distribution in South Africa. This information is vital for the development and implementation of integrated pest programmes (IPM) in agricultural ecosystems, and to ensure their compatibility with pest management programmes. Keeping *P. graminea* in a laboratory colony was also investigated, as it would be useful for providing insect hosts for laboratory biocontrol assays.

MATERIALS AND METHODS

Site identification

Approximately 350 farms with wine grapes were approached during the course of this study, located predominantly in the Western Cape, as well as various consultancies and stakeholders in the industry. Those that responded positively for katydid presence were prioritised for site visitation to confirm their occurrence, and to collect specimens for the laboratory colony and biocontrol assays (Chapter 2). Katydids were taken to the laboratory at the Department of Conservation Ecology and Entomology, Stellenbosch University, to establish a colony. Respondents were placed into two groups regarding katydid presence: reported and confirmed, and reported but unconfirmed.

Site mapping

Locations were georeferenced in Google Earth 7.1.8 (Google, 2017), exported to QGIS 2.14.22 (QGIS Development Team, 2018) and overlaid with provincial, district and administrative layers of South Africa (PlanetGIS, 2018). Sampling points were buffered to obfuscate the exact location of farms to respect anonymity, as requested by some growers.

Katydid collection

Nymphs were surveyed by searching through foliage by hand, and collected using a variety of cylindrical plastic containers, ranging between 75 ml and 250 ml in size. Caps were replaced loosely enough to allow air circulation without the insects escaping, and packed in ventilated open-top boxes prior to transport.

In addition to searching through the foliage by hand, adults were also surveyed by shaking the top wires of the vine trellis, or the vine itself. Adults were captured by hand or with the aid of a small butterfly net, and deposited in transparent acrylic containers at least 45 × 30 × 30 cm

in size, with mesh sides to allow air circulation. Vine shoots and additional leaves were placed inside for food, moisture and habitat structure.

Containers were transported in a ventilated vehicle and insects placed in the laboratory colony no later than 2 h after departure. Field collection took between 2-10 h, but care was taken to not let environmental conditions influence the fitness of individuals prior to transport. For example, fewer insects per container reduced competition, containers placed in the shade and in wind drafts reduced heat stress, and vine shoots and leaves within the container regulated humidity and supplied habitat complexity.

Laboratory colony

Rearing boxes

Four different rearing boxes were used, ranging from 60 × 60 × 45 cm to 120 × 120 × 120 cm. Five of the six sides were transparent, of which at least two sides had fine netting to allow air circulation. Rearing boxes were made of glued acrylic, while one had a wooden frame with acrylic panels and a wooden back panel. Prior to their use, rearing boxes were cleaned with lukewarm water and left to dry.

Insects

Katydidids in the laboratory colony were collected from 2016-09 to 2017-02, and again from 2017-09 to 2018-02. Sites with a high katydid abundance and close proximity to Stellenbosch University were prioritised for field collections (Fig A1.1). Newly field-collection individuals were placed in separate laboratory colony containers (quarantined) and monitored for unusual activity and mortality, prior to their utilisation in laboratory colonies or bioassays.

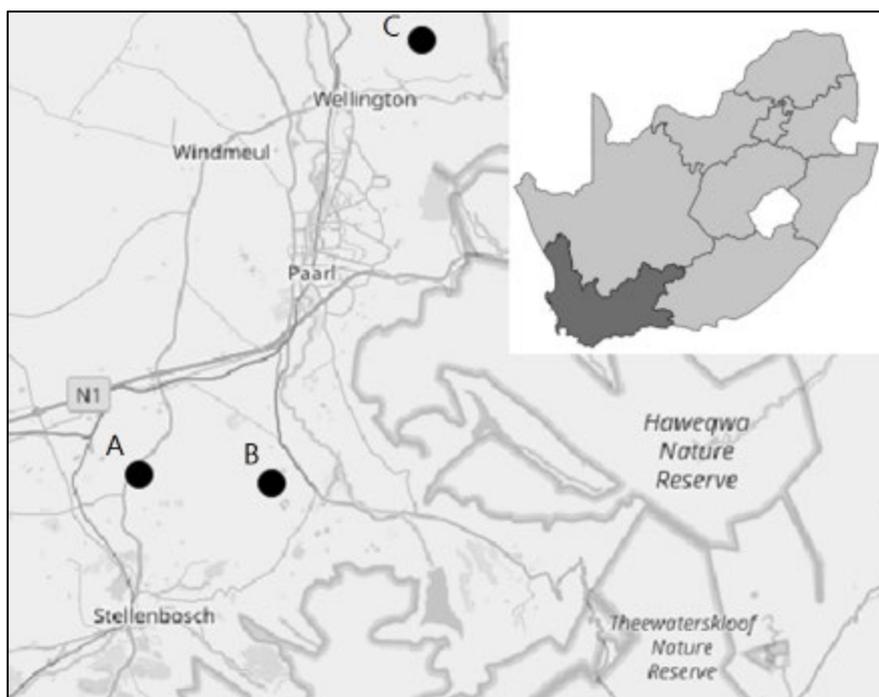


FIGURE A1.1

Map of katydid nymph collection sites in the Western Cape province, South Africa. Locations are approximate: (A) 10 km north of Stellenbosch, (B) 2 km west of Simondium, and (C) 4 km north-east of Wellington.

Food

Katydids were fed leaves collected from vineyards, supplemented with lettuce (fresh, store-brand, Food Lovers Market, Stellenbosch). Vine leaves were either placed individually, or while still on vine shoots. Leaves of lettuce were placed individually to discourage the growth of mould due to their high moisture content. High mortality necessitated the experimentation with supplementary foods, including oats (organic, store-brand, Clicks Pharmacy, Stellenbosch) and fish food flakes (Tetramin, Pet Food City, Stellenbosch), as it proved successful for rearing *L. intermedia* as in the San Diego Zoo (Chang, 2014), and carrot (fresh, store-brand, Food Lovers Market, Stellenbosch).

Fresh leaves and shoots were added every second day and removed when it started to dry out. All food (except oats and fish food flakes) were hand rinsed using tap water and patted dry using hand towel paper, prior to their introduction into containers.

Habitat

Vine shoots and twigs provided habitat structure, perching place and complexity to containers, and were typically positioned diagonally across and at an angle. Female adult katydid are

known to oviposit eggs under the bark of vine (Doubell *et al.*, 2017), for which twigs and small branches were placed. As a result of high mortality, some revisions included placing two potted vines in the largest container to exclude potential side-effects of picked leaves and vines, such as plant volatiles, however this reduced perching place and the total number of leaves available, in addition to providing a potential source of pathogens. Following the eventual death of katydids in that container, vines were removed and the container decontaminated.

Environment

Katydid were initially kept in the laboratory at room temperature (18-32°C) next to a window, where it received indirect natural sunlight during daytime, supplemented by overhead fluorescent lighting, and darkness at night. Later revisions had katydids placed outside under a tree to expose them to more natural conditions (larger temperature and humidity fluctuations, higher wind speed, more daylight), and also in the departmental insectary. In the insectary, temperature was kept constant at 25°C and light, supplied by daylight-colour overhead fluorescent lamps, at 16:8 h light:dark cycles.

RESULTS AND DISCUSSION

Distribution

Plangia graminea was primarily observed from wine grapes in the Cape Winelands region and were sporadically observed in the Breede River valley. Beyond the Cape Winelands region, they were rarely considered pestiferous and thus receive minimal attention, so their distribution is likely to be underestimated. Large parts of the Western Cape are planted with vineyard (Table A1.1) providing potential habitats for *P. graminea*, suggesting that factors which limit their distribution are likely to be more complex than only habitat availability.

TABLE A1.1

Distribution of wine grape vineyards for the top five wine regions. Adapted from Floris-Samuels & Uren (2017).

Wine region	Hectares (2017)
Stellenbosch	15 252
Paarl	15 150
Breedekloof	12 941
Robertson	12 910
Swartland	12 892
Other	25 401
Total	94 545

Of the 350 farms approached, less than 50 responded. Of those, 36 reported the presence of katydids, all in wine grapes, of which 12 were confirmed by visiting the site and collecting specimens. The Cape Winelands region had the highest concentration of reports within the Stellenbosch – Simondium – Paarl region, while fewer reports originated from the Breede River valley (Robertson district) and from the rest of the Western Cape (Fig. A1.2). Katydid are believed to be absent from the Northern Cape province (H. Burger, OWK, pers. comm., 2018) and of unknown status in the rest of South Africa. Reports of katydid presence from the towns of Lutzville (31°33'11"S 18°20'51"E) (not shown in Fig. A1.2) and Malgas were the furthest outside of the most affected regions.

Katydid have also been reported once from table grapes, but this was not confirmed. It is assumed that the more intensive management practices and agrochemical application programmes in table grapes do not provide an environment compatible with katydid persistence.

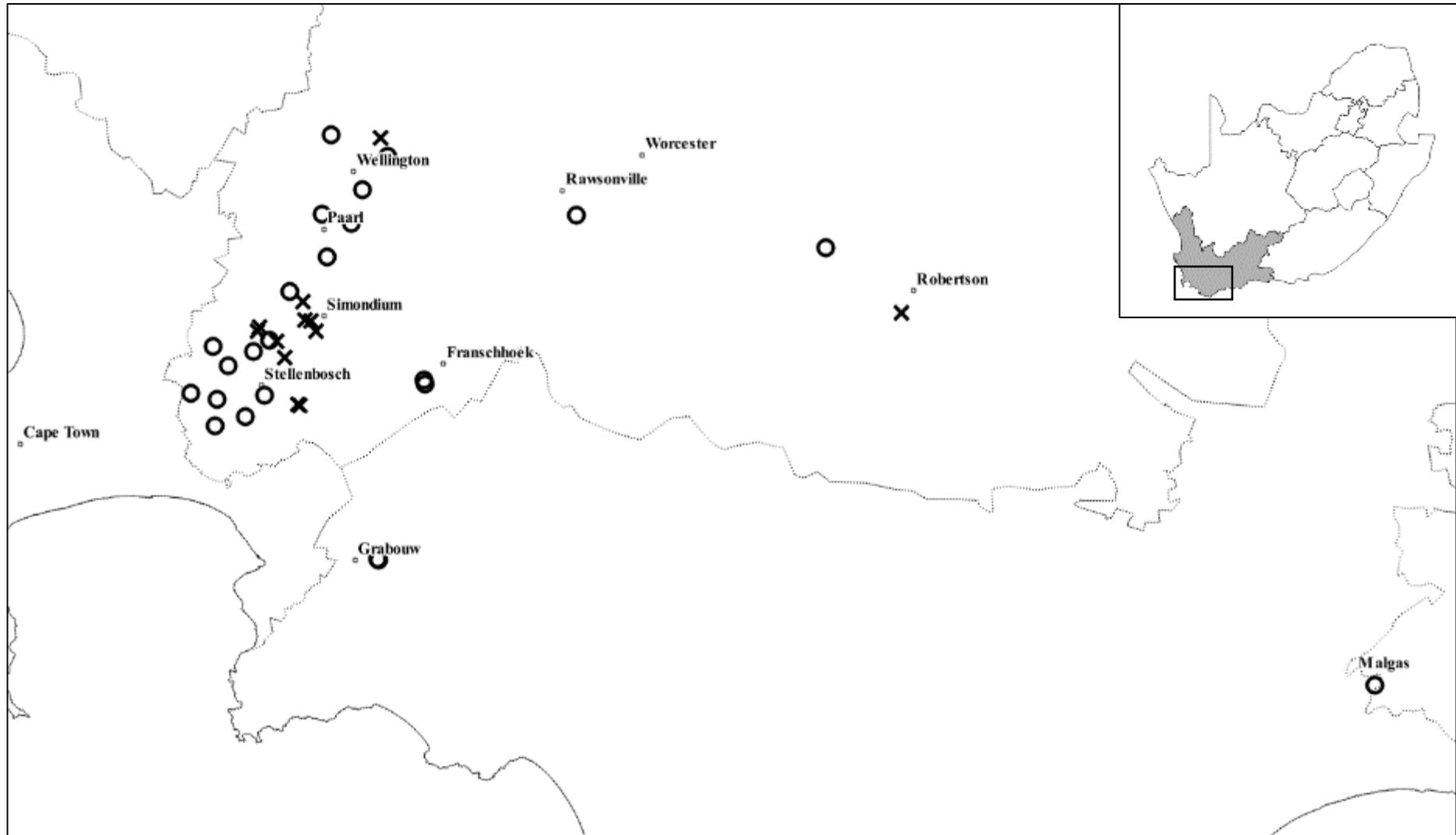


FIGURE A1.2

Distribution map of *Plangia graminea* in the Western Cape of South Africa. Circles denote reported sites, crosses denote confirmed sites.

Observations on biology

Nymphs

In the field, early nymphs were small and black, with their antennae measuring up to three times their body length. Visually they resemble small, round, black beetles, barring for their long antennae, useful as a form of mimicry for self-defence (Fig. A1.3a). Second instars developed orange and red accents on their legs, which then gradually spread to the rest of their body as they aged (Fig. A1.3b). Later instars lost the orange and red accents and progressively turned green over their entire body, with light-green and grey banding on their back, presumably to camouflage within the now foliage-rich grapevine canopy (Fig. A1.3c). Late instars were entirely green, with well-developed wing-buds and remarkably long hind legs (Fig. A1.3d). Katydid nymphs have at least three instars, but because nymphs consume their exuviae after moulting, this exact number is unknown. Researchers working on the dragon-headed katydid mentioned similar constraints, especially with regards to early instars (Chang, 2014).

Nymphs were observed to be active during daytime. They were found moving within the vine canopy, basking in the sun during early morning and actively feeding on leaves throughout the day. Nymphs are highly mobile due to their well-developed hind legs (Fig. A1.3d).



FIGURE A1.3

Nymphs of *Plangia graminea* from early (A), middle (B, C) and late (D) instars.

Adults

In the field, adults were observed to be entirely green and very well hidden in the vine canopy (Fig. A1.4). Yellow and brown variations were also observed, but very rarely, and pink variations were reported by Doubell (2017). Katydid males were reported by growers to stridulate during the dusk and early evening, but in the laboratory colony they were often heard until midnight. Adult katydids are highly mobile and were observed to fly up to 20 m, enough

to move between two or three vine rows, or when on the outer fringes of a block, between blocks.

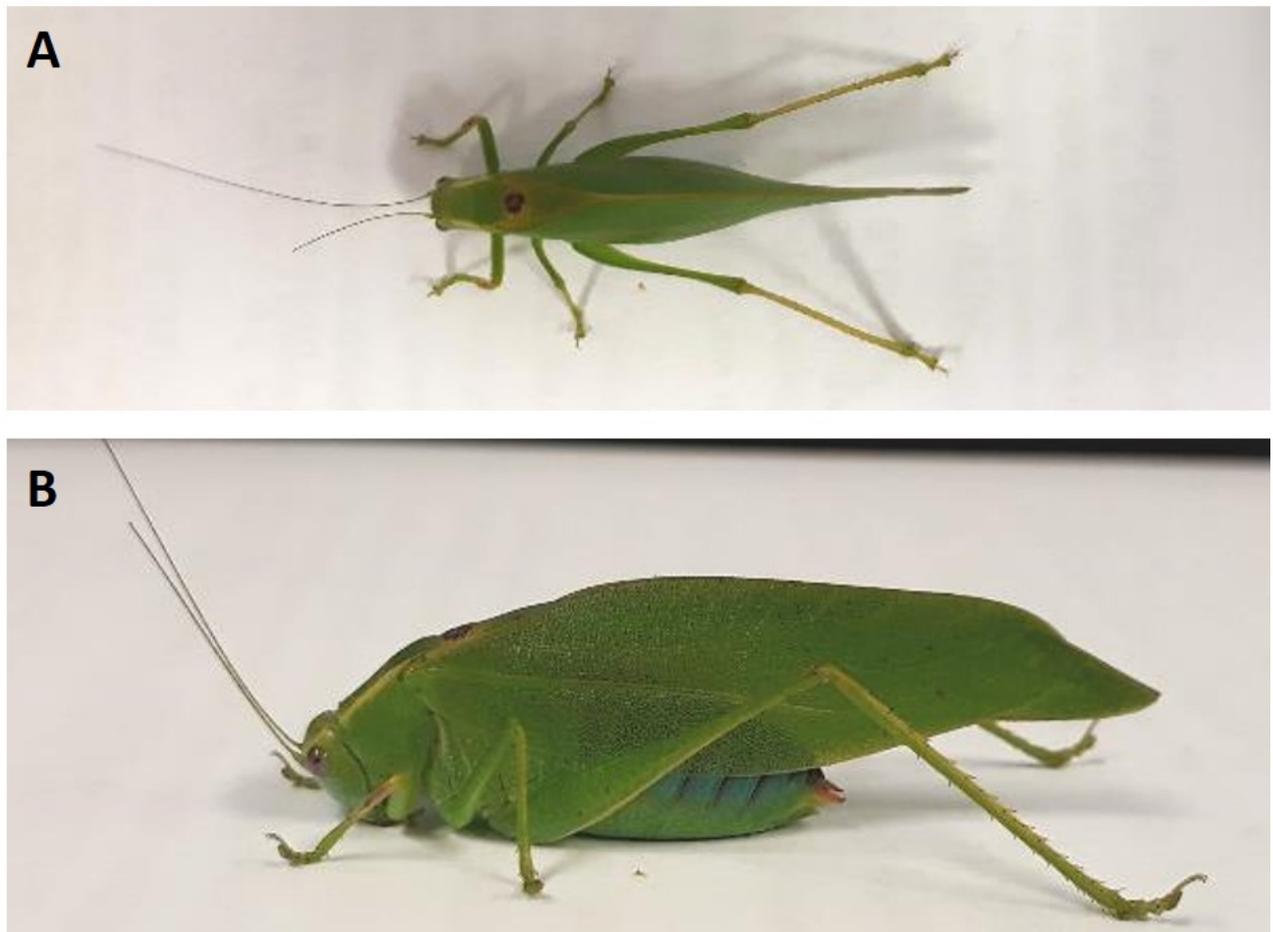


FIGURE A1.4.

Adult males of *Plangia graminea* from the top (A) and left side (B).

Observations on behaviour and agricultural impact

Katydid nymphs do not seem to have a soil stage. Nymphs, due to their small size, stay within the canopy and move within the row. Adults were often observed to fly between rows and have the ability to fly to neighbouring blocks of close proximity. This may enable adults to lay eggs in adjacent blocks after mating, potentially increasing the distribution and abundance of katydids the next season, however this was not readily observed in practice. Other than suitable habitat, many factors can influence their distribution, for example, climate sensitivities, natural enemy complexes and habitat preferences, but agrochemical use may have the greatest influence on their distribution and abundance.

Both nymphs and adults feed on leaves within the vine canopy, the damage of which were observed to be randomly placed and irregularly sized (Fig. A1.5). During large outbreaks,

damage was so severe that only the vascular tissue (veins) of leaves remained, which may result in reduced vine vigour and stunted development. Growers reported that organophosphates and pyrethroids, used for the control of weevils, among other insects, also suppressed katydids, especially when used at higher concentrations.



FIGURE A1.5

(A) Feeding damage caused by nymphs of *Plangia graminea*, (B) nymph on damaged leaf.

Observations on ecology

In natural conditions, nymphs of *P. graminea* were inconspicuous and adults well camouflaged in their environment. In grapevine, the eggs of *P. graminea* started to hatch in early September during the onset of bud break. Nymphs moulted three to four times over the course of three months and the population peaked between late-October and November. The developmental stage of katydids was often found to differ between locations, suggesting that the cues for egg hatching may trigger only in favourable environments, or alternatively that different environments may result in asynchronous development. For example, during November in a Wellington study site, mid-instar nymphs were abundant and resulted in serious foliar damage, whereas in Stellenbosch sites, nymphs were late to final instars and their population were of a much lower density.

Adult katydids were observed to emerge from November, and by late-December, at least half of the observed population were adults. The population decreased drastically from late-December to February until the end of the season, possibly due to agrochemical applications, on-farm disturbances (e.g. harvesting) or adverse weather conditions. In the summer,

heatwaves can cause day temperatures of $> 40^{\circ}\text{C}$, particularly between December and February (WOSA, 2007; Saal, 2017).

Mating took place shortly after katydids matured into adults. Females of *P. graminea* were observed to lay their eggs under the bark of vine, while most of the other Phaneropterinae katydids lay their eggs in-between the epidermal layer of leaves (Picker *et al.* 2004). Similar to *P. graminea*, *L. intermedia* were observed to oviposit their eggs are under several layers of banana plant stem (Chang, 2014). Female katydids oviposit eggs using specialised hockey-stick-like ovipositors, after which eggs overwinter until the following spring (Hartley, 1990).

Katydid males stridulate during dusk and early evening to attract females for mating, and provide a spermatophylax as nuptial gift, which the females consume for nourishment (Doubell *et al.*, 2017) and to increase the fitness of their offspring (Gwynne, 1997). Similar to other katydids of the Phaneropterinae subfamily, *P. graminea* is considered primarily herbivorous (Allsopp, 2012; Belwood, 1990), and is believed to have only one generation per year.

Observations on the laboratory colony

In the laboratory colony, nymphs of all instars were observed to prefer the topmost part of their enclosure, usually the underside of the top panel, towards the brightest light source. Researchers of the San Diego Zoo also mentioned this behaviour in nymphs of *L. intermedia* (Chang, 2014). Rotating the enclosure resulted in the katydids moving and realigning themselves to face the light source again, while temperature, time of day and the type of light did not seem to influence this preference. Nymphs, especially the early instars, were only observed on shoots and leaves while feeding. Adults, being heavier, preferred to perch on vine twigs and shoots (Fig. A1.6).



FIGURE A1.6

Nymphs and adults of *Plangia graminea* of the laboratory colony.

Plangia graminea nymphs are suspected of cannibalising one another, as individuals occasionally went missing, leaving only body parts (Fig. A1.7). Chang (2014) made similar remarks in their attempt to rear *L. intermedia*.

Katydid nymphs were supplied with lettuce and wine grape leaves, with fish food flakes and oats as protein supplement, and carrot, of which they only consumed lettuce and wine grape leaves, while preferring the latter. Adults were only given wine grape leaves, supplemented by lettuce as a source of moisture.



FIGURE A1.7

Damaged, cannibalised and malformed individuals of *Plangia graminea* from the laboratory colony.

Mortality in the laboratory colony was consistently high and averaged a decrease of approximately 40% per month. Mortality was highest the first week after collection (15- 20%), the cause of which is still unknown. There was no notable difference in mortality observed between the environments at which the colony were kept. Stressors influencing survival may include competition for food and habitat resources, rivalry, disease, or suboptimal environmental conditions. As nymphs moulted to later instars, their body colour deviated from those observed in the field, for example, the orange and red accents lasted longer for katydids in the laboratory colony, while later instars were not as green or colourful as those found in the field.

Cadavers of katydids from the laboratory colony were incubated at 25°C at close to 100% RH for 48-72 h, after which they were split into two groups, based on the cadaver's physical appearance and body rigidity. Those suspected of infection by entomopathogenic nematodes (EPN), were placed in a modified White trap (White, 1927) for two weeks, after which nematode production was evaluated. Those suspected of infection by entomopathogenic fungi

(EPF), were incubated until sporulation. Spores were then inoculated onto Sabouraud Dextrose Agar and Yeast (SDAY) plates, and incubated for two weeks at 27°C.

From cadavers suspected of EPN infection, no nematodes were produced and the odour of cadavers did not match those associated with EPN infection. From the cadavers suspected of EPF infection, only opportunistic and saprophytic EPF developed. These results thus suggest mortality by viral or bacterial infection, likely caused by a compromised immune system due to stress, or other stress factors not compatible with katydid survival.

During moulting, some katydids were also observed to be seriously malformed (Fig. A1.8). It is important to note, however, that in field environments, these individuals would likely be preyed upon and quickly removed from the population, whereas in the laboratory environment they can be more readily observed.



FIGURE A1.8

A malformed individual of *Plangia graminea* from the laboratory colony.

The main purpose of the laboratory colony was to house *P. graminea* and their offspring for use in bioassays (Chapter 2), but difficulties in obtaining enough insects, combined with significant colony mortality, required the use of field collected individuals for bioassays instead. This duality of requirements resulted in the prioritisation of katydid usage, favouring the bioassays of Chapter 2, due to low survival rates in the laboratory colony.

CONCLUSION

In this study, field observations indicated that *P. graminea* has only one generation per year and very likely no soil stage. Nymphs were observed to feed throughout the day and to exhibit both mimicry and camouflage as anti-predator defence mechanisms. Their abundance and

damage to grapevine seem to peak between October and November, which recommends the application of control measures before this time period, but only after most eggs have hatched. Katydid were mainly reported and collected from the Cape Winelands region. They have been reported from elsewhere in the province, but their impact there seem to be much less intense. Katydid did not perform well in captivity, which necessitated the use of field collected individuals for bioassays. The ability to rear these insects in an artificial environment would be beneficial for future IPM research.

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APPENDIX 2:

Observations on *Lobesia vanillana* (De Joannis) (Lepidoptera: Tortricidae) in grapevine of the Western Cape

INTRODUCTION:

Lobesia vanillana (De Joannis) (Lepidoptera: Tortricidae), is a recent, sporadic pest of grapevine in the Western Cape province of South Africa. Little is known about the biology and ecology of this species, but they have been reported to be polyphagous (Brown *et al.*, 2014) and to occur throughout the Afrotropical region (Razowski & Brown, 2009).

Morland (2015) reported *L. vanillana* as a sporadic bycatch in the Bonnievale and Robertson area ($\pm 300 \text{ km}^2$), situated in the Breede River Valley in the Western Cape province of South Africa. Pest outbreaks during the 2016/2017 season, however, resulted in economic losses for some growers in this region (K. Van Zyl, AgriRos, pers. comm., 2017). As there were no chemicals registered for its control, growers used broad-spectrum chemical insecticides, which proved successful when applied at the right time. Increasing pesticide regulation and environmental awareness from marketplaces, encourages the use of more environmentally-friendly and integrated approaches with regards to pest control. All pesticides in South Africa must be registered in accordance with the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act (Act No. 36 of 1947).

Prior to evaluating the potential of integrated pest management programmes, at least a basic understanding about the biology and ecology of the target species is required. The aim of this chapter was to investigate the basic biology, ecology and mass rearing potential of *L. vanillana*.

MATERIALS AND METHODS

Site identification

A commercial pest monitoring company (AgriRos, Robertson), used yellow delta sticky traps, baited with carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), lure (Carob Moth lure, Chempac (Pty) Ltd), to monitor the distribution and abundance of adult moths in the Breede River Valley. Since the application of pesticides to control *L. vanillana* outbreaks in 2016, economic damage in the area became less of a risk. Adult moths were captured in the Robertson/McGregor region during 2016/2017 and 2017/2018, from which two sites, located in-between Robertson and McGregor (33°53'24"S, 19°51'48"E), were selected for *L. vanillana* collection.

***Lobesia* collection**

Grape bunches were selected at random from within the vine canopy, collected by hand and placed in 2-L plastic containers, together with vines and leaves, to cushion the bunches during transport and to maintain humidity. At the insectary of the Department of Conservation Ecology and Entomology, Stellenbosch University, bunches were broken apart by hand and inspected for eggs, larvae and pupae, over the course of three days. Hand evaluation of grape bunches revealed larvae sitting in-between berries, hidden within the decaying bunch, probably feeding off sap, while none were found within the berries. Specimens were placed in separate containers and taken to the mass rearing facility at Welgevallen experimental farm in Stellenbosch.

Laboratory colony

At the rearing facility, larvae were placed in shallow, rectangular containers, and fed an agar-based modified codling moth diet (Stenekamp, 2011). The diet consisted of agar, carrageenan, wheat flour, wheatgerm, brewer's yeast, vitamins and preservatives (D. Stenekamp, pers. comm., 2018). Containers were placed in brown paper bags to limit light intensity, to preserve humidity and to limit microbial contamination. When the majority of larvae were considered final instars, crumpled paper towels were placed on top of the diet to provide a place for pupation. After emergence, adult moths were collected and placed in 2-L plastic bottles, within which females oviposited their eggs. Bottles were kept next to a window to provide moths with indirect natural sunlight, as they did not perform well under artificial light of comparatively lower intensity. A cotton ball, dipped in a 2% sugar-water solution, was placed inside the bottle for nutrition, without which oviposition was poor.

After approximately three days, adult moths were removed and bottles were cut into several large pieces, each piece containing a collection of eggs. These pieces were rinsed with room temperature tap water, before placing it partially into new diet for the eggs to hatch. Poor colony performance during the winter months prompted the use of lukewarm water instead. One to two weeks later, the larvae grew large enough to visually inspect and to handle using soft metal forceps. Plastic bottle pieces were removed from the diet, and larvae remaining on the plastic pieces had to be transferred to the diet manually. Later revisions used wire racks upon which these plastic pieces were placed, which allowed larvae to drop directly onto the diet without human intervention, saving time and limiting contamination.

The rearing room which housed the egg, larval and pupal stages of *L. vanillana*, were kept at a constant 25°C with a 18:6 h light-dark cycle, using vertically-hung daylight-colour LED tube lights.

On the day of the bioassays (Chapter 3), laboratory reared insects were collected from the rearing facility, which produced three trays per week, each with 100-200 larvae, under optimal conditions. The tray with the most final instar larvae was the primary source of insects for bioassays, while the other two were fed back into the colony to preserve genetic diversity and to prevent cross-contamination.

Eggs, larvae, pupae and adults were observed, photographed and measured using a Leica MZ75 stereo microscope fitted with a Zeiss Axiocam 105 camera device.

RESULTS AND DISCUSSION

Observations on biology

The observations in this section were made by studying the first generation of *L. vanillana* produced by the rearing facility, except where specified otherwise.

The adult moth of *L. vanillana* is small (6 mm). Wings are tan to brown and have characteristic dark patterns (Fig. A2.1). Similar to *L. botrana*, adults likely fly at dusk (Lucchi *et al.* 2018).

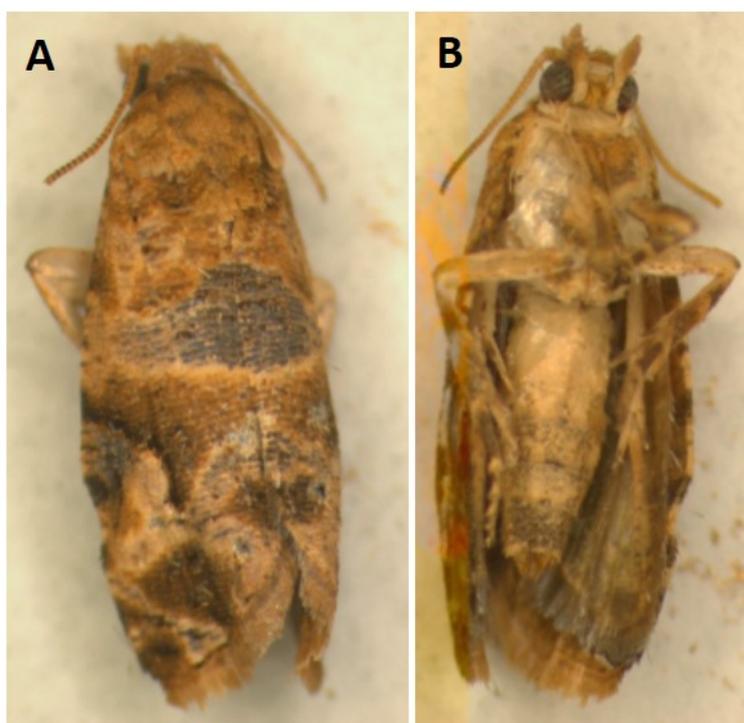


FIGURE A2.1

Adults of *Lobesia vanillana*, (A) dorsal and (B) ventral, field collected.

Eggs were observed to be slightly oval and white, turning cream in colour as they develop, with the dark head of the larvae visible through the transparent shell, prior to hatching (Fig A2.2).

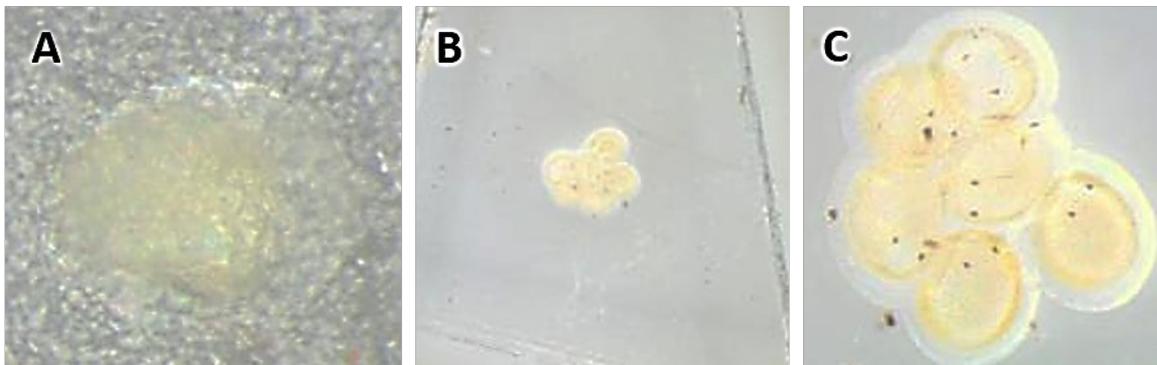


Figure A2.2

Egg development of *Lobesia vanillana*; (A) as taken on 2018/02/23 (B) eggs on plastic, 2018/03/05 and (C) a close-up of eggs on plastic, 2018/03/05.

Eggs produced cream to yellow coloured larvae (Fig. A2.3).

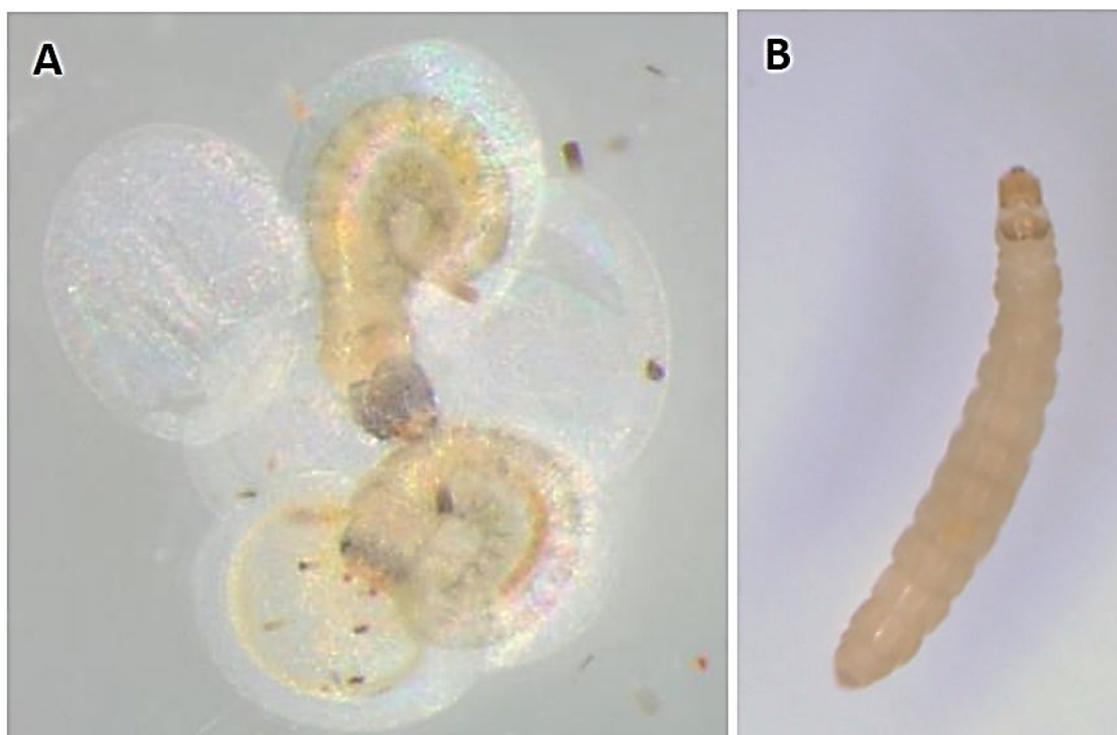


FIGURE A2.3

(A) First instar larvae of *Lobesia vanillana* hatching from eggs, as taken on 2018/03/06, and (B) developing larva, 2018/03/14.

Within two weeks, larvae developed to late instar stages, with their primary colour darkening towards brown or black in the last few days (Fig 2.4a, b), occasionally with subtle hints of blue or purple, presumably a coloration of the haemolymph (Fig 2.4c).

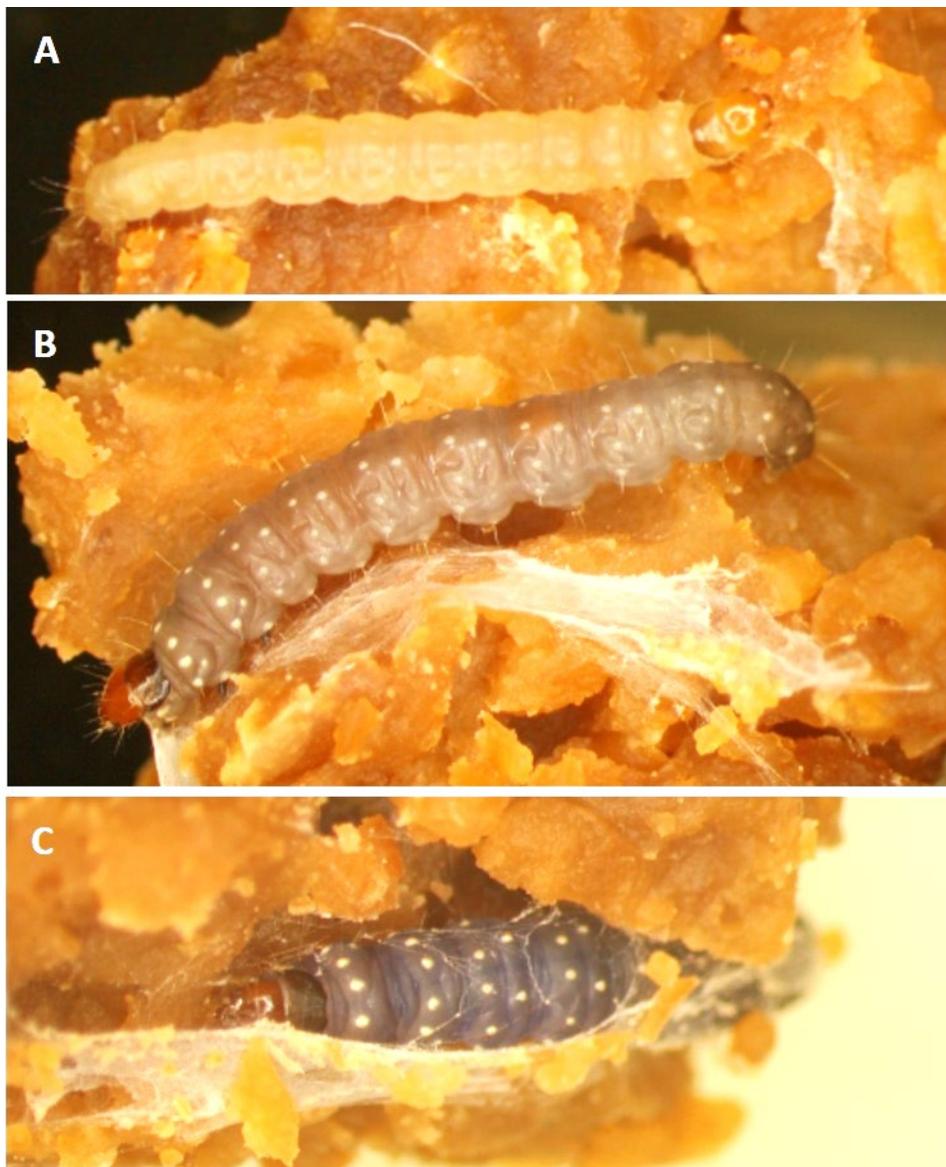


FIGURE A2.4

Development of larvae of *Lobesia vanillana*, as taken on (A) 2018/03/16, (B) 2018/03/19 and (C) 2018/03/21.

Webbing became visible in the diet during the final instars, and the transformation to pupa followed shortly after (Fig. A2.5).



Figure A2.5

Pupa of *Lobesia vanillana* as taken on (A) 2018/03/23 and (B) 2018/03/27.

TABLE A2.1

Measurements of *Lobesia vanillana* life stages.

Figure	Date	Type	Length	Width
A2.1	2018-02-22	Adults	6.0 mm	2.2 mm
A2.2c	2018-03-05	Eggs	0.6 mm	0.5 mm
A2.3a	2018-03-06	Larvae	1.45 mm	0.14 mm
n/a	2018-03-08	Larvae	2.06 mm	0.26 mm
n/a	2018-03-12	Larvae	3.43 mm	0.46 mm
A2.3b	2018-03-14	Larvae	5.97 mm	0.65 mm
A2.4a	2018-03-16	Larvae	7.75 mm	0.88 mm
A2.4b	2018-03-19	Larvae	10.07 mm	1.23 mm
A2.5a	2018-03-23	Pupae	7.2 mm	1.4 mm
A2.5b	2018-03-27	Pupae	6.3 mm	1.3 mm

Observations on ecology

The signs of infestation were observed to be similar to that of *L. botrana* (Varela *et al.* 2010), which includes: frass on the outside of berries as they feed, webbing, the spinning together of plant- or bunch parts to form a nest, circular entry holes and hollowed out berries, and bunch-rot, the progressive decay of an entire bunch due to damage and secondary infections. During

scouting, webbing may be a useful indicator to predict damage, as it precedes infestations of future, and often larger, generations.

Once infestation has taken place, individual berries start to rot and may infect healthy berries or neighbouring bunches with secondary pathogens. Ant and vinegar fly infestations, mould and sometimes botrytis were observed to follow soon after. This material may encourage the proliferation and spread of other airborne plant pathogens and secondary insect pests.

In the field, *L. vanillana* was observed to have three to four flights per year, each separated by 4-5 weeks, depending on environmental conditions. The first flight of adults emerged intermittently during late spring, probably from overwintering pupae similar to *L. botrana* (Deseo *et al.* 1981), or from alternative hosts. Subsequent larvae are believed to have fed on flowering parts, undeveloped berries and other nutritional soft plant tissues, like *L. botrana* (Stavridis & Savopoulou-Soultani, 1998), which may handicap berry formation and bunch development.

The second larval generation emerged 4-5 weeks after the first. Control programmes (either ovicides or larvicides) for *L. botrana* typically target this generation to mitigate pest damage, before veraison sets in and berries grow too tightly packed for insecticidal penetration (Cooper *et al.* 2010). Adults of the second generation were observed to oviposit eggs directly on berries, which after hatching, provides direct access to food and habitat resources offered by the grape bunch. Larvae of the third generation were observed to nest within the bunch, which may accelerate and intensify injury, though it is unknown at this stage whether multiple larvae will feed on a single berry, or whether single larva will feed on multiple berries.

It is believed that the life cycle of *L. vanillana* does not include a soil stage. In vineyard, eggs are oviposited on berries, where larvae feed and develop until they pupate within the bunch, in plant crevices or in-between the bark of vine. There may be a fourth or even fifth generation, depending on the season length and associated factors (average temperature, daylight hours, locality, etc.), but more research is needed, especially with regards to the development of a degree day model and life history tables.

Observations on rearing

Under laboratory conditions, the developmental time of *L. vanillana* was 4-5 weeks. Eggs were observed to hatch within one week of their oviposition, after which larvae developed for 2-3 weeks until the final instar stage. Adults emerged 1-2 weeks after pupation. After mating, adults were observed to lay eggs for approximately one week, with peak egg production after two

days. In laboratory colonies, the population of *L. vanillana* appeared stressed during the winter months, with high mortality rates and inconsistent development times, especially of the early instars. It is assumed that their poor performance was caused by inhibiting their transition to the diapause stage, as the rearing conditions for *L. vanillana* were kept constant throughout the year. Once spring arrived, the health of the colony improved to or above pre-winter levels. Except for the adults placed next to a window, none of the other life stages were exposed to conditions outside of the rearing room, suggesting that this may have been a cued genetic response. Alternatively, the colony was stressed and high mortality in the winter months coincidental.

Insects of the laboratory colony were observed to lack the brownish pigmentation, presumably of the cuticle, of insects in the field, which made their haemolymph shine through their mostly transparent cuticle. This resulted in completely blue or purple larvae (Fig. A2.6a, b). The reason for this strange coloration is unknown, but it did not seem to affect fitness in the laboratory colony or in bioassays. Green and purple colourations have also been observed in the larvae *L. botrana* (Steinitz *et al.*, 2016).

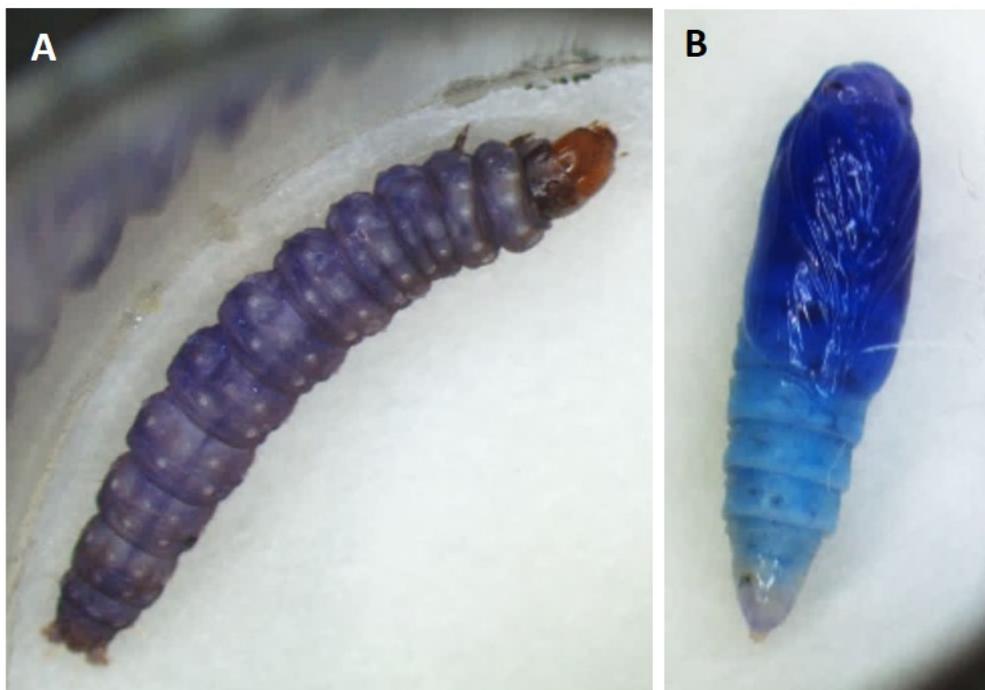


FIGURE A2.6

Blue/purple colouration of a (A) larva and (B) pupa, sourced from the *Lobesia vanillana* laboratory colony.

CONCLUSION

Knowledge on the biology and ecology of *L. vanillana* was expanded upon by laboratory and field observations, which may prove useful for pest management considerations or for more comprehensive research in the future. *Lobesia vanillana* proved relatively easy to rear in the laboratory, following experimentation and adaptations based on previous rearing experience with codling moth (D. Stenekamp, pers. comm., 2018). Reduced colony production during winter months suggests a diapausing stage, a phase not promoted in the laboratory colony. Future research on *L. vanillana* should aim to further expand upon the ecological data currently available, including host range, degree day models and economic damage thresholds. The development of a lure specific to *L. vanillana* may improve the sensitivity of monitoring traps, as lure for carob moth is currently used. The geographical distribution of *L. vanillana* is likely to be underestimated due a lack of monitoring outside of existing problem areas.

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