Damage to citrus and vegetables by *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) and prospects for control with entomopathogenic fungi

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Thesis accepted in fulfilment of the requirements for the degree *Doctor of Philosophy in Environmental Sciences* at the North-West University

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DEDICATION

To my beloved wife Mwanjia Hassani, our sons Dhaky and Anwar for their love, support and encouragement. I really love you.

To my father, Mohamed Mkiga, my mother, Regina Ekonga and entire Mkiga family, thanks for your love and prayers, you were honest partners in this struggle.

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ABSTRACT

Globally, orange is one of the major fruit crops contributing to nutrition and monetary income. False codling moth (FCM), *Thaumatotibia leucotreta*, is one of the major constraints of orange production. Before this study, little was known regarding the bio-ecology of FCM in orange and vegetables in Kenya and Tanzania and the potential use of dry conidia of entomopathogenic fungi for control of *T. leucotreta* moths has not been tested. There is also no IPM strategy available for FCM in East Africa. This PhD study therefore aimed at generating information on these aspects. Field surveys on damage inflicted by the pest on orange and vegetables were conducted in Kenya and Tanzania. The spatial-temporal population dynamics and genetic diversity of FCM were evaluated in citrus orchards in these two countries. The highest FCM larval incidence (46%) was recorded on orange produced at high altitudes in Kenya while the lowest (33%) was recorded at low altitudes in Tanzania. The highest FCM infestation amongst the vegetables was recorded on African eggplant (12%) while the lowest was on okra (3%). A similar spatio-temporal pattern of FCM was observed in both countries, with the highest catches being recorded in August, during the 2017 and 2018 orange fruiting seasons in these regions. Microbial control of the pest was tested by screening dry conidia of entomopathogenic fungi isolates of *Metarhizium anisopliae* and *Beauveria bassiana* species. Dry conidia of these EPF isolates were found to be pathogenic to the moths, the ICIPE 69 isolate caused the highest mortality of 94.2%. Fecundity was reduced by 33.6 and 25.9% for the donor (fungal contaminated moths) and recipient (fungus-free moths allowed to mate with fungal contaminated moths) FCM females, respectively after horizontal transmission. Compatibility of the potent entomopathogenic fungal isolate, ICIPE 69 and the FCM sex pheromone was tested in an auto-inoculation device. The fungus remained viable and was therefore compatible with the pheromone. The fungus in the autoinoculation device was integrated with other control tactics and evaluated in citrus orchards in Machakos and Makueni counties in Kenya. In this trial, a lower percentage of infested fruit (4.67% and 6.67%) was recorded in orchards where the treatment combination "ICIPE 69 campaign + dry conidia of ICIPE 69 applied in the autoinoculation device + Last call FCM" was applied, compared to the untreated orchards (48.67% and 54.33%) at Machakos and Makueni respectively. The effect of FCM infestation was also reflected on marketable yield, with the highest yield (10,880.68 and 11,192.26 kg orange fruit/ha) recorded in orchards where this treatment combination was applied, while the lowest yield was recorded in untreated orchards (5,944.28 and 5,458.63 kg orange fruit/ha) at Machakos and Makueni, respectively. The findings from this study indicated that *T. leucotreta* is present and causes significant losses in Kenya and Tanzania thus control tactics need to be implemented. The compatibility of ICIPE 69 and FCM sex pheromone in the auto-inoculation device provide for its use in integrated management strategies for the pest. A significant reduction in the *T. leucotreta* population and fruit infestation as well as an increase in marketable orange fruit yield were obtained with the combined use of entomopathogenic fungi and Last Call FCM. This combination can therefore be used as an integrated management strategy for FCM. Low genetic diversity of FCM specimens from Kenya, Tanzania, Uganda, Sudan and the Republic of South Africa, as well as from different hosts was determined. Similar management strategies for control of *T. leucotreta* can therefore be used across Africa.

Keywords: citrus, entomopathogenic fungi, Kenya, Tanzania, *Thaumatotibia leucotreta*, vegetables

RESEARCH ETHICS CLEARANCE: 2019

FNAS Ethics Committee

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Date: 22-May- 2019

Dear Researcher,

Re: Ethics waiver

This letter serve as confirmation that based on the scientific committee assessment of the research proposal concluded that:

Student: A Mkiga

Title:

Does not require ethical clearance as the study has no/low risk.

Supervisor (s)

Hannalene Du Plessis

Signature: Chairperson of FNAS Ethics Committee

Prof Lesetja Motadi

PREFACE

This thesis follows the article format style as prescribed by the North-West University. Therefore, articles appear in published format, while manuscripts are adjusted according to the instructions to authors of internationally accredited, scientific journals. As an additional requirement by the North-West University, Table A details the contributions of authors for each article/manuscript and provides permission for use as part of this thesis.

The following Chapters were included in this work:

Chapter 1 – Introduction (NWU Harvard, Reference Style of the Faculty of Law, published by the Library Services of the NWU)

Chapter 2 – Literature review (NWU Harvard, Reference Style of the Faculty of Law, published by the Library Services of the NWU)

Chapter 3 – Article 1 (published) ***Insects*** **(MDPI)**

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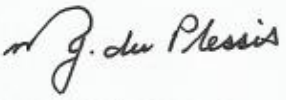
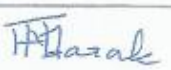
Chapter 5 – Article 3 (prepared): ***Journal of Pest Science*** **(Springer)**

Chapter 6 – Article 6 (prepared): ***Journal of Applied Entomology*** (**Wiley Online Library)**

Chapter 7 – General discussion, conclusions and recommendations (NWU Harvard, Reference Style of the Faculty of Law, published by the Library Services of the NWU)

Table A: Contributions of authors and consent for use.

Author	Article	Contribution	Consent*
AM Mkiga	1, 2, 3 and 4	Conducted experiment, responsible for data collection, analysis and result interpretation, wrote articles and thesis.	
MJ Du Plessis	1, 2, 3 and 4	PhD promotor, supervision, provided intellectual input, execution of the study, data analysis, articles and thesis review.	
SA Mohamed	1, 2, 3 and 4	PhD co-promotor, supervision, provided intellectual input, execution of the study, data analysis, articles and thesis review.	
FM Khamis	1, 2, 3 and 4	PhD assistant-promotor, supervision, provided intellectual input, execution of the study, data analysis, articles and thesis review.	
KS Akutse	2 and 3	Provided intellectual input and review of articles.	
S Ekesi	1, 2, 3 and 4	PhD co-promotor, supervision, intellectual input, execution of the study, articles and thesis review, secured funding for the project.	

*I declare that the stated contributions are accurate and have approved the use of this article/manuscript as part of the thesis of Mr. AM Mkiga.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Citrus, (Rutaceae: Aurantioideae) is a major fruit crop and is widely grown in tropical and subtropical regions (Reykande *et al.,* 2013). The major citrus fruit is sweet orange (*Citrus sinensis* L.), tangerines (*Citrus reticulate* Blanco.), grapefruit (*Citrus paradis* Macf.), lime (*Citrus aurantifulia* L.) and lemon (*Citrus limonum* Burm. f.) (Okwu, 2008). Sweet orange fruit are consumed fresh and approximately a third is processed globally, mostly as juice (Liu *et al.,* 2012). Citrus contributes to the human diet (Liu *et al.*, 2012) and nutritional security by providing vitamins (Lv *et al.,* 2015). Apart from providing vitamin C (Turner & Burri, 2013), the fruit also contains macro- and micronutrients (Economos & Clay, 1999). Brazil, China mainland, the United States of America, Mexico and India are the top five world citrus producers (FAOSTAT, 2017).

Kenya and Tanzania are citrus producing countries in East Africa (Makorere, 2014). Citrus production in Tanzania is largely concentrated in the North East Coast. Tanga and Coast region have the largest planted area of citrus followed by Morogoro, Mwanza and Ruvuma (Makorere, 2014). Sweet orange is a major cash crop, with the fruit marketed inside and outside the country. In Kenya, citrus is a source of income for small-scale farmers and employs the rural population (Olubayo *et al.,* 2011). The highest production is in Coast, Eastern and Rift Valley provinces (Mounde *et al.,* 2009).

1.2 Problem statement and justification

Citrus is attacked by many insect pests and diseases with the false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) amongst the major pests of the crop. FCM is a polyphagous pest (Venette *et al.,* 2003; Timm *et al.,* 2010) that attacks a range of cultivated and uncultivated plants. The pest also jeopardizes access to quarantine sensitive markets (Mazza *et al.*, 2014). Losses due to *T. leucotreta* attack are reported to be about 46% on orange and 12% on solanaceous vegetables in East Africa (Mkiga *et al.,* 2019). The pest is widely controlled with synthetic insecticides which can result in insecticide resistance (Hofmeyr and Pringle, 1998) and disruption of control by natural enemies, causing outbreaks of secondary pest populations (Steinmann *et al.*, 2011).

The injuriousness of *T. leucotreta* to citrus in South Africa was studied by Newton (1998) and its host plant range by Kirkman and Moore (2007). The incidence and damage inflicted on citrus by this pest in Kenya and Tanzania are unknown. Information on the

incidence of the pest on other crops, preference for oviposition by FCM and suitability of the hosts for offspring development that may serve as alternative host plants between successive seasons is scanty.

Several studies have been conducted assessing the potential use of entomopathogenic fungi against *T. leucotreta* (Goble *et al.*, 2011; Coombes *et al.*, 2013; Moore *et al.*, 2015). However, information on the virulence of dry conidia of entomopathogenic fungi on adult *T. leucotreta*, the fungal transmission and the impact on the reproduction potential of FCM are not available. No integrated pest management (IPM) strategy is available for sustainable control of the FCM in East Africa. Knowledge to fill the information gaps mentioned above is crucial for sustainable control of FCM on orange and other hosts.

Molecular studies on different *T. leucotreta* populations have been conducted for southern Africa (Timm *et al.*, 2010; Mazza *et al.*, 2014) and western Africa (Onah *et al.*, 2016). There is, however, limited information on the genetic structure of *T. leucotreta* populations from eastern Africa at different altitudes. In studies that were done, *T. leucotreta* were sampled with pheromone traps in Nigeria and to a limited extent, from incubated infested orange fruit (Onah *et al.*, 2016). Little is known on the genetic structure of *T. leucotreta* that infests vegetables such as peppers. Population diversity and differentiation of closely related individuals can be done with molecular techniques (Deverno *et al.*, 1998). Understanding the genetic variability among the different *T. leucotreta* populations will guide the implementation of proper management approaches.

1.3 Objectives

1.3.1 General objective

The main objective of this study was to investigate the bio-ecology of *T. leucotreta*, as well as the efficacy and implementation of entomopathogenic fungi for the control of *T. leucotreta.*

1.3.2 Specific objectives

The specific objectives of the study were to:

i. assess the damage levels, larval incidence and host preference of *T. leucotreta*, as well as the host suitability of orange and vegetables to *T. leucotreta* in Kenya and Tanzania.

ii. determine the efficacy of different isolates of entomopathogenic fungi for the control of *T. leucotreta* moths and their effect on the reproduction potential of the pest.

iii. study the compatibility of a potent entomopathogenic fungal isolate with a *T. leucotreta* sex pheromone and their use in the integrated management of *T. leucotreta.*

iv. study the spatial-temporal population dynamics and genetic diversity of *T. leucotreta* in Kenya and Tanzania.

1.3.3 Research Hypotheses

i. *Thaumatotibia leucotreta* infests orange and other crops grown near or intercropped with orange in Kenya and Tanzania.

ii. Entomopathogenic fungi affect the reproductive potential of *T. leucotreta.*

iii. There are potent entomopathogenic fungal isolates against *T. leucotetra* which are compatible with the *T. leucotreta* sex pheromone and can be used in the integrated management of the pest.

iv. *Thaumatotibia leucotreta* populations change over time and space and there is genetic variability among different populations of the pest.

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CHAPTER 2

LITERATURE REVIEW

2.1 Citrus production, nutritional and health importance

Citrus (Rutaceae: Aurantioideae) is an important fruit globally, which is grown in more than 100 countries in tropical, subtropical and Mediterranean climates (Shafieizargar *et al.*, 2012). Sweet orange (*Citrus sinensis* L.) is the most consumed citrus fruit grown commercially worldwide (Fu *et al.*, 2011), and it provides microelements to the human diet (Economos & Clay, 1999).

The functional ingredients and antioxidant nutraceuticals or phytochemicals are nutritionally important (Etebu & Nwauzoma, 2014). Citrus fruit are rich in vitamin C (Turner & Burri, 2013) and contains carotenoids and other compounds with nutritional properties, such as vitamin E, provitamin A, flavonoids, limonoids, polysaccharides, lignin, fibers, phenolic compounds and essential oils (Economos & Clay, 1999; Iglesias *et al.*, 2007). Citrus fruit are therapeutic and have anti-tumor, anti-inflammatory and anti-cancer properties, due to the phyto-vitamins and nutrients it contains (Aslin Sanofer, 2014). The non-nutrient compounds of Mandarin peel, such as, hesperidin and narirutin are used as safe food additives with antioxidant activity (Tumbas *et al.*, 2010). The soluble and insoluble dietary fibres of citrus contribute to reducing the risk of many chronic diseases like arthritis, obesity and coronary heart diseases (Crowell, 1999).

2.2 Botany of citrus

The trees of sweet orange are small, evergreen and 7.5 m to 15 m high (Etebu & Nwauzoma, 2014). The use of a vigorous and healthy rootstock is a key element that affects the quality and yield of citrus fruit (Shafieizargar *et al.*, 2012). The leaves are leathery, evergreen and elliptical, oblong or oval in shape and range from 6.5 – 15 cm long and 2.5 – 9.5 cm wide (Etebu *et al.*, 2014). Flowers are white in colour and with a strong scent (Liu *et al.*, 2012), either singly or in whorls of six, with five petals and 20 – 25 yellow stamens.

2.3 Constraints to citrus production

Citrus production is constrained by both abiotic and biotic factors. Environmental conditions influence blooming and flower development and may hamper the natural processes of these stages (Iglesias *et al.*, 2007). Several biotic factors limit the production and productivity of citrus. Examples of diseases that influence citrus are Citrus Variegated

Chlorosis, caused by *Xylella fastidiosa* (Alves *et al.,* 2009), Citrus greening by *Candidatus liberibacter* (Doddapaneni *et al.,* 2008), sweet orange scab caused by *Elsinoe australis* (Chung, 2011) and *Citrus tristeza* virus (Dawson *et al.,* 2013).

Citrus is also infested by various pests. Fruit infesting pests like Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (QFF) and Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritedae) have been reported (De Lima *et al.,* 2007). The False codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) is one of the most destructive pests of citrus fruit in Africa (Gilligan *et al.,* 2011). Damage is caused through larval feeding and development which can also lead to the development of secondary infection mediated by fungi and bacteria (Mazza *et al.,* 2014).

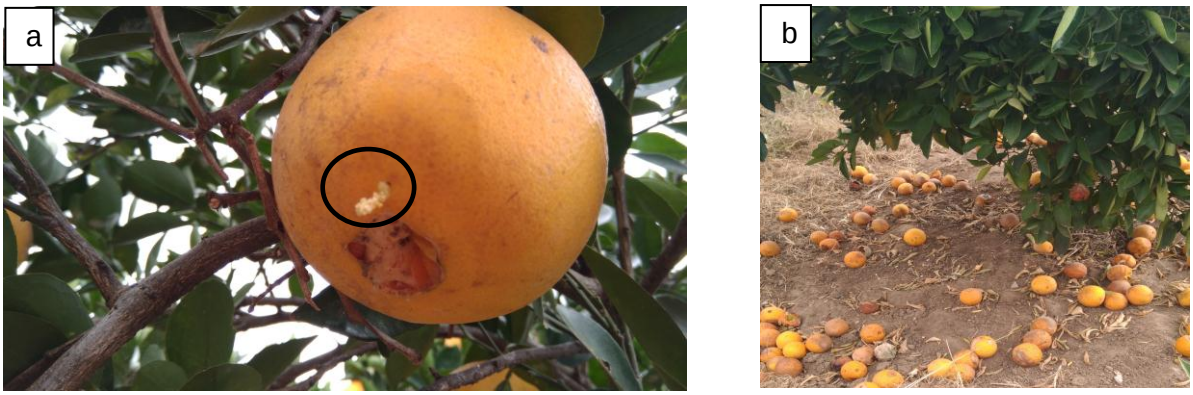

Fig. 2.1: Damaged orange fruit (a) on a tree with frass from a *Thaumatotibia leucotetra* larva feeding inside the fruit, and (b) fruit that dropped to the soil surface as a result of *Thaumatotibia leucotetra* larva feeding.

Source: *icipe.*

2.4 *Thaumatotibia leucotreta*

The false codling moth (FCM), *T. leucotreta,* is native to sub-Saharan Africa and is a key pest of citrus (Venette *et al.,* 2003; Gilligan *et al.,* 2011). The pest is present in most sub-Saharan areas of Africa and nearby islands in the Atlantic and Indian oceans (Newton, 1998). *Thaumatotibia leucotreta* infests its host crops throughout the year (without diapause), with overlapping generation (Mazza *et al.,* 2014). As many as five *T. leucotreta* generations per year have been reported on citrus in South Africa (Venette *et al.,* 2003).

Table 2: Classification of *Thaumatotibia leucotreta.*

Phylum	Arthropoda
Class	Insecta
Order	Lepidoptera
Family	Tortricidae
Tribe	Grapholitin
Genus	*Thaumatotibia* (Meyrick)
Species name	*leucotreta* (Meyrick)
Synonym	*Cryptophlebia leucotreta*
Common name	False Codling Moth

Source: Stibick *et al*. (2008).

2.4.1 Biology of *Thaumatotibia leucotreta*

2.4.1.1 Eggs

The females lay eggs during the night in the depression of the rind of fruit, on foliage, on fallen fruit or on smooth non-pubescent surfaces (Stibick *et al*., 2008). According to Daiber (1978) females lay between 100 and 250 eggs/female on fruit or foliage. The white to cream, flat, oval-shaped eggs (0.77 mm long by 0.60 mm wide) are laid individually. Hatching always occurs during the day with incubation periods of 9 to 12 days in winter and 6 to 8 days in summer on citrus (Newton, 1998). However, only a few of these eggs will survive, due to cannibalism (Stibick *et al*., 2010). The egg incubation period is both temperature and relative humidity dependent. For example, incubation period at 15, 20 and 25 °C are 14.5, 9.8 and 5.1 days respectively, no development at 10 °C and high a mortality rate at 13 ºC and 30% RH, compared to at 60% and at 90% RH, respectively (Daiber, 1979a). According to Johnson and Neven (2010), white eggs become red within 3 – 4 days and finally develop into the blackhead stage at 5 – 6 days after oviposition, when kept at 26 °C.

Fig. 2.2: a) *Thaumatotibia leucotreta* eggs, b) larva and c) pupae.

Source: *icipe.*

2.3.1.2 Larvae

After hatching, neonate larvae penetrate the fruit and larval development is completed inside the fruit (Carpenter *et al.*, 2004). According to Stibick *et al*. (2008), young larvae feed near the surface of fruit, produce frass and cause discolouration of the rind. Mature larvae feed more to the centre of the fruit. Daiber (1979b) reported that larval developmental of *T. leucotreta* to last long (35 – 67 days) in cool conditions compared to 12 – 33 days in warm conditions. Daiber further reported that at 15, 20 and 25 °C the larval durations are 46.6, 18.8 and 11.6 days respectively.

2.3.1.3 Pupae

The fully-grown larvae exit the fruit through holes and drop onto the soil surface where they spin a cocoon of silken threads that bind to the soil particles (Stofberg, 1954) and detritus (Newton, 1998) for pupation. Pupal development consists of light-brown pre-pupal (Newton, 1998) and brown pupal stages (Stofberg, 1954), respectively. The pupal stage is the most sensitive phase in the development cycle of FCM and its duration at 15, 20 and 25 °C is 37.3, 18.6 and 10.8 days for females and 42.4, 20.1 and 11.8 days for males (Daiber, 1979c).

2.3.1.4 Adult

Thaumatotibia leucotreta moths are small, inconspicuous, and dark-brown to grey with a wingspan of 16 to 20 mm (Newton, 1998). The forewing length of males is 7 – 8 mm, and 9 – 10 mm for females (Gilligan *et al.*, 2011). Males have a semi-circular pocket of opalescent scales on the distal end of the vein CuA2 of the hind wing (Gilligan *et al.*, 2011), which is used as the distinguishing characteristic during sexing. According to Stofberg (1954) females mate and commence with egg-laying 2-3 days after emergence

from pupae. The average lifetime fecundity is 460 eggs at 25 ^{o}C and the adult lives longest at 15 ^{o}C (Daiber, 1980).

Fig. 2. 3: *Thaumatotibia leucotreta* moths a)female, b) male

Source: *icipe.*

2.3.2 Global distribution of *Thaumatotibia leucotreta*

Thaumatotibia leucotreta is indigenous to southern Africa (Malan *et al.*, 2017) and occurs in most of the sub-Saharan African countries (Venette *et al.*, 2003). It has also been reported on the islands of the Indian and Atlantic oceans (Newton, 1998).

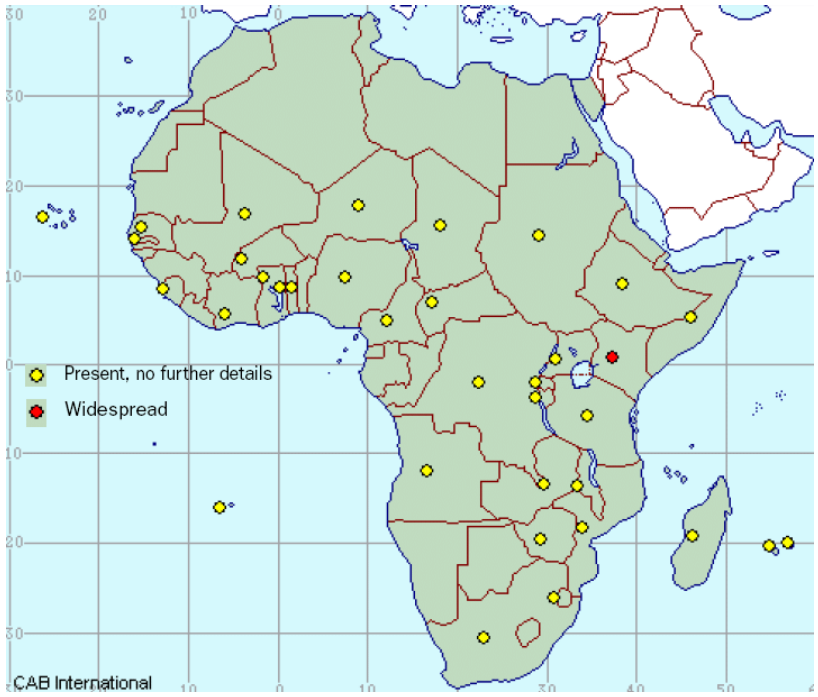

Fig. 2.4: Distribution of *Thaumatotibia leucotreta* in Africa.

Source: CABI.

2.3.3 Host plants

Thaumatotibia leucotreta is highly polyphagous, infesting both cultivated and uncultivated plants (Timm *et al.*, 2010), thus complicating its control. The known host plants of *T. leucotreta* are listed in Table 3.

Table 3: Host plants of *Thaumatotibia leucotreta* reported by Stotter (2009).

Family	***Scientific name***	**Commom name**
Anacardiaceae	*Mangifera indica* L.	Mango
Anacardiaceae	*Sclerocarya birrea* (A. Rich.) Hochst.	Marula
Annonaceae	*Annona muricata* L.	Soursop
Annonaceae	*Annona reticulata* L.	Custard apple
Asclepiadaceae	*Calotropis procera* (Aiton) W. T. Aiton	Roostetree
Bombacaceae	*Ceiba pentandra* (L.) Gaertn.	Kapoktree
Bromeliaceae	*Ananas comosus* (L.) Merr.	Pineapple
Capparaceae	*Capparis* fascicularis L.	Caper
Celastraceae	*Catha edulis* (Vahl) Forssk. ex Endl.	Khat
Clusiaceae	*Garcinia mangostana* L.	Mangosteen
Combretaceae	*Combretum apiculatum* Sond. red	Bushwillow
Combretaceae	*Combretum zeyheri* Sond. large-fruited	Bushwillow
Crassulaceae	*Crassula helmsii* L.	Pygmyweed
Ebenaceae	*Diospyros kaki* L.	Diospyros
Ebenaceae	*Diospyros virginiana* L.	Common persimmon
Euphorbiaceae	*Ricinus communis* L.	Castor bean
Fabaceae	*Acacia karroo* Hayne	Sweet thorn
Lauraceae	*Persea americana* Mill.	Avocado
Malvaceae	*Abelmoschus esculentus* (L.) Moench	Okra
Malvaceae	*Gossypium hirsutum* L.	Cotton
Malvaceae	*Hibiscus moscheutos* L.	Rosemallow
Myrtaceae	*Eugenia monticola* L.	Stopper

Myrtaceae	*Psidium guajava* L.	Guava
Olacaeae	*Ximenia caffra* Sond.	Sour
Oleaceae	*Olea europaea* L.	Olive
Oxalidaceae	*Averrhoa carambola* L.	Carambola
Poaceae	*Saccharum officinarum* L.	Sugarcane
Poaceae	*Sorghum* Moench	Sorghum
Poaceae	*Zea mays* L.	Corn
Proteaceae	*Macadamia integrifolia* Maiden & Betche	Macadamia nut
Punicaceae	*Punica granatum* L.	Pomegranate
Rosaceae	*Prunus persica* (L.) Batsch	Peach
Rubiaceae	*Coffea arabica* L.	Arabian coffee
Rubiaceae	*Vangueria infausta* Burch.	Medlar
Rutaceae	*Citrus sinensis* L.	Orange
Sapindaceae	*Sapotaceae litchi chinensis* Sonn. *Englerophytum magaliesmontan*a (Sond.) lychee T. D. Penn.	Stem fruit
Solanaceae	*Capsicum annuum* L.	Cayenne pepper
Solanaceae	*Solanum melongena* L.	Eggplant
Stericulaceae	*Cola nitida* (Vent.) A. Chev. ghanja	Kola
Theaceae	*Camellia sinensis* (L.) Kuntze	Tea

2.3.4 Population dynamics and molecular characteristics of *Thaumatotibia leucotreta*

Stibick *et al*. (2008) reported that *T. leucotreta* adults can disperse over several hundred meters, with their numbers being controlled by temperature and host availability. This may result in population changes in the pest from different agricultural systems. Agricultural and forest landscapes are heterogeneous environments characterized by a range of different quality of host plant patches (Fuentes-Contreras *et al.,* 2014). The heterogeneity of the environment can occur naturally or artificially by human activities which influence

the distribution of different insect species. High levels of genetic variation have been shown among field populations of *T. leucotreta* in South Africa (Timm *et al*., 2010). The levels of genetic diversity and structure of new invasive populations can, therefore, be affected by recent expansions of the species and passive dispersal (Watts *et al.*, 2010).

2.3.5 Management of *Thaumatotibia leucotreta*

Given that *T. leucotreta* is a quarantine pest and that it causes high fruit losses, citrus producers must suppress *T. leucotreta* using different control tactics. These include cultural, biological and chemical control methods.

2.3.5.1 Culture control

Cultural control is mainly done through orchard sanitation to reduce FCM infestation levels by removing infested fruit both fruit on the ground and hanging from trees. According to Moore & Kirkman (2008) weekly orchard sanitation can remove up to 75% FCM larvae in affected citrus orange orchards in South Africa. Other cultural practices include sanitation of other host plants grown near the orchards (Mkiga *et al*., 2019). It is also advised to cultivate and apply heavy irrigation to eliminate the hibernating insects and kill the soil-dwelling stages of the pest (De Jager, 2013).

2.3.5.2 Monitoring and chemical control

Pheromone trapping is the most common method for monitoring FCM. Weekly trap checking is recommended and 10 moths per trap, is regarded as the threshold for treatment with chemical insecticides (Grout *et al.*, 1998). Although synthetic pyrethroids are the most common chemicals applied for control of FCM, their use is not recommended due to the detrimental effects on the environment and resistance developed towards these insecticides by insect pests (Hofmeyr & Pringle 1998; Carpenter *et al*., 2007).

2.3.5.3 ‘Attract and kill’ and mating disruptants

Pheromones are used to attract and pesticides are used to kill. The attract and kill product, Last Call FCM®, is used commonly. This attractant contains the insecticide (permethrin) and false codling moth pheromone (Anonymous, 2008). Mating disruptants contain a pheromone only, without the killing agent. The pheromone confuses wild FCM males, preventing them from finding females for mating (Carde & Minks, 1995).

2.3.5.4 Biological control

Some organisms control FCM. Egg and larval parasitoids are known to suppress pest populations effectively (Ghimire & Phillips, 2010; Wang *et al*., 2014). For example, the egg parasitoid, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae) can parasitize up to 80% of the eggs in citrus orchards while *Agathis bishopi* (Nixon) (Hymenoptera: Braconidae) has been reported to parasitize up to 40% FCM larvae (Moore, 2012). *Orius* beetles, assassin bugs and ants have also been noted to prey on FCM eggs, larvae and pupae (Moore, 2012). The virulence of a naturally occurring virus (Moore *et al*., 2015), as well as fungi (Goble *et al*., 2011) and nematodes (Malan *et al*., 2011) have been tested for the control of FCM.

2.3.5.5 Entomopathogenic fungi

This is a group of fungi that kills an insect by infecting its host through contact. Over 700 species of entomopathogenic fungi (EPF) have been reported to infect insects (Wraight, 2007). EPF has been identified as a promising biocontrol agent in the regulation of insect pest populations without harming non-target insects. Most of EPF infect their hosts primarily through the external cuticle, although a few taxa such as *Culicinomyces* spp. are able to attack through the alimentary canal (Inglis *et al.*, 2001). According to Roy *et al.* (2006), a fungus-infected insect reduces its feeding activity. Fecundity is also reduced and leads to the reduction of the pest status. Most of the commercially produced fungi are *Beauveria* spp., *Metarhizium* spp. and *Lecanicillium* spp. (Shahid *et al*., 2012). The efficacy of fungi, especially, *Beauveria* spp. and *Metarhizium* spp. has been evaluated for control of various lepidopterans, including immature stages of *T. leucotreta* (Furlong & Pell, 2001; Goble *et al*., 2011; Coombes *et al*., 2013; Opisa *et al*., 2018). The susceptibility of arthropods to entomopathogenic fungi can be influenced by different factors such as developmental stages of the host (Inglis *et al*., 2001).

2.3.5.5.1 Mode of action of entomopathogenic fungi

Several studies have described the pathogenicity mechanism of EPF and their life cycle (Wraight & Ramos, 2005; Zimmermann, 2007; Sandhu *et al*., 2012; Shahid *et al*., 2012). Entomopathogenic fungi conidia infect the insect cuticle. The infection process is then initiated by host recognition and attachment to the cuticle through the secretion of mucilage (Wraight *et al*., 1990). This is normally followed by conidial germination, germ tube and appressorium formation. Thereafter, a penetrating hypha breaches the cuticular layers by secreting enzymes such as proteases, esterases, lipases and chitinases that

hydrolyze the epidermis of the insect (Ortiz-Urquiza & Keyhani, 2013). The germ tube then reaches the hemocoel where chitinous walls (hyphal bodies) are formed which spread throughout the insect to obtain nutrients. A disruption in the metabolic activities of the host occurs, and it is also possible that toxic metabolites are produced, which eventually causes death 3 – 7 days after infection (Shahid *et al*., 2012).

2.3.5.5.2 Factors influencing the pathogenicity of entomopathogenic fungi in biological control

There are several biotic and abiotic factors affecting the pathogenicity of entomopathogenic fungi (Bueno *et al*., 2015). Biotic factors include germination, growth, the ability of entomopathogenic fungi to induce disease and their persistence have been reported to affect the efficacy of the fungus (Elghadi, 2016). Soil moisture, temperature, relative humidity and solar radiation (UV light) are important factors influencing the survival and persistence of fungal pathogens (Inglis *et al.*, 2001; Meikle *et al.*, 2003; Yeo *et al.*, 2003). Temperature is also amongst the key factors affecting the performances of entomopathogenic fungi through conidial germination, mycelia growth, sporulation and survival (Inglis *et al*., 2001; Dimbi *et al*., 2003; Yeo *et al*., 2003; Faria & Wraight, 2007). For example, germination, growth and spore formation of Hyphomycetes perform well at an optimum temperature between 20 and 30 °C (Rangel *et al*., 2010) and at a relative humidity of 50% (James *et al*., 1998). Direct sunlight also affects the persistence of the propagules of EPF. For example, the UV-B spectrum ranges between 285 – 315 nm, which may damage the DNA, RNA, as well as proteins and other cell constituents (Griffiths *et al.*, 1998; Inglis *et al.*, 2001). Hence, proper application of the EPF reduces the challenges with regard to the performance of the fungal inoculum in terms of interaction with other factors, such as, the environment, insect host and time (Inglis *et al*., 2001)

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CHAPTER 3: ARTICLE 1

Field and laboratory performance of false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Troticidae) on orange and selected vegetables.

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Article

Field and Laboratory Performance of False Codling Moth, *Thaumatotibia Leucotreta* (Lepidoptera: Troticidae) on Orange and Selected Vegetables

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Abstract: False codling moth (FCM), *Thaumatotibia leucotreta* is a key pest of citrus orange and other plants causing fruit loss through larval feeding. Although this pest is native to sub-Saharan Africa little is known on its performance on orange and vegetables in Kenya and Tanzania. Our objective was to assess the incidence, oviposition preference and offspring performance of FCM on orange and vegetables, namely, okra, African eggplant, chili and sweet peppers. A higher percentage of orange with FCM damage symptoms was recorded from the ground than from the tree sampled fruit. However, FCM larval incidence was higher for the latter (tree sampled fruit). The highest FCM larval incidence amongst the vegetables was recorded on African eggplant (12%) while the lowest was on okra (3%). Orange was the most while African eggplant was the least preferred for oviposition by FCM. Among the vegetables tested, strong oviposition preference was found for sweet pepper; however, larval survival was lowest (62%) on this crop. Highest larval survival (77%) was recorded on orange. Most demographic parameters (i.e., intrinsic rate of increase, doubling time) were comparable among the studied host plants. The results are discussed in line of FCM management.

1. Introduction

Fruit and vegetable production are important source of income for East African growers. Production of these crops is, however, constrained by insect pests and diseases resulting in yield loss and poor quality. The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), one of the pests of these crops, is native to sub-Saharan Africa [1] and has been recorded on 24 cultivated and 50 wild species in different plant families [2]. It is a key pest of citrus (Rutaceae) [3,4], avocado, *Persea americana* (Mill) (Lauraceae) [5] macadamias, *Macadamia* spp. (Proteaceae) [6] and cotton, *Gossypium* spp. (Malvaceae) [1]. *Thaumatotibia leucotreta* is a multivoltine pest [7] which does not enter diapause leading to year-round overlapping generations on host plants [8]. The female moths lay eggs on fruit, often near the stylar end [9]. The hatched larvae penetrate and feed inside the fruit resulting in fruit dropping. Damage symptoms caused by *T. leucotreta* vary with the host plant. For example, scull on avocado [5] and a yellowish-brown rind around a penetration hole on citrus orange [4] have been documented. Larval incidence on orange can be up to 75% [10]. In addition to direct losses, *T. leucotreta* infestations also cause financial losses due to quarantine restrictions imposed on exporting countries and detection of a single larva can result in rejection of an entire consignment [9].

Although South Africa and Egypt are the largest citrus producing countries in Africa, Tanzania and Kenya are considered as the leading countries in citrus production in East Africa [11]. Citrus production in Tanzania is largely concentrated on the North East Coast. The main production areas are in Tanga and Coast region, followed by Morogoro, Mwanza and Ruvuma. In Kenya, citrus production is concentrated in Coast, Eastern and Rift valley provinces [12]. Although *T. leucotreta* has been reported in Kenya and Tanzania [13], little is known about the larval incidence of the pest especially during the citrus orange fruit harvesting period. Orange is produced from low to high altitudes in these countries. Altitudinal gradients and vegetation had been reported to influence distribution and abundance of moths [14–16], which are highly diverse and ecologically important herbivorous insects [17]. Odanga et al. [13] reported similar *T. leucotreta* infestations on avocado grown at different altitudinal gradients in Kenya and Tanzania. Knowledge on the effect of altitude on *T. leucotreta* infestation on orange will contribute to management of the pest. The incidence of the pest on other crops which may serve as alternative host crops between successive orange fruiting seasons is not
well known.

The ovipositional preference and offspring performance of *T. leucotreta* on orange in a laboratory study was reported by Love et al. [18]. The ovipositional preference of the pest on orange and vegetables has not been determined. According to Thompson and Pellmyr [19], the plant selection made by egg laying females may often provide the initial basis for divergence of insect populations onto different plant species and it may drive the evolution of some plant defences. Developmental biology and adult life parameters of *T. leucotreta* reared on artificial diet have been reported [8, 20–22] and to a limited extend on orange, grapes and apple [23]. However, no detailed study on the offspring performance of *T. leucotreta* on other key host plants has been reported. The field dynamics of *T. leucotreta* in a mixed cropping system, a common practice in sub-Saharan Africa, need to be investigated to develop better management strategies. The aims of this study were therefore to determine *T. leucotreta* larval incidence on ripe orange as well as on mature vegetables of okra (*Abelmoschus esculentus* (L.) Moench var. Clemson), African eggplant (*Solanum aethiopicum* L., var. Tengeru white), chili pepper (*Capsicum anuum* L., var. Jalapeno) and sweet pepper (*Capsicum anuum* L., var. California Wonder). These vegetables are mainly grown near or within orange orchards. The developmental performance and life table parameters of the pest on three solanaceous vegetables *viz*. chili, sweet pepper and African eggplant were also determined and compared to that of orange, the most common host.

2. Materials and Methods

2.1. Study Sites

Field surveys were carried out between June and September 2017. In Kenya, the survey was conducted in three citrus producing areas namely Kilifi (3° 13^{0}6.3” S, 40° 6^{0}54.49” E), Makueni (1° 47^{0}11.38” S, 37° 57^{0}51.99” E) and Machakos (1° 16^{0}1.23” S, 37° 19^{0}12.64” E) representing low (0–500 masl), mid (501–1200 masl) and high (1201 masl and above) altitudes, respectively (Figure 1). Rainfall in these regions is bimodal. The short rains occur from November to December and the long rains are between March and May. In Tanzania, the study was carried out in Tanga (5° 5^{0}19.95” S, 39° 6^{0}8.36” E), Morogoro (8° 3^{0}50.31” S, 36° 57^{0}14.79” E) and Mwanza (2° 52^{0}40.81” S, 32° 43^{0}5.3” E) regions (Figure 1) representing low, mid and high altitudes respectively. The rainfall pattern in the former and latter region is bimodal and occurs during the same months as that in the Kenyan sites. Morogoro lies in a transition zone between a monomodal and bimodal rainfall pattern.

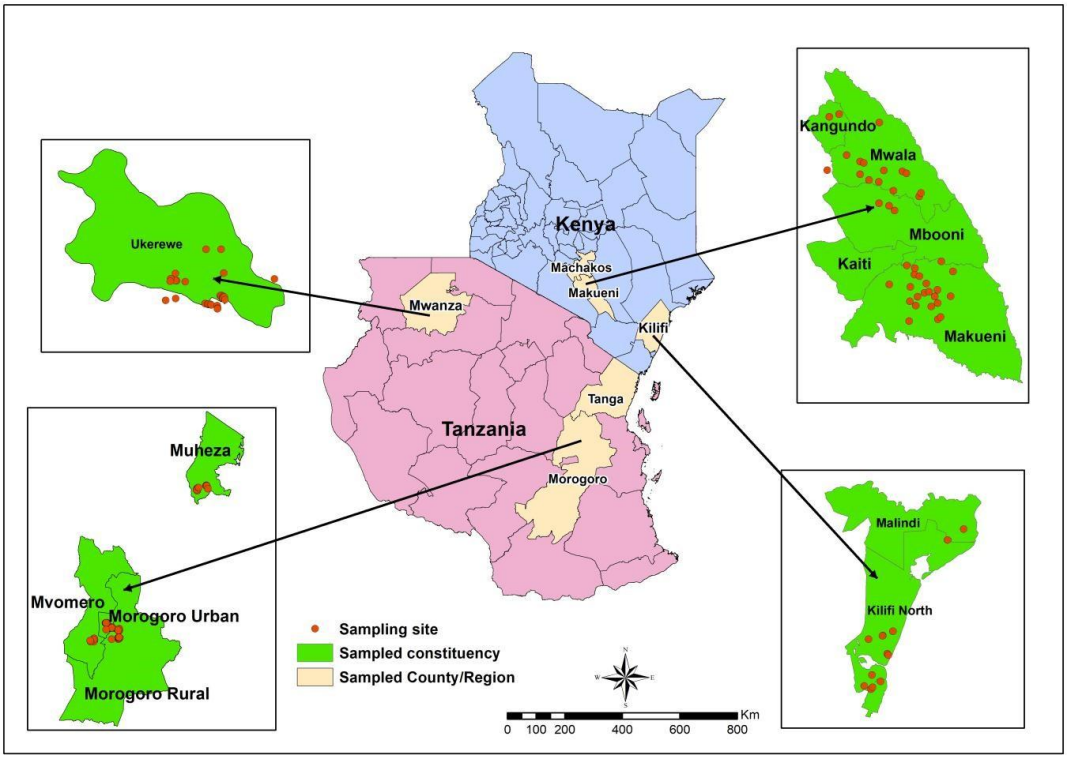

Figure 1. Study sites within Machakos, Makueni and Kilifi in Kenya, Mwanza, Morogoro and Tanga in Tanzania.

2.2. Assessment of Thaumatotibia leucotreta Incidence on Orange in Kenya and Tanzania

Ripe orange fruit sampling was conducted from June to September 2017 in the three rainfed citrus producing areas of Kenya and Tanzania (Figure 1). The fruit were sampled from 25 orchards in each of the selected altitudes in the two countries. Ten trees were randomly selected from each orchard, from which 10 fruit were sampled for each sampling method. Each sampling method was executed on each of the selected trees. Firstly, fruit were sampled from the ground, followed by sampling from the tree without shaking and lastly by shaking the branches of the tree. In total, 100 fruit were sampled per method per orchard. *Thaumatotibia leucotreta* damage symptoms were recorded for each fruit, washed using a non-caustic liquid dish washing soap and incubated for three weeks. The incubated fruit were dissected and the *T. leucotreta* larvae or pupae counted. The last instar larvae collected were placed in plastic containers (Kenpoly) (2 L) with a thin layer of soft sand covering the bottoms. Smaller larvae were transferred to glass jars containing an artificial diet developed by Moore et al. [24] and reared until pupation. Identification of *T. leucotreta* adults enclosing from the pupae were confirmed using standard keys. The percentage of orange fruit with *T. leucotreta* damage symptoms as well as percentage fruit infested with *T. leucotreta* larvae were calculated.

2.3. Assessment of Thaumatotibia leucotreta Incidence in Vegetables from Morogoro, Tanzania

Vegetable sampling was conducted at Mlali ward in Morogoro rural (Figure 1, Table 1) between November and December 2017 following the orange fruiting season. The study site was determined by the availability of vegetable fields near the citrus orchards. Eight fields planted with only one vegetable crop were selected for sampling. These fields were at least 0.1 ha in size and planted with okra, sweet peppers, chili pepper, and African eggplant respectively. The fields were selected on western side and within 100 m from orchard(s). Three hundred matured vegetables per species were randomly collected from each field. The vegetable samples were incubated separately in plastic containers (Kenpoly) (2 L) covered with fine mesh at the top for ventilation. These containers were kept in a laboratory at ambient

conditions (23–28 ◦ C) for three weeks before dissection of the vegetables and counting of *T. leucotreta* larvae or pupae. Percentage *T. leucotreta* larval incidence was calculated only. Since *T. leucotreta* damage symptoms on vegetables were not as clear as on oranges, assessment of the percentage of vegetables with damage symptoms caused by this pest, was excluded from the study.

Table 1. Vegetable samples collected near citrus orchards for *Thaumatotibia leucotreta* incidence in mid altitude, Mlali, Morogoro, Tanzania.

Village/Orchard	Latitude	Longitude	Vegetables Sampled Nearby the Orchard	Distance from the Orchard
Mlali	S06◦ 56^{0}55.3”	E037◦ 31^{0}55.4”	Sweet pepper, African eggplant, okra, chili pepper	67–72 m
Mlali	S06◦ 56^{0}33.6”	E037◦ 31^{0}59.1”	Sweet pepper, African eggplant, okra, chili pepper	69–75 m
Mlali	S06◦ 57^{0}20.5”	E037◦ 32^{0}02.9”	Sweet pepper, African eggplant, okra, chili pepper	58–69 m
Mlali	S06◦ 57^{0}46.8”	E037◦ 32^{0}14.1’^{0}	Sweet pepper, African eggplant, okra, chili pepper	65–70 m
Mlali	S06◦ 57^{0}35.2”	E037◦ 31^{0}51.8”	Sweet pepper, African eggplant, okra, chili pepper	68–74 m
Mkuyuni	S06◦ 58^{0}41.9”	E037◦ 31^{0}58.0”	Sweet pepper, African eggplant, okra, chili pepper	63–69 m
Kipera	S06◦ 56^{0}14.3”	E037◦ 31^{0}40.2”	Sweet pepper, African eggplant, okra, chili pepper	66–74 m
Vitonga	S06◦ 57^{0}15.9”	E037◦ 29^{0}35.9”	Sweet pepper, African eggplant, okra, chili pepper	55–67 m
Vitonga	S06◦ 58^{0}36.8”	E037◦ 29^{0}23.0”	Sweet pepper, African eggplant, okra, chili pepper	66–72 m
Vitonga	S06◦ 57^{0}52.4”	E037◦ 30^{0}38.5”	Sweet pepper, African eggplant, okra, chili pepper	56–63 m

2.4. Laboratory Performance of Thaumatotibia leucotreta on Orange and Solanaceous Vegetables

2.4.1. Insect Cultures and Host

Insects were obtained from the mass rearing room at the Animal and Quarantine Containment Unit at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. This colony was established from field-collected larvae from citrus orange, *Citrus sinensis* L. var Washington Navel fruit in Wote, Makueni county (1◦ 47^{0}11.38” S, 37◦ 57^{0}51.99” E) and had been maintained continuously for over 33 generations. The colonies were maintained on artificial diet described by Moore et al. [24] and kept in preserve jars (corked with cotton wool). The procedures for diet preparation and the inoculation of eggs and larvae as well as moth collection were adopted from Opoku-Debrah et al. [25] with slight modifications as described below. The diet in the jars was autoclaved at 121 ◦ C for 20 min then cooled in a laminar-flow cabinet. To obtain eggs, newly emerged moths were transferred to a small rectangular Perspex cage (37 × 22 × 6 cm). The cage was made at *icipe*. A wax paper sheet was inserted into the cage through a slit in the floor to serve as an oviposition substrate for the moths. Wet cotton wool as a source of water for the moths, was plugged into a hole cut at one side of the cage. The oviposition sheet was removed daily, cut into equal pieces, surface-sterilized with 25% formaldehyde (35%–40%, *v*/*v* stock solution, Minema®) and placed onto the diet for egg hatching. The neonates fed on the diet and pupated in the cotton wool plug, which was then transferred into a wooden moth emergence box (40 × 40 × 40 cm) made at *icipe*. Eclosing moths were attracted through an exit hole on the side of the emergence box by light, entering through

glass vials (32.6 cm^3). The colony was maintained at 25.0 ± 2.0 °C, 60% relative humidity and 12-h photoperiod (L12:D12).

2.4.2. Oviposition Preference of Thaumatotibia leucotreta for Orange and Solanaceous Vegetables

Choice Test

Newly emerged naive *T. leucotreta* moths were obtained from a colony reared on artificial diet. The moths were sexed based on the presence of densely packed black fringes of hair on the hind tibia of male *T. leucotreta* as described by Gilligan et al. [1]. One male-female *T. leucotreta* pair was placed in a vial (32.6 cm^3) closed with moist cotton wool to serve as a source of water [25] and kept for 24 h to mate. A choice test was conducted by placing one fruit each of orange, African eggplant, chili and sweet peppers per corner of the experimental cage (50 × 50 × 50 cm). The cage was also made at *icipe* and had two openings (diameter 10 cm) on two opposite sides. One of the openings was covered with netting cloth; and the other was fitted with a fine netting sleeve for easy handling of the insects and fruit [26]. Eight moths (1:1 ♀:♂) were released at 20:00 h in the cage and left to oviposit for four hours. The fruit were then removed and the number of eggs on each fruit was counted. The fruit were incubated as described above. The experiment was replicated 12 times, for each replicate the position of the fruit was rotated ensuring that every fruit type was placed in each position of the cage an equal number of times.

No-choice Test

The no-choice test was done similar to the choice test but with only one type of fruit placed separately per Perspex cage (20 × 15 × 15 cm). Four moths were released in each cage. A replicate consisted of four cages with one fruit per cage. This no-choice test was replicated 12 times.

2.5. Developmental Duration and Larval Mass of Thaumatotibia leucotreta Reared on Orange and Solanaceous Vegetables

2.5.1. Insect Colonies and Hosts

Eggs obtained from the rearing colony were incubated in 2 L plastic containers (Kenpoly) until hatching. Two neonate larvae were transferred onto individual fresh fruit of orange, var Washington Navel, African eggplant, chili and sweet peppers of similar cultivars as above, collected from farmers' fields at Wote, Makueni and Kangundo, Machakos. The inoculated fruit were incubated until final instar larvae exited. The larvae from each fruit type were transferred into separate ventilated plastic containers (Kenpoly) (2 L) with a thin layer of sterilized soft sand for pupation. The containers with the pupae were placed in separate emergence boxes. Adult collection was done following the procedures described above.

2.5.2. Egg Stage

Four Perspex oviposition cages (37 × 22 × 6 cm) were used to obtain *T. leucotreta* eggs from moths of which larvae were reared on the respective host plants, *viz*. orange, African eggplant, chili and sweet peppers. Newly emerged moths were transferred to separate cage containing wax paper sheet for oviposition. Wax paper sheets with eggs were removed and cut into four strips containing at least 100 eggs. The eggs were counted under a stereomicroscope (Leica WILD M3Z). Each strip of wax paper with eggs from moths for each host plant was individually placed in plastic containers (Kenpoly) (2 L) to allow for hatching. The eggs were checked under a microscope twice a day for emergence of neonates. Egg incubation period was recorded as time in days taken for 50% of eggs to hatch as described by Mkiga and Mwatawala [27]. The experiment was replicated four times.

2.5.3. Larval Stage

Duration of *T. leucotreta* larval development was determined on each host fruit type. Upon hatching, a cohort of 100 one day old neonates originating from eggs laid by *T. leucotreta* reared from orange, African eggplant, chili and sweet pepper were inoculated onto a fruit placed in a plastic container following the procedures described by Love et al. [18]. A total of 50 fruit for each replicate were used per each host. The inoculated fruit were incubated individually in ventilated plastic containers (Kenpoly) (2 L) on sterile sand. The fruit were observed daily until final instar larval exited from the fruit. Duration of *T. leucotreta* larval

development was recorded as time in days taken for 50% of final instar larvae to exit from the fruit for pupation. The experiment was replicated four times.
To determine the effect of the rearing host on larval mass, recently exited *T. leucotreta* larvae from each host plant were weighed individually using a digital analytical balance (Sartorius CPA225D, Germany). Ten larvae were weighed per replicate for each host plant and the experiment was replicated four times.

2.5.4. Pre-Pupal Stage

To obtain final instar *T. leucotreta* larvae from each host, 300 neonates originating from eggs laid by *T. leucotreta* reared from orange, African eggplant, chili and sweet pepper were transferred onto orange fruit and the respective vegetables. The neonates were collected following the procedures described above. A cohort of 100 final instar larvae exited from each fruit type on the same day were introduced into the four ventilated plastic containers (25 larvae/container) containing a thin layer of soft sterilized sand as pupation media. The larvae were monitored every 12 h until cocoon formation. Duration of the pre-pupal stage was recorded as time in days taken for 50% of final instar larvae to form cocoons. The experiment was replicated four times and each replicate contained 100 larvae.

2.5.5. Pupal Stage

Pupae were obtained by rearing larvae, inoculated as neonates onto the fruit of each host until cocoon formation following the procedures above. A cohort of 100 cocoons were carefully collected and placed in a Perspex cage (15 cm × 15 cm × 10 cm) manufactured at *icipe* and monitored daily for adult eclosion. Pupal duration was recorded as time in days taken for *T. leucotreta* adults to eclose from 50% of the cocoons.

2.5.6. Survival of *Thaumatotibia leucotreta* Reared on Orange and Solanaceous Vegetables

Thaumatotibia leucotreta stage specific survival was determined using the cohorts from the developmental duration bioassays above to calculate the percentage of eggs laid on the wax paper strips for each plant species that successfully hatched, percentage of neonates that successfully developed into final instars larvae, percentage of cocoons formed from the final instar larvae and percentage of adults that eclosed from cocoons.

2.5.7. Adult Life History of *Thaumatotibia leucotreta* Reared on Orange and Solanaceous Vegetables

Adult *T. leucotetra* life history was assessed using a cohort of newly emerged moths from larvae reared on orange, sweet pepper, African eggplant and chili pepper. The sex ratio was determined by counting the number of males and females from a sample of 100 moths emerging from pupae from which the larvae were reared on each host plant species. One male-female pair of moths was kept in a vial (32.6 cm^3) corked with wet cotton wool serving as a source of water. A total of 15 vials were used for each host. The pre-oviposition period was recorded as the number of days from adult eclosion until the first eggs were laid. The pair of moths were carefully removed from the vial and transferred into a new vial using the protocol described by Opoku-Debrah et al. [25]. The number of eggs laid by the female per vial was counted using a magnifying lens. Mortality of the moths was also recorded daily. The experiments were replicated four times.
The net reproductive rate (R0), intrinsic rate of natural increase (rm), mean generation time (T), doubling time (DT) and finite rate of increase (λ) were calculated using the equations from Carey [28].

2.6. Data Analysis

The percentage of orange fruit and vegetables (African eggplant, okra, chili and sweet peppers) with *T. leucotreta* damage symptoms as well as fruit containing *T. leucotreta* larvae were arcsine transformed before analysis. Data on percentage of orange fruit with *T. leucotreta* damage symptoms as well as fruit with larvae of the pest were analysed with a three-way ANOVA followed by Tukey's HSD test to determine statistically significant differences between countries, altitudes and sampling methods. Data on percentage of infested vegetable fruit of the respective host plants were analysed by means of one-way ANOVA

followed by Tukey's HSD test. *Thaumatotibia leucotreta* development time, larval mass and adult life history parameters were analysed by means of one-way ANOVA followed by Tukey's HSD test. Data on *T. leucotreta* survival in the developmental experiments were analysed using logistic regression models followed by Tukey's HSD test. Likelihood ratio test on a GLM (family: negative binomial link: log) was used to analyse the number of eggs laid per fruit in the oviposition preference assays as well as the number of eggs laid per day by each female reared from the respective hosts. Pairwise comparison of Least Squares Means (LSMeans; package lsmeans,' function 'lsmeans') of number of eggs laid on choice and no-choice tests and daily oviposition of moths reared on the respective hosts was done with Tukey's HSD ($\alpha = 0.05$). All statistical analyses were performed using R-version (3.5.2) statistical software packages (R Development Core Team [29]).

3. Results

3.1. Percentage of Orange Fruit with Thaumatotibia leucotreta Damage Symptoms and Larvae

Significantly more *T. leucotreta* damaged fruit ($F = 39.28$, df = 1,444, $p < 0.001$) and higher larval incidence were recorded in Kenya than in Tanzania ($F = 26.43$, df = 1,444, $p < 0.001$) (Table 2). The percentage of fruit with damage symptoms also differed significantly between the respective sampling methods ($F = 277.43$, df = 2,444, $p < 0.001$). Significantly more fruit with symptoms were sampled from the ground compared to those sampled from the tree by branch shaking or without shaking (Table 2). The percentage of damaged fruit sampled from the ground did, however, not differ significantly between low, mid and high altitudes in both countries (Table 2). There were, a significantly lower percentage of damaged fruit found on trees without branch shaking at low, compared to mid and high altitudes in Tanzania but not in Kenya. No significant difference in percentage of damaged fruits sampled after the branches of trees were shaken at low, mid and high altitudes existed in Tanzania but a significantly lower percentage of *T. leucotreta* damaged fruit were sampled at low altitude in Kenya (Table 2). Significantly higher percentages of damaged fruit were also sampled from high and mid altitudes compared to low altitudes ($F = 31.53$, df = 2,444, $p < 0.001$). Significantly fewer larvae were found in damaged fruit sampled from the ground at low compared to high altitudes in Kenya.

In Tanzania, the percentage of larval infestation per sampling method was similar, regardless of altitude (Table 2). The percentage larval incidence in fruit sampled from the trees as well as from the ground after branch shaking did, however, not differ significantly at the respective altitudes in both countries. Larval incidence was significantly higher in high and mid compared to low altitude (Table 2).

Table 2. Percentage of fruit with *Thaumatotibia leucotreta* damage symptoms (A) and larvae (B) sampled from the ground and from trees (with and without branch shaking) at the respective altitudes in Kenya and Tanzania.

Parameter	Altitude	Fruit Sampling Method	Country		Average
			Kenya	Tanzania	
A% fruit with damage symptoms	>1201 masl	Ground	42.16 ± 3.40 h	33.92 ± 3.05 fgh	38.04 ± 2.29 e
		Tree without shaking	12.72 ± 1.69 bcd	9.36 ± 1.35 bc	11.04 ± 1.10 b
		Tree shaking	28.48 ± 2.88 fg	22.08 ± 2.12 def	25.28 ± 1.75 c
	501–1200 masl	Ground	40.24 ± 2.28 gh	28.58 ± 2.72 fgh	34.38 ± 1.91 de
		Tree without shaking	7.72 ± 1.26 abc	5.52 ± 1.96 bc	6.62 ± 0.80 a
		Tree branch shaking	27.56 ± 2.20 efg	21.40 ± 1.95 def	24.48 ± 1.49 c
	0–500 masl	Ground	35.88 ± 1.87 gh	21.76 ± 2.49 def	28.82 ± 1.82 cd
		Tree without shaking	5.76 ± 1.21 ab	2.88 ± 0.76 a	4.32 ± 0.74 a
		Tree shaking	16.08 ± 1.82 cde	14.16 ± 1.69 cd	15.12 ± 1.21 b
Average			24.07A	17.73B	
B% Larval infested fruit	>1201 masl	Ground	33.26 ± 3.59 efg	21.00 ± 2.75 cde	27.12 ± 2.41de
		Tree without shaking	16.00 ± 0.99 bcd	12.08 ± 0.75 abc	14.04 ± 0.68 bc
		Tree shaking	46.48 ± 4.38 g	41.80 ± 3.69 fg	44.14 ± 2.85 g
	501–1200 masl	Ground	29.00 ± 3.02 def	16.40 ± 1.84 bcd	22.62 ± 1.96 cd
		Tree without shaking	14.93 ± 1.27 bc	8.96 ± 0.63 abc	11.90 ± 0.82 ab

		Tree shaking	45.04 ± 3.75 g	37.44 ± 3.75 fg	41.24 ± 2.64 fg
	0–500 masl	Ground	15.46 ± 1.80 bc	11.44 ± 1.27 abc	13.40 ± 1.13 b
		Tree without shaking	8.27 ± 0.83 ab	4.92 ± 0.49 a	6.64 ± 0.54 a
		Tree shaking	34.24 ± 3.99 efg	33.00 ± 3.78 efg	33.62 ±2.72 ef
Average			26.93A	20.79B	

Means for *Thaumatotibia leucotetra* damage symptoms and larval incidence in the same column followed by the same lower-case letter and means within a row followed by the same upper case letter do not differ significantly at $p = 0.05$ (Tukey's HSD).

3.2. Incidence of Thaumatotibia leucotreta Larvae in Vegetables

The incidence of *T. leucotetra* differed significantly among the vegetables ($F = 9.812$, df = 3, 28, $p < 0.001$). There was no significant difference in percentage *T. leucotetra* larval infested African eggplant, chili and sweet pepper. Significantly fewer okra was, however, infested compared to the other vegetables (Table 3).

Table 3. Mean percentage of *Thaumatotibia leucotreta* larval infested vegetables sampled from fields near orange orchards in the mid-altitude growing region of Tanzania.

Host Plant	Scientific Name	Percentage Infestation
Okra	*Abelmoschus esculentus*	3.08 ± 0.90 a
Sweet pepper	*Capsicum* spp.	8.72 ± 1.81 b
African eggplant	*Solanum aethiopicum*	12.00 ± 1.46 b
Chili pepper	*Capsicum* spp.	10.96 ± 1.28 b

Means within the column followed by the same letter do not differ significantly at $p = 0.05$ (Tukey's HSD).

3.3. Oviposition Preference of Thaumatotibia leucotreta for Orange and Solanaceous Vegetables

3.3.1. Choice Test

The mean number of eggs laid per fruit in the choice test varied significantly among the host plants (LR = 153.21, df = 3, $p = 0.001$) (Figure 2A). The highest number of eggs was recorded on orange followed by sweet and chili pepper. The lowest number of eggs was laid on African eggplant, with no significant difference in oviposition choice between African eggplant and chili pepper.

3.3.2. No-choice Test

The highest number of eggs were also laid on orange in the no-choice test with African eggplant and chili pepper being the least preferred for oviposition (Figure 2B). The number of eggs laid on sweet pepper was significantly less than laid on orange but sweet pepper was significantly more preferred for oviposition than chili pepper and African eggplant (LR = 146.97, df = 3, $p = 0.001$).

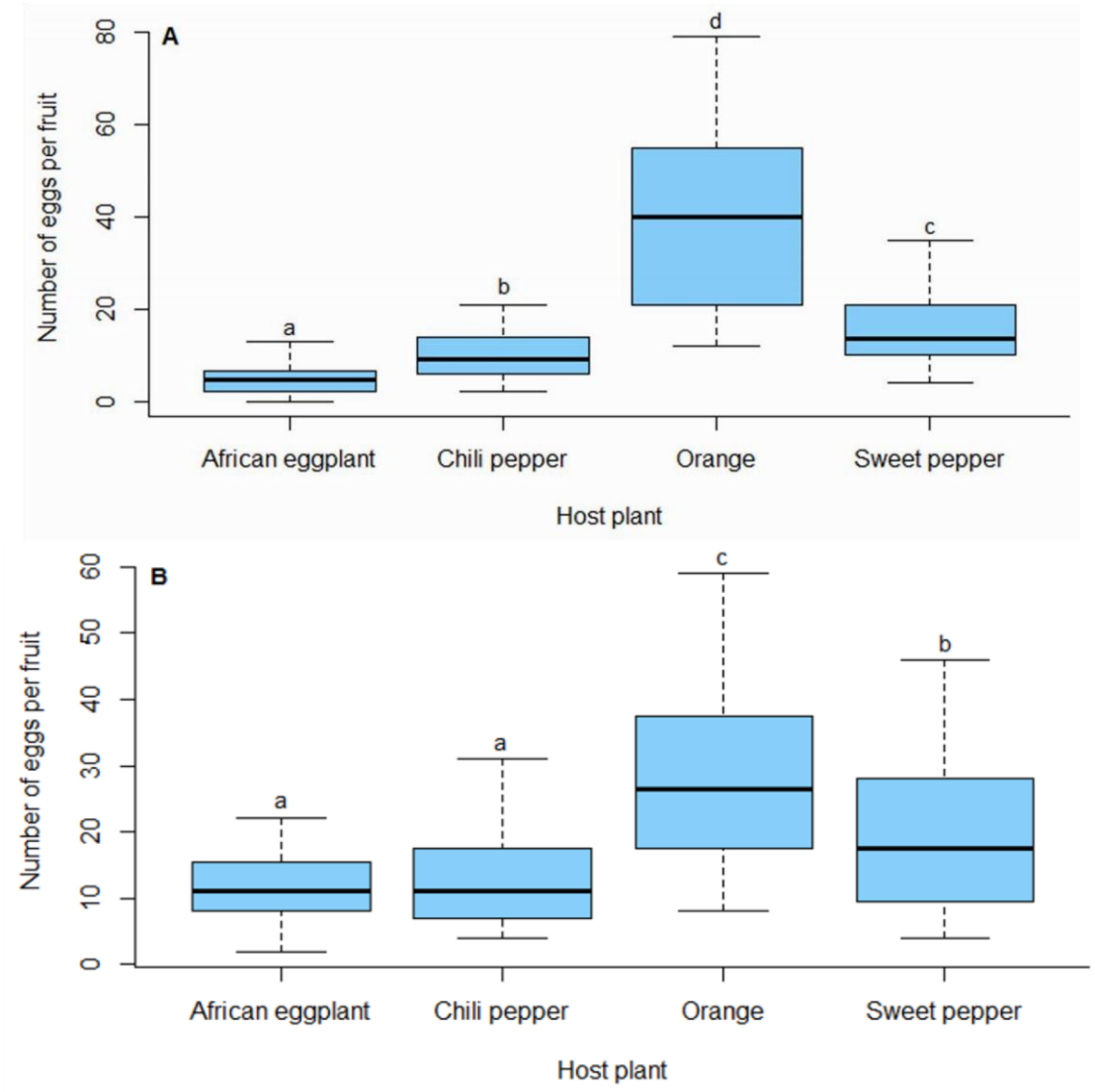

Figure 2. Number of eggs laid by *Thaumatotibia leucotreta* on different host plants in choice (**A**) and no-choice (**B**) experiments. Boxes caped with the same letter are not significantly different (p = 0.05, Tukey's HSD).

3.4. Development Time of Thaumatotibia leucotreta Immature Stages and Final Larval Instar Mass when Reared on Orange and Solanaceous Vegetables

The host plant on which a specific generation was reared had no significant effect on the egg incubation period of *T. leucotreta* (F = 0.13; df = 3, 12; p = 0.937). The larval development time (neonates to final instars) differed significantly among the respective solanaceous vegetables (F = 5.63; df = 3, 12; p < 0.001) (Figure 3A). Development was significantly shorter on orange compared to sweet and chili peppers. Larval development time on African eggplant, did, however, not differ significantly from development time on orange. The prepupal period from larvae reared on the respective host plant species did not differ significantly (F = 0.96; df = 3, 12; p = 0.444) but duration of the pupae originated from larvae reared on these solanaceous vegetables differed significantly (F = 4.84; df = 3, 12; p < 0.040) (Figure 3B). Pupal duration from which the larvae were reared on orange was significantly shorter compared to sweet pepper (Figure 3B). There was, however, no significant difference in duration of pupae originating from larvae reared on African eggplant, chili and sweet pepper.

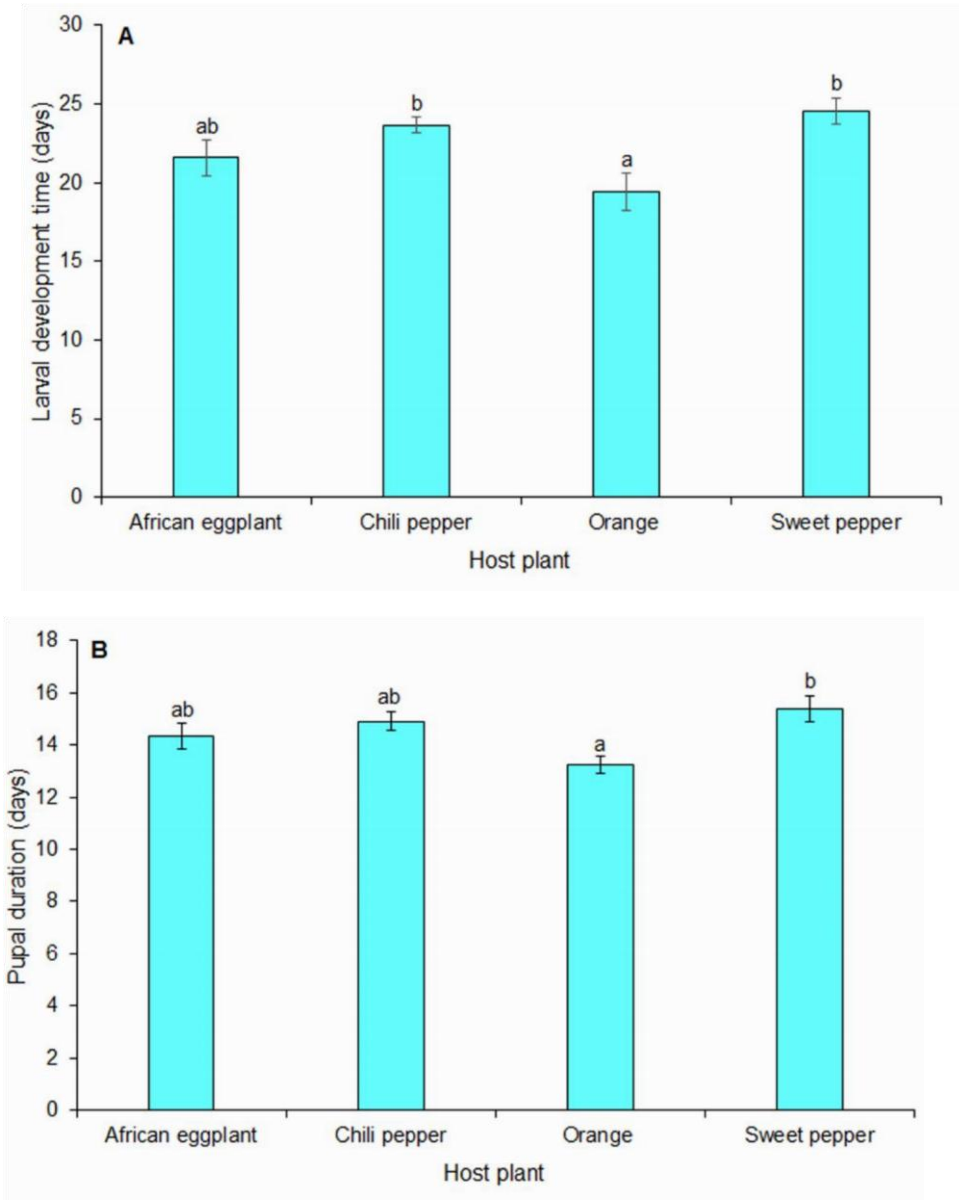

Figure 3. Mean development times (±SE) of *Thaumatotibia leucotreta* larvae (**A**) and pupae (**B**) on orange and solanaceous vegetables. Bars caped by the same letter are not significantly different (p = 0.05, Tukey's HSD).

Mass of final instar larvae reared on orange was significantly higher compared to larvae reared on vegetables (African eggplant, chili and sweet pepper) (F = 5.84; df = 3, 12; p = 0.011). It did, however not differ significantly between the vegetables (Figure 4).

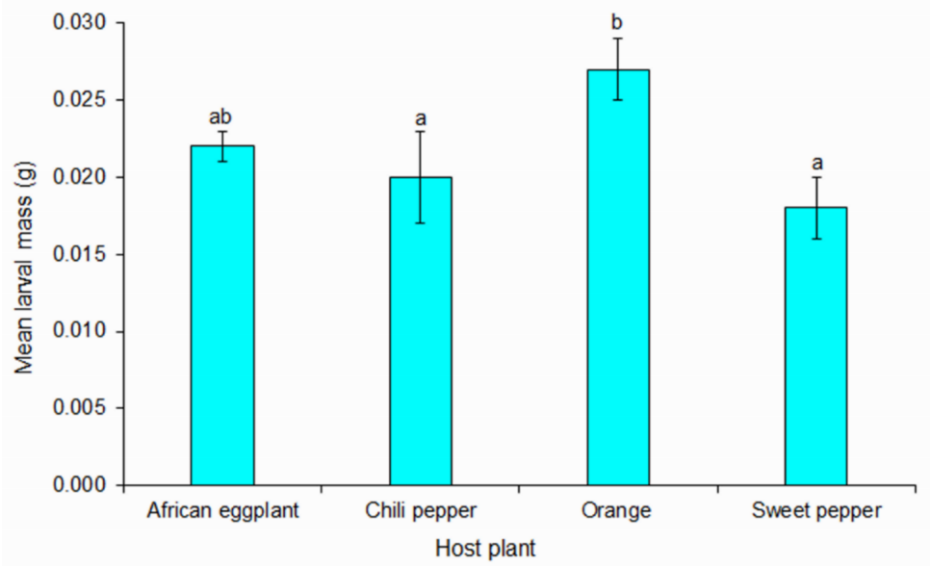

Figure 4. Mean mass (±SE) of final instar *Thaumatotibia leucotreta* larvae from orange and solanaceous vegetables. Bars caped with the same letter are not significantly different (p = 0.05, Tukey's HSD).

3.5. Development and Survival of Thaumatotibia leucotreta Immature Stages Reared on Orange and Solanaceous Vegetables

The percentage fertility of eggs laid by moths of which larvae were reared on orange, sweet pepper, chili pepper and African eggplant in the previous generation did not differ significantly (χ^2 = 26.436, df = 3, 12; p < 0.916). The percentage of larvae that survived differed significantly between the rearing hosts (χ^2 = 65.049, df =3,12; p < 0.001). A significantly higher percentage of larvae reared on orange survived compared to sweet and chili peppers (Figure 5). The percentage surviving larvae reared on orange and African eggplant did, however, not differ significantly. The percentage pupae that survived from larvae reared on these four host plants did not differ significantly (χ^2 = 8.018, df = 3, 12; p = 0.904) as well as the percentage of adults that eclosed from these pupae (χ^2 = 16.456, df = 3, 12; p = 0.937).

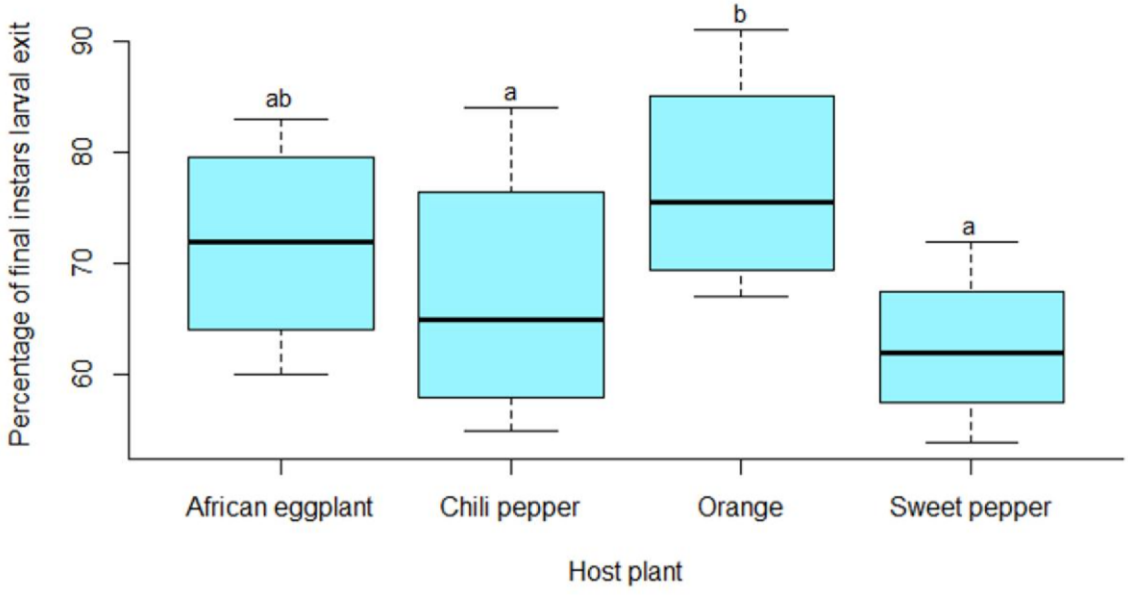

Figure 5. Percentage of surviving *Thaumatotibia leucotreta* larvae reared on orange and solanaceous vegetables. Boxes caped by the same letter are not significantly different (p = 0.05, Tukey's HSD).

3.6. Reproductive Parameters for Thaumatotibia leucotreta Reared on Orange and Solanaceous Vegetables

The host plant on which the larvae were reared did not significantly affect the pre-oviposition period (F = 0.08; df =3, 12; p = 0.971), oviposition period (F = 0.48; df = 3,12; p = 0.703), female longevity (F = 0.16; df = 3,12; p = 0.923) and male longevity (F = 0.04; df = 3,12; p = 0.990) (Table 4). The fecundity of the moths

from larvae reared on the different host plants was, however, significantly different (F = 32.32; df = 3,12; p < 0.001). Moths originating from larvae that were reared on orange laid significantly more eggs than moths from larvae reared on African eggplant as well as on sweet and chili peppers. Moths from African eggplant reared larvae were also more fecund than those reared on sweet peppers but fecundity of moths reared on African eggplant and chili pepper was similar.

Table 4. Sex ratio, oviposition, female longevity, male longevity and fecundity of *Thaumatotibia leucotreta reared on* orange and solanaceous vegetables.

Parameter	Host Plant			
	Orange	**Sweet Pepper**	**African Eggplant**	**Chili Pepper**
* Sex ratio (male: female)	0.38:0.62	0.32:0.68	0.37:0.63	0.34:0.66
* Pre-oviposition period	1.20 ± 0.08	1.21 ± 0.09	1.17 ± 0.12	1.23 ± 0.06
* Oviposition period	8.00 ± 0.4	7.75 ± 0.29	8.00 ± 0.41	7.75 ± 0.25
* Female longevity (days)	16.02 ± 0.89	16.21 ± 0.71	16.61 ± 0.73	15.90 ± 0.82
* Male longevity (days)	15.67 ± 0.69	15.34 ± 0.60	15.51 ± 0.90	15.61 ± 0.81
Fecundity	415.00 ± 3.70c	341.00 ± 5.37a	378.00 ± 8.03b	364.00 ± 3.53b

Means followed by the same letter within the row for fecundity are not significantly different (p = 0.05, Tukey's HSD), * No significant difference between hosts.

3.6.1. Fecundity per Female per Day

There was no significant interaction (LR = 200.0, df = 3, 24; p = 0.205) between moth age and host plant in terms of daily oviposition (Figure 6). Host plant used for rearing the larvae did, however, had a significant effect on egg laying (LR = 3556.4, df = 3, p < 0.001). Moths from larvae reared on orange laid the most eggs per day. The number of eggs laid per day varied considerably with female age (LR = 229.4, df = 8, p < 0.001). The most eggs were laid by moths from larvae reared on orange, five days after moth eclosion. Egg laying for females reared on the other host plants peaked on the fourth day after moth eclosion. On the days where oviposition was high, females from the larvae reared in orange laid more egg followed by females from larvae reared from African eggplant, chili and sweet peppers.

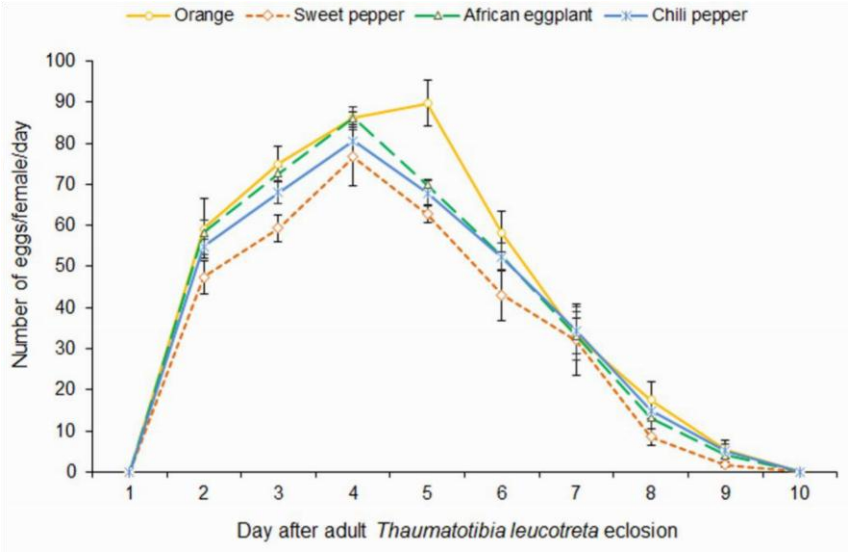

Figure 6. Mean (±SE) daily oviposition of *Thaumatotibia leucotreta* reared from orange and solanaceous host fruit.

3.6.2. Pairwise Comparison on Fecundity Rate of *Thaumatotibia leucotreta* Reared from Orange and Solanaceous Hosts

Pairwise comparison of the three days where oviposition was highest (Table 5), for *T. leucotreta* reared from orange and solanaceous hosts shows that oviposition of females reared from the tested hosts on day three was comparable. On day four, the females reared from orange oviposited significantly more eggs when compared to each solanaceous host. However, the number of eggs oviposited by the females reared

from solanaceous hosts did not differ significantly when compared to each other. On day five, the number of eggs laid by *T. leucotreta* females reared from orange was significantly higher than from females reared on sweet pepper.

Table 5. Pairwise comparison on mean number of eggs laid by *Thaumatotibia leucotreta* reared from orange and solanaceous fruit on three peak days of egg laying.

	Orange	**Chili Pepper**	**African Eggplant**	**Sweet Pepper**
Day 3 Orange				
	-	0.823	1.000	0.460
Chili pepper		-	0.829	0.928
African eggplant			-	0.459
Day 4 Orange				
	-	**0.003**	**0.009**	**0.001**
Chili pepper		-	0.988	0.822
African eggplant			-	0.630
Day 5 Orange				
	-	0.6815	0.177	**0.010**
Chili pepper		-	1.000	0.182
African eggplant			-	0.177

p-Values indicating significant differences are in bold (Tukey HSD, $\alpha = 0.05$).

3.7. Life History Parameters of Thaumatotibia leucotreta Reared from Orange and Solanaceous Vegetables

Net reproductive rate of *T. leucotreta* reared on the respective hosts differed significantly ($F = 30.61$; df = 3, 12; $p < 0.001$) (Table 6). The highest net reproductive rate was recorded for females reared from larvae reared on orange followed by those reared on eggplant while the lowest was from larvae on sweet pepper. Other demographic parameters; mean generation time ($F = 0.50$; df = 3, 12; $p = 0.688$), intrinsic rate of increase ($F = 1.23$; df = 3, 12; $p = 0.341$), doubling time ($F = 1.43$; df = 3, 12; $p = 0.284$) and finite rate of increase ($F = 1.77$; df = 3, 12; $p = 0.207$) were similar for females reared from larvae on the respective host plants.

Table 6. Life history parameters of *Thaumatotibia leucotreta* recovered from orange and solanaceous vegetables.

Parameter	**Host Plant**			
	Orange	**Sweet Pepper**	**African Eggplant**	**Chili Pepper**
Net reproductive rate	419.7 ± 3.844c	348.00 ± 5.484a	385.90 ± 7.722b	371.5 ± 3.611b
* Mean generation time	48.19 ± 1.103	48.90 ± 1.301	47.29 ± 0.807	48.42 ± 0.261
* Intrinsic rate of increase	0.13 ± 0.002	0.12 ± 0.003	0.13 ± 0.002	0.12 ± 0.001
* Doubling time	5.53 ± 0.128	5.79 ± 0.162	5.50 ± 0.086	5.67 ± 0.024
* Finite rate of increase	1.40 ± 0.002	1.13 ± 0.004	1.13 ± 0.002	1.12 ± 0.002

Means followed by the same letter within the row indicating the net reproductive rate are not significantly different ($p = 0.05$, Tukey's HSD), * No significant difference between hosts.

4. Discussion

Thaumatotibia leucotreta occurred in all surveyed sites of Kenya and Tanzania from low to high altitudes. The higher percentage of damaged fruit recorded in Kenya compared to Tanzania could be attributed to the different farming practices within each country. In Kenya, most citrus trees are intercropped with

peppers and maize which are also recorded as crop hosts of *T. leucotreta* [1,6] while such practices are not common in Tanzania. Pests and diseases are generally controlled by means of pesticide application, which may result in a reduction of the natural enemy populations in the citrus orchards in Tanzania. According to Hofmeyr and Pringle [30], excessive use of broad spectrum chemical pesticides causes pest outbreaks due to disruption of natural enemies and it could also lead to the development of insecticide resistance. The fruit sampled from the ground had a higher percentage of *T. leucotreta* damage symptoms than those sampled from trees (with or without branch shaking). Higher *T. leucotreta* larval incidence was, however, recorded from fruit sampled after shaking of the tree branches. This could be ascribed to *T. leucotreta* laying eggs mainly on fruit while they are still on the trees [31] and the larvae completing their development while the fruit is still on the tree [9]. When the fruit drop to the ground, the larvae would have already completed their development and exited the fruit to pupate in the soil. Lower larval incidence was therefore recorded in the fruit sampled on the ground. Although. *T. leucotreta* infested fruit do often not show damage symptoms, final instar larvae may be present in the fruit mainly in the centre cores. This may have implications for *T. leucotreta* management especially in terms of orchard sanitation and post-harvest treatment. Proper orange orchard sanitation and harvesting should therefore also take in consideration infested fruit that is still on the tree by shaking of the branches. Higher *T. leucotreta* larval incidence on orange fruit was recorded in high than low altitudes of both countries. In contrast, Odanga et al. [13] reported a similar population density and number of infested avocado fruit in different altitudes of Mount Kilimanjaro in Tanzania and Taita Hills in Kenya. This difference could possibly be ascribed to the difference in crop and altitudinal ranges.

Amongst the vegetables sampled, the highest percentage of *T. leucotreta* infested fruit was on African eggplant compared to okra, sweet and chili peppers. This could be due to the lower moisture content of the fruit of this host plant, since infested African eggplant produced very little fluid under field and laboratory conditions. According to Jaenike [32], the suitability of a plant for larval development is a function of many variables, including its chemical and physical properties, microhabitat and degree of infestation. During dissection of fruit, final instar larvae were also mainly found in the central core of orange and placenta of peppers where very little fluid was produced due to insect feeding and decay. African eggplant, okra, sweet and chili pepper serve as reservoirs for *T. leucotreta* when grown in the vicinity or in mixed cropping with orange orchards. These vegetables are also grown throughout the year in irrigated fields which provides a continuous availability of host plants for this pest.

Although vegetables can serve as host plants for *T. leucotreta*, oranges were the most preferred host plant for oviposition in both choice and no-choice tests. The *T. leucotreta* moths do prefer certain orange varieties above others for oviposition. For example, Love et al. [18] reported that Fischer Navels were the least preferred early maturing variety for oviposition. Navel oranges were reported to be more preferred for oviposition than Valencia oranges and guava [33]. Most of the available literature report on the oviposition preference of *T. leucotreta* for different citrus varieties and guava under laboratory and field conditions [18, 33]. This study provides additional information on *T. leucotreta* ovipositional preference for and performance on solanaceous vegetables, with chili and sweet pepper more preferred for oviposition than African eggplant.

In general, insects are reported to oviposit on hosts that maximize the performance of their progeny, a hypothesis referred to as “mother knows best” [19, 32, 34, and 35]. In this study, oviposition preference by *T. leucotreta* did not mirror host suitability for development of the immatures and survival on these host plants. The sweet and chili peppers which were more preferred than African eggplant for oviposition were less suitable in terms of developmental time, larval mass and survival of immature stages (larvae and pupae) as well as female fecundity and net reproductive rate. The mismatch between preferred vegetable hosts for oviposition and host suitability of peppers could be explored for use as trap crop. A similar finding was reported for the butterfly, *Anthocharis cardamines* (L) (Lepidoptera:

Pieridae). The oviposition preference of females did not correlate with the fitness parameter of the offspring reared on 51 populations of two ploidy types of the perennial herb *Cardamine pratensis* L. (Brassicales: Brassicaceae) [36]. The suitability of the African eggplant for development of the immature stages of *T. leucotreta* could be explained by the higher protein and lower moisture content of African eggplant compared to sweet and chili pepper [37–39]. Rearing host quality has also been reported to

influence the developmental duration of other lepidopteran species. For example, Traore et al. [40] reported the larvae of *Maruca vitrata* (Fabricius) (Lepidoptera: Pyralidae) reared on cowpea flowers to have a short developmental time compared to those reared on the other plant parts. The effect of host plants on the life table parameters is dependent on the quality of certain components, such as carbon, nitrogen and defensive metabolites [41].

In this study, adult life table parameters for moths from larvae reared on the respective host plants are similar except for fecundity and net reproductive rate. Fecundity of *T. leucotreta* ranged from a total of 341 to 415 eggs per female for those reared on sweet pepper and orange, respectively. Both these values are within the range reported for fecundity of *T. leucotreta* from larvae reared on artificial diet [8, 25]. Females reared on solanaceous vegetables laid fewer eggs than moths from larvae reared on orange. Daily egg laying peaked at day three and four after eclosion, for females reared on solanaceous vegetables and orange, respectively. *Thaumatotibia leucotreta* oviposition from larvae reared on artificial diet reached a peak, 2 and 3 days earlier than for the females reared on solanaceous vegetables and orange, respectively [8]. Generally, the highest daily fecundity was recorded for females reared from orange while the lowest was for females reared from sweet pepper. Oviposition peaked one day earlier (day three) for those of which larvae were reared on solanaceous vegetables than those reared on orange (day four) after moths emerged.

The intrinsic rate of natural increase, mean generation time, doubling time and finite rate of increase are important biotic parameters of insect performance [42, 43]. Considering these parameters, survival of *T. leucotreta* is equal on orange, sweet and chili pepper as well as African eggplant. This has implications for management strategies of the pest. According to Birch [44] the intrinsic rate of increase is a basic parameter for insect population development, which is an indicator of the species developmental speed, longevity and fecundity [28].

Although oranges sampled from the ground had more *T. leucotreta* damage symptoms compared to fruit sampled from the trees, the latter hosted a higher number of *T. leucotreta* larvae. It is therefore recommended that citrus trees should be subjected to gentle shaking prior to fruit harvesting to reduce citrus orange post-harvest fruit decay and minimize indirect losses due to quarantine measures. Infested orange fruit from both ground and tree after branch shaking should be collected and removed using sustainable sanitation measures such as the use of augumentorio which sequesters the moth while conserving any parasitoids. This control tactic should be integrated with other *T. leucotreta* control measures such as the use of biopesticides *viz*. *Cryptophlebia leucotreta* granulovirus (CrleGV), entomopathogenic nematode, *Heterorhabditis bacteriophora*, entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana*, attract and kill through the use of Lastcall® and the Sterile Insect Technique. The potential of solanaceous vegetables to act as *T. leucotreta* reservoirs between successive citrus orange seasons for rainfed orange production, the common cropping systems in Kenya and Tanzania were determined. The strong preference of *T. leucotreta* to chili and sweet peppers for oviposition and relatively higher mortality of the larvae on these crops could be explored for management of this pest using attract and kill approaches. Although there is currently a very potent male moth attractant commercially available, a need to identify semio-chemicals that can attract female *T. leucotreta* moths for both monitoring and suppression of the pest, is needed.

5. Conclusions

More ripe orange fruit with *T. leucotreta* damage symptoms are on the ground but the larvae are abundant in the fruit still hanging on the tree. Orange is strongly preferred for oviposition and highly suitable for *T. leucotreta* larval survival when compared to solanaceous vegetables.

Thaumatotibia leucotreta has a strong oviposition preference to sweet and chili pepper compared to African eggplant. African eggplant is, however, highly suitable for larval survival. Fecundity is the only affected adult *T. leucotreta* fitness trait from larvae reared from orange and solanaceous vegetables. The findings from this study will contribute to sustainable management of this pest.

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S.M. and H.P.; writing—original draft preparation, A.M.; supervision, S.M., H.P., F.K. and S.E.; project administration, S.M. and S.E.; funding acquisition, S.E.

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CHAPTER 4: ARTICLE 2

Metarhizium anisopliae and *Beauveria bassiana*: Pathogenicity, horizontal transmission and their effects on reproductive potential of *Thaumatotibia leucotreta*

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4.1 Abstract

The polyphagous moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) is among the major constraints to the horticultural industry in East Africa. In a search of sustainable control methods, the pathogenicity of the dry conidia of 17 *Metarhizium anisopliae* and five *Beauveria bassiana* isolates were tested against *T. leucotreta* moths. The conidial uptake by a single moth, horizontal transmission and effect of fungal inoculant on fecundity and fertility of the pest were also assessed. The findings from this study showed that tested fungi were virulent to the moths, with 12 isolates causing mortality that ranged between 58.8 and 94.2% for *M. anisopliae* and three isolates between 57.6 and 84.6% for *B. bassiana.* The entomopathogenic fungi isolates, *M. anisopliae* ICIPE 69 and *B. bassiana* ICIPE 279 were highly virulent with low lethal times to 50% mortality (LT_{50}) of 3.81 and 5.13 days, respectively. The highest, ICIPE 69 conidia acquisition by a single moth were approximately 4.58 x 10^6 for males and 3.51 x 10^6 for females The highest mortality rate (96.7%) caused by inoculum transmission was recorded in donor males compared to 83.3% in recipient males. Fecundity was reduced by 33.6 and 25.9% for donor and recipient females, respectively. Virulence of dry conidia of *M. anisopliae* and *B. bassiana* against adult *T. leucotreta* demonstrate their potential use in the sustainable management of the pest. The ability of the moths to transmit the potent ICIPE 69 fungus through horizontal transmission suggest the use of this fungus through the auto-dissemination technique. The auto-dissemination technique requires the use of pheromone incombination with an enotomopathogenic fungus. We therfore recommend further investigation on the compatibility of ICIPE 69 with FCM sex pheromones.

Keywords: Dry conidia, entomopathogenic fungi, fertility, mortality

4. 2 Introduction

The false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) attacks a range of cultivated and wild vegetation (Timm et al. 2010; Venette et al. 2003). Citrus, particularly orange (*Citrus sinensis* L.) is among the most preferred host plants (Mazza et al. 2014). The infestation of citrus fruit generally results in yield losses caused by premature and harvestable fruit falling (Kirkman and Moore 2007). The pest causes enormous losses due to quarantine restrictions on citrus export to lucrative export markets (Moore 2012). The use of chemical pesticides, often results in fruit infected with pesticide residues which are detrimental to end-users and the environment (Carvalho 2017). Excessive use of synthetic chemicals for controlling the pest leads to the development of pesticide resistance (Hofmeyr 1998), disruption of natural enemies and the development of secondary pests (Steinmann et al. 2011). Sustainable eco-friendly management strategies are therefore needed to control *T. leucotreta.*

Fungal-based biopesticides are gaining more recognition for arthropod pest control. Biopesticides have advantages over conventional insecticides with fewer effects on non-target insects (Zimmermann 2007). Entomopathogenic fungi (EPF) as bio-control agents can remain in the environment and infect the targeted pests (Hajek and Mcmanus 2007). Another advantage of EPF is that they can attack pesticide-resistant pests, for example, insecticide-resistant mosquitoes infected by *B. bassiana* were vulnerable to pesticides (Farenhorst et al. 2009). *Metarhizium anisopliae* ICIPE 7, cause up to 100% mortality of the amitraz-resistant tick strain of *Rhipicephalus decoloratus* (Murigu et al. 2016).

Entomopathogenic fungi showed virulence to various lepidopteran pest species. For example, *Beauveria bassiana* (Bals.) Vuill.) isolates ICIPE 725, ICIPE 284 and USDA 2729, as well as *Metarhizium anisopliae* (Metschn.) Sorokin isolates ICIPE 18, ICIPE 69 and ICIPE 30, have shown high efficacy against *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae) (Furlong and Pell 2001). Virulence has also been shown by *M. anisopliae* ICIPE 18 and ICIPE 69 against *Maruca vitrata* (Fabricius) (Lepidoptera: Plutellidae) (Tumuhaise et al. 2015) and *M. anisopliae* isolate ICIPE 30 and *B. bassiana* isolate ICIPE 725 against *Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae) (Opisa et al. 2018). Entomopathogenic fungus transmission from infected to fungal-free insects have been established using *M. anisopliae* isolate ARSEF 2521 against cabbage flies, *Delia radicum* L. (Diptera: Anthomyiidae) (Meadow et al. 2000). Fungal transmission has also been demonstrated with *M. anisopliae* ICIPE 30 against the malaria vector, *Anopheles gambiae* s.s. (Diptera: Culicidae) (Meadow et al. 2000; Scholte et al.

2004; Toledo et al. 2007) and two *B. bassiana* products LCPP and Bassianil against Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae) (Toledo et al. 2014). However, to the best of our knowledge, effectiveness of EPF against *T. leucotreta* was evaluated to a limited extent with *M. anisopliae* isolates (G 11 3 L6 , Ar 23 B3, G OL R8, G Ar 17 B3 and G OL R8) and *B. bassiana* isolates (G Ar 17 B3, G 14 2 B5, FCM 10 13 L1 and G Moss R10) against fifth instar *T. leucotreta* (Coombes et al. 2013; Coombes et al. 2015; Goble et al. 2011). Coombes (2013) also reported persistence and efficacy of the two isolates (G Ar 17 B3 and Ar 23 B3) for control of *T. leucotreta*, with a reduction in fruit infestation of up to 82% in citrus orchards. The larvae of the pest feed most of the lifetime inside fruit. There is, however, no information available on the use of dry EPF conidia for the control of adult FCM in autodissemination devices. This technique consists of an autoinoculation device charged with the synthesized female-based pheromone of FCM, to attract male moths (Newton et al. 1993). Pell et al. (1993) and Furlong et al. (1995) reported on a pheromone containing autoinoculation device for control of *Plutella xylostella* (Lepidoptera: Plutellidae) moths. The aims of this study were to determine the pathogenicity of dry conidia of several isolates on the adult FCM, and to use the most virulent isolate to determine conidial acquisition, retention and transmission of these conida by moths. The effect of transmitted conidia on fecundity and fertility of *T. leucotreta* eggs were also studied.

4.3 Materials and methods

Insect culture

Thaumatotibia leucotreta were obtained from a rearing colony at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The origin, mass production and maintenance of the rearing colony used in this study were as described by Mkiga et al. (2019). Field collected moths were added regularly to the mass rearing colony to limit inbreeding (Boardman et al. 2012). The colonies were reared on the artificial diet developed by Moore et al. (2014). The diet was placed in glass jars which were covered with cotton wool and kept at 25.0 ± 2.0 ˚C, 60% relative humidity and a 12L:12D photoperiod. Cohorts of *T. leucotreta* moths were established following the protocols of Opoku-Debrah et al. (2014) and Mkiga et al. (2019).

Preparation of *Metarhizium anisopliae* and *Beauveria bassiana* isolates

Twenty-two *icipe* isolates of entomopathogenic fungi, 17 of *M. anisopliae* and 5 of *B. bassiana* were used in this study. Origin, host and year of isolation of the fungi are

presented in Table 1. Sabouraud Dextrose Agar (SDA) was use to culture *M. anisopliae* and Potato Dextrose Agar (PDA) to culture *B. bassiana* isolates, at 25 ± 2 °C in darkness.

To test for their viability, conidia were collected from three week old sporulating fungus cultures with a clean scalpel and suspended in 10 ml distilled water with 0.05% Triton X-100 in universal bottles containing glass beads following standard procedures according to Maniania (1992). A homogeneous conidial suspension was obtained by vortexing the mixture for five minutes at 100 rpm. Quantification of conidial concentrations was done by using a haemocytometer under a light microscope (Leica, United Kingdom) at 400 x magnification. The conidial concentration was diluted to 3×10^6 conidia/ml, and 0.1 ml of this suspensione mixture was spread plated on SDA for *M. anisopliae* and on PDA for *B. bassiana* isolates following procedures described by Goettel and Inglis (1997). Four sterile coverslips were then placed randomly onto each inoculated plate. The plates were sealed with Parafilm and incubated at 25 ± 2°C in darkness for 18 hours. At 18 hours after inoculation, lactophenol cotton blue was dropped onto each plate to stop conidial growth and to stain the conidia for germination assessment. The viability of fungal isolates (percentage germination) was assessed by collecting 100 conidia randomly from under each coverslip. The viable conidia was counted under a light microscope and the percentage germination was calculated. Acceptable viability of conidia was considered when the length of the germ tube was at least twice the diameter of the conidium (Goettel and Inglis 1997). Germination of between 92.67 and 99.00% was obtained (Table 1).

Table 1: Percentage germination of *Metarhizium anisopliae* on sabouraud dextrose agar and *Beauveria bassiana* isolates on potato dextrose agar medium, 18 h post-inoculation at 25 ± 2 °C.

Fungal species	**Strain**	**Year of isolation**	**Source**	**Country**	**% germination ± SE**
Metarhizium anisopliae	ICIPE 30	1989	*Busseola fusca*	Kenya	95.58 ± 0.85 ab
	ICIPE 18	1989	Soil	Kenya	93.00 ± 1.38 ab
	ICIPE 78	1990	*Temnoschoita nigroplagiata*	Kenya	97.42 ± 0.74 b
	ICIPE 62	1990	Soil	(DRC)	93.85 ± 0.71 ab
	ICIPE 69	1990	Soil	(DRC)	98.36 ± 0.49 b
	ICIPE 63	1990	Soil	(DRC)	97.00 ± 1.04 ab
	ICIPE 20	1996	Soil	Kenya	92.67 ± 0.33 ab
	ICIPE 7	1996	*Amblyoma variegatum*	Kenya	92.57 ± 0.30 ab
	ICIPE 74	1990	Soil	Kenya	94.59 ± 0.43 ab
	ICIPE 656	2008	Soil	Kenya	97.00 ± 0.58 ab
	ICIPE 68	1990	Soil	(DRC)	99.00 ± 0.14 b
	ICIPE 40	1990	Soil	Kenya	97.08 ± 0.08 ab
	ICIPE 315	2006	*Tetranychus urticae*	Kenya	94.84 ± 0.09 ab
	ICIPE 31	2003	*Locusta migratoria*	Madagascar	94.31 ± 0.75 ab
	ICIPE 22	1999	*Schistocerca gregaria*	Sudan	89.30 ± 0.88 a
	ICIPE 725	2015	Soil	Kenya	98.17 ± 0.58 b
	ICIPE 676	2008	Soil	Kenya	92.67 ± 0.58 ab
	ICIPE 720	2015	Soil	Kenya	98.17 ± 0.85 b
Beauveria bassiana	ICIPE 283	2005	Soil	Mauritius	96.17 ± 1.75 ab
	ICIPE 273	2006	Soil	Kenya	96.08 ± 0.22 ab
	ICIPE 279	2005	Coleopteran larvae	Kenya	99.00 ± 0.08 b
	ICIPE 647	2005	Soil	Mauritius	98.25 ± 0.25 b

Means within the column followed by the same letter do not differ significantly at $P < 0.05$ (Tukey's HSD).

For use in experiments, mass production of EPF, *M. anisopliae* and *B. bassiana* isolates was done on a long rice substrate, in Milner bags (600 mm x 350 mm) (Maniania 1998). Rice was autoclaved for one hour at 121 °C and cooled in a laminar flow cabinet and inoculated with three-day-old blastopores (Jenkins et al. 1998). The inoculated rice in the bags were incubated for three weeks at 20 – 26 °C and 40 – 70% relative humidity (RH). The rice substrate with fungal conidia was dried at room temperature for five days. The conidia were collected by sifting the substrate through 295 μm mesh sieve and kept in plastic bags in a refrigerator (4-6 °C) until use.

4.3.1 Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* isolates on *Thaumatotibia leucotreta* moths

For each of the 17 *M. anisopliae* and 5 *B. bassiana* isolate (Table 1), 20 *T. leucotreta* moths, one-day-old, were infected with conidia according to a method described by Dimbi et al. (2003). Twenty two plastic jars were lined with velvet and evenly coated with 0.3 g dry conidia. The bottoms of these jars were removed and replaced with nylon netting. The moths were exposed to dry fungus conidia in the jars for two minutes before being transferred to sterilized aerated Perspex cages (300 × 300 × 300 mm). Water was provided by means of wet cotton balls. Other cohorts of moths were also exposed to fungus-free velvet lined plastic jar which served as the control treatment. Mortality of moths was recorded on a daily basis for 10 days in both control and treatment cages. The dead moths were observed for mycosis by microscopic examination for external fungal growth on the moths. Each treatment was replicated three times.

4.3.2 Conidial acquisition of *Metarhizium anisopliae* ICIPE 69 isolate by *Thaumatotibia leucotreta* moths

Conidial acquisition of *M. anisopliae* ICIPE 69 isolate by *T. leucotreta* moths from a treated substrate was investigated with 100 newly eclosed male and female moths, respectively. These moths were exposed to dry conidia in plastic jars following the procedures as described above (see 4.3.1). The moths were transferred separately from these jars into sterilized Perspex cages (300 × 300 × 300 mm). Immediately after exposure (0 h), 10 moths were transferred individually to plastic vials (2 ml) with 1 ml water to which 0.05% Triton X-100 was added. Glass beads were placed in these vials which were vortexed for 5 minutes to remove the conidia from the moths. Conidia acquired by the moths were quantified using a hemocytometer (Dimbi et al. 2003). The moths which remained in the cage were used to assess conidia retention by moths over time. Ten moths were removed from the cages with 2 hours intervals, up to 8 hours after acquisition and to quantify conidia retention as described above. Each of these 10 moths served as a replicate and the experiment was repeated three times.

4.3.3 Horizontal transmission of *Metarhizium anisopliae* ICIPE 69 conidia between male and female *Thaumatotibia leucotreta* moths

Three sets of 50 male moths (one day old), were infected with the fungus *M. anisopliae* ICIPE 69 following the procedures described above. Each set of moths was maintained separately in Perspex cages (300 × 300 × 300 mm). The fungal exposed moths served as

"donors". The males were kept in the cages for 1 hour before 50 one-day-old fungus-free females ("recipients") were released into the cage to mate with the donor males. When mating took place, twenty mating pairs from each cage were carefully removed with a vial and transferred into another clean cage of the same size. The pairs were observed until mating ceased, which generally lasted for two days. After mating, 10 moths from each sex were removed. The number of conidia from each moth was quantified one day after mating ceased. Moths from the other two cages were individually assessed for conidia retention two and three days after mating. The procedures were repeated with the same numbers of females serving as "donors" and males as "recipients". Each moth served as one replicate per sex for the respective days after mating and the experiment was repeated three times.

4.3.4 Effect of horizontal transmission of fungal conidia on adult mortality, fecundity and fertiliy

Male moths (one day old) were infected with the fungus *M. anisopliae* ICIPE 69 (donors), according to the procedure of Dimbi et al. (2003). Fifty of these moths were kept in each of six cages (300 × 300 × 300 mm). Fifty fungus-free females (recipients) were transferred to six of the cages with donor males for horizontal transmission of the fungus. When females acted as donors, female moths were initially infected and males were transferred to cages with female donors. The moths were grouped in two sets of three cages, each containing 50 males and females for assessment of survival and fecundity and also for assessment of fertility per treatment group. There were also sets of six cages with fungus-free moths, that served as controls.

To assess survival and fecundity one set of the three cages was used, 20 mated moths from each treatment and control cages were transferred individually to vials and kept for 10 days at room temperature (23 – 28 $^{\circ}$C). A piece of moist cotton wool was used to close each vial and to serve as a source of water for the moths as well. Female moths oviposited in the vials. Each female moth was transferred to a clean vial every 24 hours (Opoku-Debrah et al. 2014). The eggs laid in each vial were counted with a magnifying lens. Mortality was recorded daily until all moths per group died. The three cages per treatment served as replicates.

To determine *T. leucotreta* fertility of the respective treatment groups, 30 mated females from another set of the remaining three cages were carefully removed three days after fungal transmission and transferred to a small cage (20 × 15 × 10 cm) containing a wax

paper sheet. The moths were allowed to lay eggs for 4 hours. After this period, the oviposition substrate (wax paper) containing the eggs was detached from each cage and the eggs were counted under a microscope (Leica WILD M3Z). Four strips of wax paper with more than 100 eggs each were cut individually and placed in ventilated plastic containers (220 x 150 x 80 mm). The eggs were incubated for six days at 25 ± 2 ˚C. Hatching of larvae from eggs was recorded twice daily using a microscope and fertility was calculated for the respective groups. The three containers containing *T. leucotreta* eggs served as replicates and the experiment was repeated three times.

4.3.5 Statistical analysis

Percentage conidial viability of *M. anisopliae* and *B. bassiana* and percentage mortality obtained from the screening for pathogenicity of EPF isolates against *T. leucotreta* were analysed using logistic regression models, with isolate as the explanatory variable. Percentage mortality data were corrected for natural mortality (Abbott 1925) before analysis. Lethal time to 50% mortality (LT_{50}) values were estimated for each replicate by logit analysis method for correlation data using SPSS version 22. The slope from the resultant LT_{50} values was subjected to ANOVA. The LT_{50} was only evaluated for the entomopathogenic fungal isolates that caused mortality of *T. leucotreta,* higher than 50% of the cohort. Data on the number of conidia acquired and retained by *T. leucotreta* moths did not meet the assumption of normality and were therefore fitted with GLM using the negative binomial regression analysis, with *T. leucotreta* sex (2 levels: male and female) and time (5 levels: 0, 2, 4, 6 and 8 hours post fungal infection) as the explanatory variables. Data on the number of conidia retained over time by the male and female donor and recipient moths were also not normal distributed and were also fitted into GLM using a negative binomial regression analysis, with *T. leucotreta* sex (2 levels: male and female), *T. leucotreta* status (2 levels: donor and recipient) and time (3 levels: 1, 2 and 3 days post horizontal transmission), being explanatory variables. Kaplan Meier Survival Analysis was used to analyze the number of *T. leucotreta* moths that survived after being infected with *M. anosipliae* ICIPE 69 through horizontal transmission. Data on the daily fecundity of *T. leucotreta* were analyzed using the logistic linear mixed model with intercept slope using the 'glmer' function from the lme4 package to test the interaction effect between *T. leucotreta* female status and time in days. Pairwise comparison of Least Squares Means (LSMeans; package lsmeans,' function 'lsmeans') of the number of eggs laid by *T. leucotreta* at different days was conducted using Tukey's HSD (P = 0.05). Data on the total number of eggs laid by *T. leucotreta* were also not normally distributed and were

fitted into GLM using the negative binomial regression analysis, with *T. leucotreta* status being explanatory variable. Percentage *T. leucotreta* fertility were analyzed by means of a one-factor logistic regression model, with *T. leucotreta* female status as the explanatory variable. Tukey's HSD test was used to separate all means where there were significant differences among the treatments. All data analyses were done by using R (version 3.2.5) statistical software packages (R Development Core Team 2016) except for LT_{50}.

4.4 Results

4.4.1 Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* isolates to *Thaumatotibia leucotreta* moths

All the fungal isolates tested were pathogenic to *T. leucotreta* moths, 10 days after contamination. Significant differences in mortality of *T. leucotreta* moths was caused by the candidate fungal isolates ($\chi 2 = 272.43$, df = 21, $P < 0.001$). Percentage mortality caused by the *M. anisopliae* isolates varied between 29.41 and 94.23% and for *B. bassiana* isolates between 47.06 and 84.62%. The highest mortality was caused by ICIPE 30 and 69 as well as by *B. bassiana* ICIPE 279. Percentage mortality caused by ICIPE 30 and 270 did however, also not differ from that caused by ICIPE 20, 7, 279 and 273. Amongst the isolates, *M. anisopliae* ICIPE 69 had the highest pathogenicity and slope ($F (15, 32) = 2.815$; $P < 0.001$). The slope was also not significantly different from the slopes of ICIPE 20, 7, 273 and 279. ICIPE 69 had the shortest LT_{50} in days (Table 2).

Table 2: Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* isolates to *Thaumatotibia leucotreta* moths, 10 days post-inoculation.

Fungal species	Isolate	% Mortality ± SE	LT_{50} (days) (95%FL)	Regression line	
				Slope (± SE)	Intercept
Metarhizium anisopliae	ICIPE 30	84.90 ± 4.99 jk	.467 (4.51-6.33)	0. 67 ± 0.06 ab	-3.64
	ICIPE 18	60.36 ± 3.27 efg	7.97 (7.04-9.29)	0.44 ± 0.05 a	-3.46
	ICIPE 78	69.80 ± 1.89 fghi	6.98 (5.96-8.32)	0.46 ± 0.06 a	-3.21
	ICIPE 69	94.23 ± 3.33 k	3.81 (2.73-4.67)	1.04± 0.35 b	-3.83
	ICIPE 63	71.16 ± 3.33 fghi	6.90 (6.04-7.96)	0.39± 0.05 a	-3.12
	ICIPE 20	76.93 ± 3.32 hij	6.11 (5.17-7.16)	0.58± 0.04 ab	-3.53
	ICIPE 7	74.07 ± 1.85 ghij	6.860 (5.84-8.14)	0.58± 0.02 ab	-3.95
	ICIPE 74	66.67 ± 3.21 fgh	7.15 (6.07-8.56)	0.51± 0.04 a	-3.61
	ICIPE 676	63.46 ± 1.92 fgh	7.87 (6.85-9.16)	0.42± 0.05 a	-3.28
	ICIPE 315	58.82 ± 5.19 ef	7.08 (7.081-9.60)	0.45± 0.04 a	-3.61
	ICIPE 656	62.75 ± 3.92 fg	7.27 (6.24-8.72)	0.38± 0.01 a	-2.76
	ICIPE 68	65.39 ± 3.34 fgh	7.72(6.58-9.47)	0.53± 0.02 a	-4.11
	*ICIPE 720	41.18 ± 3.40 abc	-	-	-
	*ICIPE 725	47.06 ± 3.40 cde	-	-	-
	*ICIPE 40	31.37 ± 1.96 ab	-	-	-
	*ICIPE 62	43.37 ± 3.27 bcd	-	-	-
	*ICIPE 22	39.22 ± 3.92 abc	-	-	-
	*ICIPE 31	29.41 ± 3.40 a	-	-	-
Beauveria bassiana	*ICIPE 647	47.06 ± 3.92 cde	-	-	-
	ICIPE 283	57.70 ± 1.92 def	7.75 (6.5-9.62)	0.43± 0.02 a	-3.34
	ICIPE 273	81.12 ±1.89 ij	6.02 (5.09-7.03)	0.64 ± 0.07 ab	-3.81
	ICIPE 279	84.62 ± 1.92 jk	5.13 (4.19-6.03)	0.61± 0.02 ab	-3.13

Means within the same column followed by the same letter do not differ significantly at $P < 0.05$ (Tukey's HSD). * LT_{50} values not estimated (less than 50% mortality caused). FL represents 95% fiducial limits.

4.4.2 Conidial acquisition of *Metarhizium anisopliae* ICIPE 69 isolate by *Thaumatotibia leucotreta* moths

There was no interaction effect between exposure times and sex of the exposed *T. leucotreta* moths ($\chi2 = 101.07$, df = 4, P = 0.896). The number of *M. anisopliae* ICIPE 69 conidia acquired and retained by *T. leucotreta* moths differed significantly between exposure times ($\chi2 = 144.54$, df = 4, $P < 0.001$) and also between male and female *T. leucotreta* moths ($\chi2 = 102.16$, df = 1, $P < 0.001$). There was no significance difference in the number of conidia retrieved from female *T. leucotreta* moths immediately, 2, 4, and 6 hours after the moth has being exposed to the fungal substrate. Significantly more conidia were retrieved immediately after exposure compared to 8 hours after exposure of female moths to conidia. There was no difference in the number of conidia retrieved from *T. leucotreta* males, from directly after exposure until 8 hours after exposure. However, significantly more conidia were retrieved from male *T. lecuotereta* moths than from females at 4, 6 and 8 hours after the moths were exposed to the fungal treated substrate (Table 3).

Table 3: Mean number of conidia recovered from *Thaumatotibia leucotreta* moth over time after exposure to entomopathogenic fungi *Metarhizium anisopliae* ICIPE 69 isolate (0-8 hours).

Thaumatotibia leucotreta sex	Post-fungal exposure time (h)	Mean number of conidia recovered from a single moth x 10^6 ± SE
Female	0	3.51 ± 0.25 bcde
	2	2.90 ± 0.21 abcd
	4	2.54 ± 0.34 abc
	6	2.46 ± 0.23 ab
	8	2.35 ± 0.26 a
Male	0	4.58 ± 0.30 e
	2	3.78 ± 0.17 de
	4	3.68 ± 0.31 de
	6	3.56 ± 0.34 cde
	8	3.45 ± 0.20 bcde

Means within a column followed by the same letter are not significantly different at $P < 0.05$.

4.4.3 Horizontal transmission of *Metarhizium anisopliae* ICIPE 69 conidia between male and female *Thaumatotibia leucotreta* moths

The interaction between *T. leucotreta* sex and its status as donor or recipient was significant ($\chi2 = 47.20$, df = 1, $P < 0.001$). However, interactions between sex and time ($\chi2 = 44.33$, df = 2, P = 0.238), *T. leucotreta* status and time ($\chi2 = 40.97$, df = 2, P = 0.187) and sex, *T. leucotreta* status and time ($\chi2 = 36.31$, df = 2, P = 0.097) were not significant. Both male and female *T. leucotreta* moths were able to acquire and transmit the fungal conidia to conspecifics. After horizontal transmission, a significantly higher number of conidia was retained by males than females ($\chi2 = 366.65$, df = 1, $P < 0.001$). Donor moths retain more conidia than recipient moths ($\chi2 = 185.06$, df = 1, $P < 0.001$). Retention of conidae by *T. leucotreta* moths over time differed significantly ($\chi2 = 85.46$, df = 2, $P < 0.001$), with fewer conidia retained by the moths at day three compared one and two days after after horizontal transmission (Table 4).

Table 4: Mean number of conidia recovered from donor and recipient *Thaumatotibia leucotreta* male compared to female moths after horizontal transmission of *Metarhizium anisopliae* ICIPE 69. Donors are fungal infected moths and recipients are fungus-free moths. Horizontal transmission occurred during mating of donor and recipient moths.

Thaumatotibia leucotreta		Post-horizontal transmission time (days)	Mean number of conidia recovered from single moths x 10^6 ± SE
Sex	Status		
Female	Donor	1	1.22 ± 0.10 ef
		2	0.94 ± 0.06 de
		3	0.41 ± 0.03 ab
	Recipient	1	0.81 ± 0.03 cde
		2	0.46 ± 0.03 bc
		3	0.35 ± 0.02 ab
Male	Donor	1	1.73 ± 0.06 f
		2	1.53 ± 0.05 f
		3	0.94 ± 0.07 de
	Recipient	1	0.55 ± 0.05 bcd
		2	0.36 ± 0.06 ab
		3	0.17 ± 0.02 a

Means within a column followed by the same letter are not significantly different at $P < 0.05$.

4.4.4 Effect of horizontal transmission of fungal conidia on adult mortality, fecundity and fertiliy

Survival of *T. leucotreta* moths differed significantly between fungal infected moths (donors) and those who received the fungus from the donors during mating (recipients) (χ^2 = 24.5, df = 4, $P < 0.001$). Among the fungal infected moths, the daily survival of fungus infected moths was the highest for recipient males until 7 days after horizontal transmission. The lowest daily percentage survival was recorded for donor males from 2 to 10 days after horizontal transmission (Fig. 1).

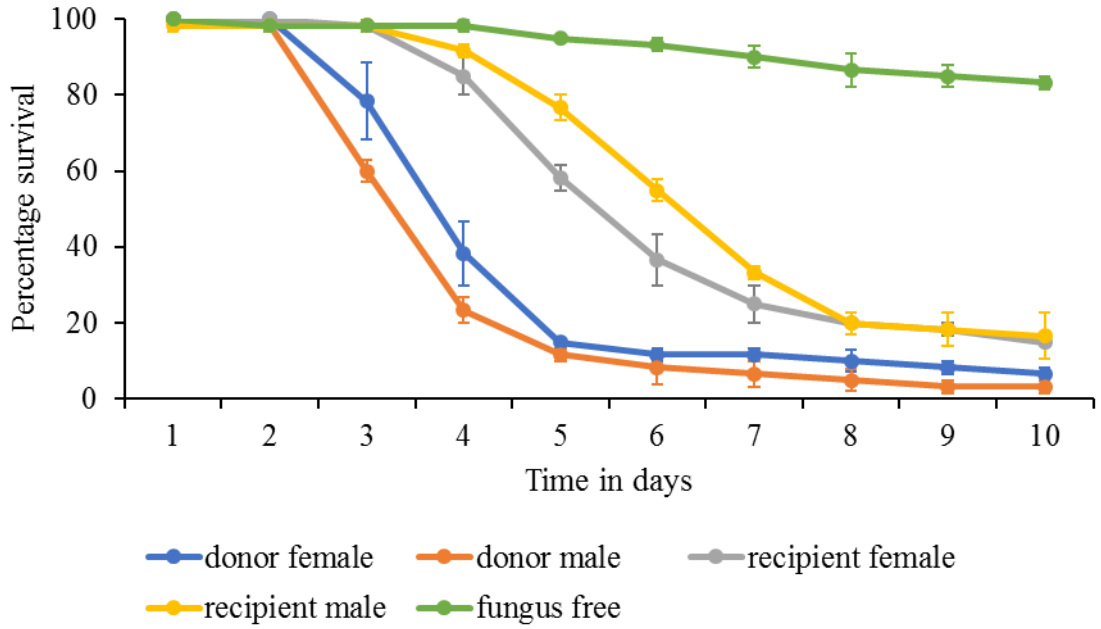

Fig. 1 Percentage survival (±SE) of *Thaumatotibia leucotreta* donors and recipient moths over time. Donors are fungal contaminated moths and recipients are fungus-free moths allowed to mate with each other, while the control motha were fungus-free.

There was a significant female status (in terms of fungal contamination) and time (after conidial acquisition) interaction in terms of fecudity of *T. leucotreta* moths ($\chi2$ = 163.125, df = 12, P < 0.001) (Fig. 2). Fecundity of females was significantly affected by *M. anisopliae* ICIPE 69 infection ($\chi2$ = 96.325, df = 2, P < 0.001). Fewer eggs were laid by donor females compared to recipient and fungus free females (Fig. 2). The number of eggs

laid also differed significantly between the days after conidia contamination ($\chi 2$ = 658.820, df = 6, P < 0.001). The daily fecundity did not differ significantly between donor females and recipient females, donor females and untreated females and between recipient females and untreated females from day 1 to 3after horizontal transmission (P > 0.05). The daily fecundity of *T. leucotreta* donor and recipient female moths did also not differ significantly on day 4 and 5 (P > 0.05). Fecundity of donor females was, however, significantly lower than for recipient females at day 6 (P < 0.001) (Fig. 2). The daily fecundity of donor and recipent moths was significantly lower than daily fecundity of fungus-free females from day 4 to 6 after horizontal fungus transmission (P < 0.001).

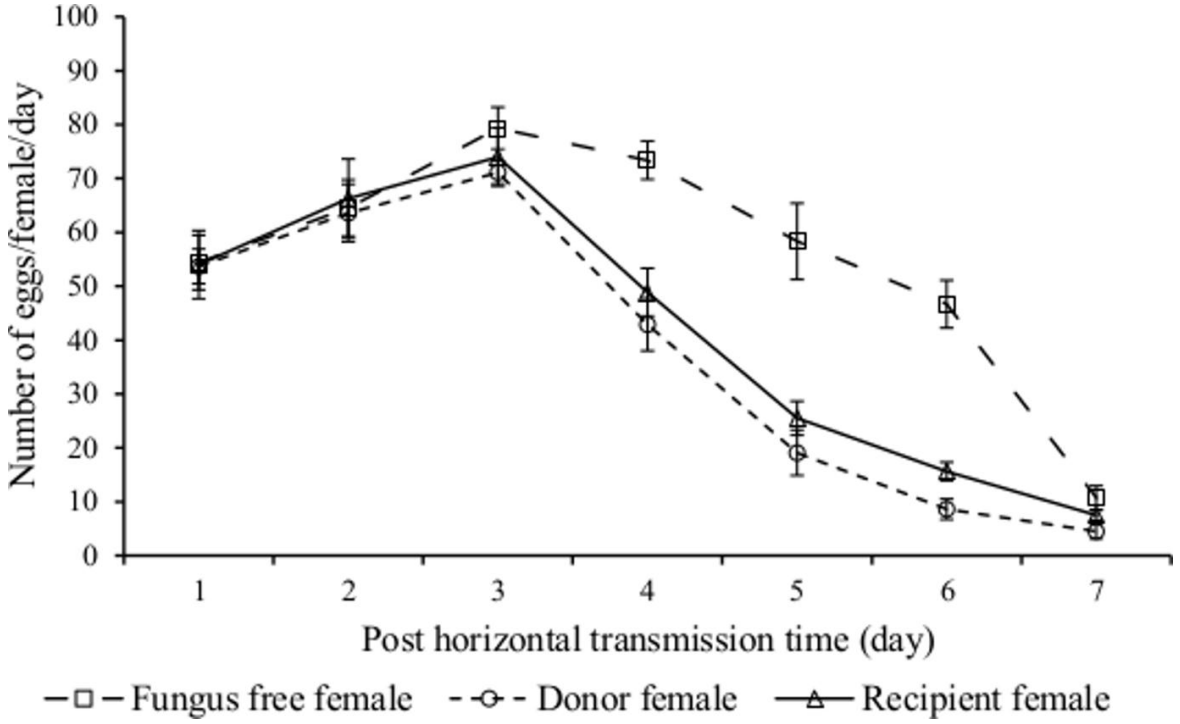

Fig. 2: Fecundity of *Thaumatotibia leucotreta* moths. Donors are *Metarhizihium anisopliae* ICIPE 69 infected moths and recipients are females allowed to mate with *Metarhizihium anisopliae* ICIPE 69 infected moths, while fungus-free moths are the control moths.

The total fecundity of *T. leucotreta* fungus-free, donor or *M. anisopliae* ICIPE 69 recipient moths, differed significantly ($\chi 2$ = 5.897, df = 2, P < 0.001) (Fig. 3). Fecundity of fungus-free female moths was significantly higher compared to donor and recipient females (Fig. 3). The fecundity of donor and recipient females did not differ significantly. Fertility of eggs was, however, not affected by the fungus infection of the female moths ($\chi 2$ = 17.017, df = 2, P = 0.178)

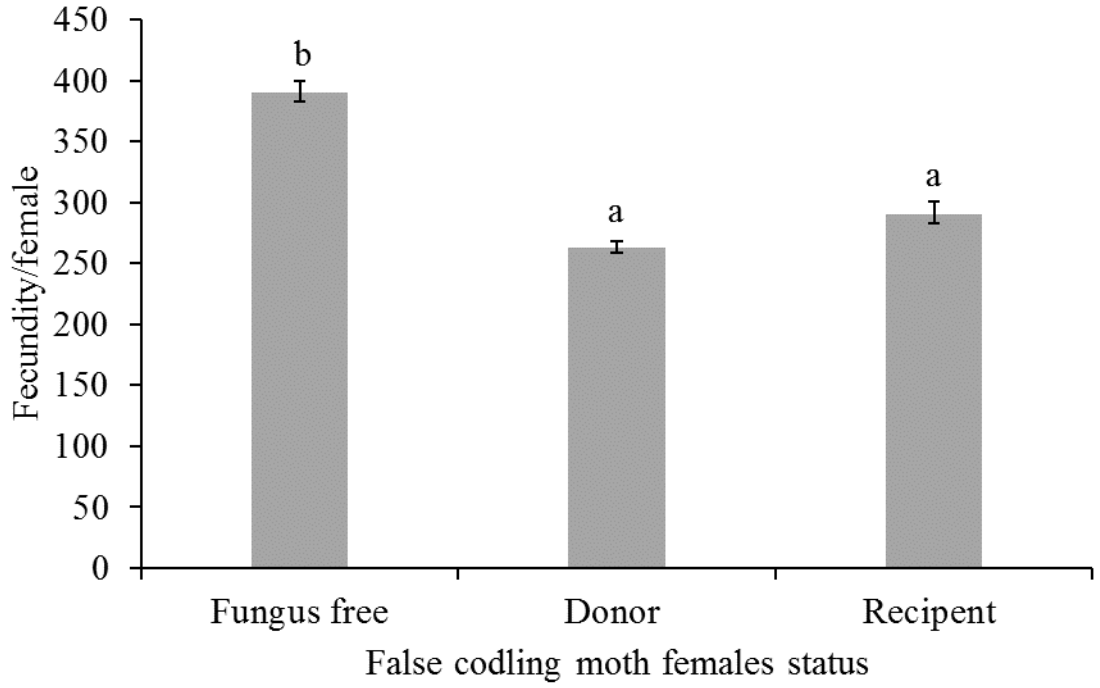

Fig. 3: Effect of *Metarhizium anisopliae* ICIPE 69 on *Thaumatotibia leucotreta* fecundity. Bars caped by the same letter are not significantly different ($P < 0.05$, Tukey's HSD). Donors are fungal treated moths and recipients are fungus-free moths allowed to mate with fungal treated moths, while fungus-free moths are the controls.

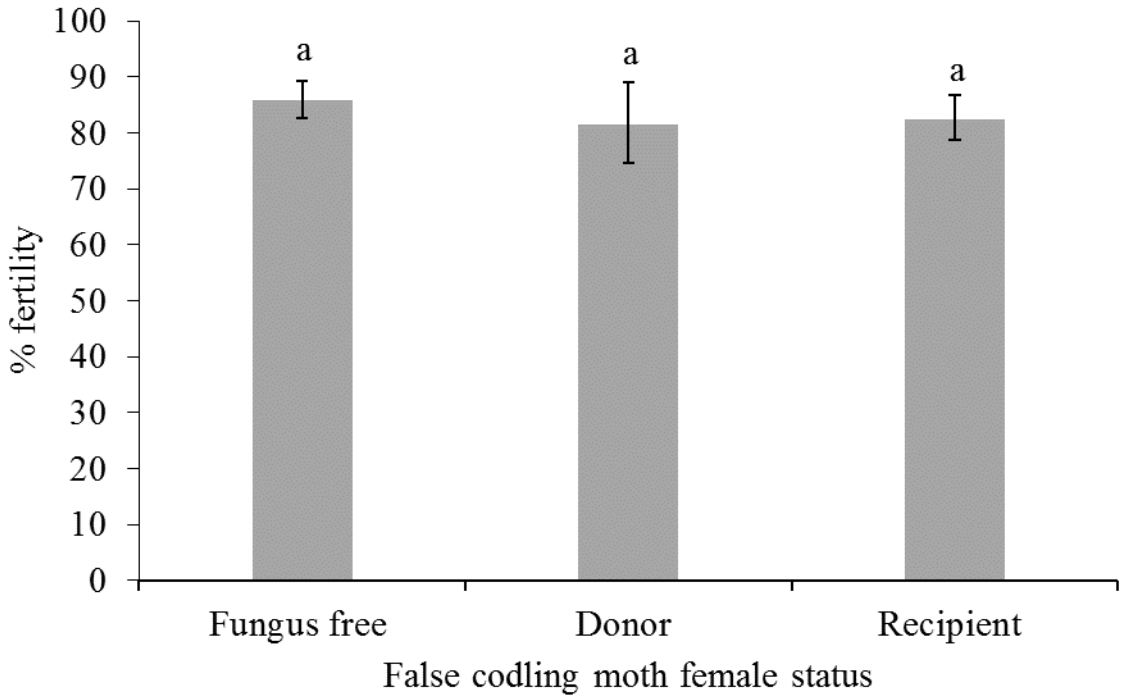

Fig.4: Effect of *Metarhizium anisopliae* ICIPE 69 on *Thaumatotibia leucotreta* fertility. Bars caped by the same letter are not significantly different ($P < 0.05$, Tukey's HSD). Donors are fungal treated moths and recipients are fungus-free moths allowed to mate with fungal treated moths. Fungus-free moths served as the controls.

4.5 Discussion

The use of entomopathogenic fungi for the control of different insect pests has been well documented (Easwaramoorthy and Jayaraj 1978; Ramesh et al. 1999; Sahayaraj and Namachivayam 2011; Senthilraja et al. 2010). This study highlights the pathogenicity of dry conidia of *M. anisopliae* and *B. bassiana* isolates for use in controlling *T. leucotreta*. All screened entomopathogenic fungi isolates caused mortality of *T. leucotreta* moths. There was, however, a difference in pathogenicity between the species as well as between the isolates. It could be attributed to inter- and intraspecific disparities in pathogenicity between the tested fungi as discussed by Ekesi et al. (1998). Four of the isolates evaluated caused moth mortality higher than 80%, with *M. anisopliae* ICIPE 69 causing the highest mortality. Pathogenicity of *M. anisopliae* ICIPE 69 isolate on *T. leucotreta* was also reported for the larval and pupal stages by Coombes et al. (2013). The results from this study proved that dry conidia of entomopathogenic fungi can be used in control of *T. leucotreta* moths.

Thaumatotibia leucotreta moths were able to acquire conidia from the surface of the inoculated jars and successfully transmit the fungal inoculum to conspecifics. It therfore showed potential to be used in autodissemination techniques for control of this pest. Male moths acquired more conidia from a fungal treated substrate compared to female moths. This could be explained by morphological differences of the tibia between the two sexes., The inner apical spur on the hind tibia of males is enlarged with a batch of scales while there is no such feature to the hind tibia of the females (Gilligan et al. 2011). This is beneficial for horizontal transmission of entomopathogenic fungi because males which will be attracted by the female pheromone to an auto-dissemination device, will acquire the conidia and transmit it. Both male and female *T. leoucotreta* were able to acquire and transmit the dry fungal conidia through mating and were also able to retain the conidia for at least three days after horizontal transmission. Recipient females acquired more conidia when mated with donor males, which is an important aspect in the auto-dissemination of fungal conidia to other individuals and the consequent spread of the fungal infection in *T. leucotreta* populations. Males, unlike females, are known to be polygynous (Zagatti and Castel 1987) and can therefore contribute to more inoculum transmission for suppression. Intraspecific auto-dissemination of dry fungal conidia has also been reported for another lepidopteran pest, *P. xylostella* (Furlong and Pell 2001). In subsequent studies by Pell et al. (1993) and Furlong et al. (1995), evidence was provided for the effective use of an auto-dissemination device for fungal dispersal and mortality of *P. xylostella* moths. The authors reported that *Plutella xylostella* male moths visit the auto-inoculation trap baited with a pheromone and containing conidia of *Z. radicans* which resulted in mortality caused by the fungus of 76 – 100%. Survival of *T. leucotreta* "donors" and "recipients" for a few days after horizontal transmission provides time to transmit the fungus between individuals.

Fecundity of *T. leucotreta* females was also negatively affected by *M. anisopliae* (ICIPE 69) from four days after horizontal transmission onwards. A reduction in fecundity after EPF infection has also been reported for other fruit infesting pests. For example, *C. capitata* was reported to lay fewer eggs with a 58.4 to 72.1% reduction in oviposition after being infested by *M. anisopliae* (Quesada-Moraga et al. 2006). A reduction in fecundity after *M. anisopliae* infection was also reported for *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) (Sookar et al. 2014). The reduction in fecundity of fungal infected moths recorded in this study, therefore, also provides more evidence of the potential of

entomopathogenic fungi for control of *T. leucotreta* which can be included in IPM strategies of this pest.

The similarity in the fertility of *M. anisopliae* infected and fungus-free female moths could be explained by the short pre-oviposition period (approx. 1 day) (Daiber 1980; Mkiga et al. 2019), as well as the time lag for the infection mechanism of entomopathogenic fungi to kill target organisms. It could be explained by the fungal infection process which generally takes some time (3 – 7 days) from contact with infective conidia until initiation of disruption of the metabolic processes (Sandhu et al. 2012; Shahid et al. 2012; Vega et al. 2009; Zimmermann 2007). In another study with *M. anisopliae* ICIPE 69, Dimbi et al. (2013) reported that the fungus did not affect the fertility of *M. anisoplae* infected *C. capitata*. This further supports the use of the auto-inoculation strategy with dry conidia incorporated into a device with a pheromone in an integrated control management strategy for *T. leucotreta*. Moths can be targeted with EPF through an autodissemination device, while another control tactic should be used against the immature stages of the pest. The low fertility of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) eggs following infection by *B. bassiana* reported by Kaur et al. (2011) is in contrast to the findings of this study. A possible explanation for this discrepancy in the fertility from fungus-infected females could be the fungal dosage applied, formulation and the life stage of the pest. For example, we used dry fungal formulation while liquid formulation was used in the study conducted by Kaur et al. (2011).The use of dry conidia of entomopathogenic fungal isolate *M. anisopliae* ICIPE 69 proved to be a viable control option for *T. leucotreta* which should be investigated further with the auto-dissemination device under field conditions. Using dry fungal conidia in the auto-dessimination technique could provide for the development of a novel management strategy for use of fungal microbes in IPM programs targeting the adult stage of *T. leucotreta*. The compatibility of dry conidia of *M. anisopliae* ICIPE 69 with the sex pheromone of *T. leucotreta* should, however, be investigated and confirmed before using it in the auto-inoculation device.

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CHAPTER 5: ARTICLE 3

An IPM approach for the management of *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae) in citrus orchards in Kenya

Prepared for submission to ***Journal of Pest Science***

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5.1 Abstract

Compatibility of *Metarhizium anisopliae* ICIPE 69 with *Thaumatotibia leucotreta* (FCM) sex pheromone was investigated for use in the integrated management of this pest. Dry conidia of the fungus were placed in an autoinoculation device (AID) with and without the pheromone, for 8 days and four weeks under laboratory and field conditions, respectively. Conidial acquisition and FCM mortality were assessed by transferring the autoinoculation devices from the field to a screen house, where moths were introduced. These moths were captured after 24 h. The compatible package was integrated with other control tactics against FCM in citrus orchards, namely ICIPE 69 campaign + dry ICIPE 69 in AID, Last Call FCM and ICIPE 69 campaign + dry ICIPE 69 in AID + Last Call FCM. Fungal germination was not affected by the pheromone, but it decreased with an increase in exposure time. Under laboratory conditions, the fungal germination reduced from 95.12 to 86.50% and from 92.33 to 65.33% under field conditions. The number of conidia acquired by the moths ranged from 18.65 x 10^5 to 11.90 x 10^5 while mortality ranged from 87.99 to 69.81% at the onset of the experiment and four weeks after exposure, respectively. The highest marketable yield (10 880.68 and 11 192.26 kg orange fruit/ha) was recorded in orchards where ICIPE 69 campaign + ICIPE 69 in AID + Last Call FCM were applied and the lowest was in the untreated orchards (5 944.28 and 5 458.63 kg orange fruit/ha) for Machakos and Makueni respectively. Low *T. leucotreta* damage on orange fruit and high estimated marketable fruit yield in the orchards applied with the combined use of Last Call FCM and entomopathogenic fungi demonstrate the potential of the sustainable control for *T. leucotreta* in orange and other crops across Africa.

Keywords

Autoinoculation, attractant, ICIPE 69, suppression, virulence

5.2 Key message

- *Metarhizium anisopliae* ICIPE 69, an entomopathogenic fungus and the synthetic false codling moth (FCM) sex pheromone are compatible and can be used in an IPM strategy for control of the pest.
- *Metarhizium anisopliae* ICIPE 69 in combination with the pheromone can persist and is virulent against *T. leucotreta* under field conditions for up to 4 weeks.
- Combined application of ICIPE 69 campaign®, dry ICIPE 69 in AID and Last Call FCM® significantly reduced FCM population, fruit infestation and consequently increased marketable fruit yield.

5.3 Author contribution statement

AMM, SAM, KSA and SE conceived and designed research. AMM conducted experiments. AMM analysed data. AMM wrote the manuscript. SAM, HDP, FMH, KSA and SE reviewed the manuscript, SAM was the project administrator, SE acquired research funds". All authors read and approved the manuscript.

5.4 Introduction

The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), is one of the economically important pests of fruit and vegetables in sub-Saharan Africa. It is phytophagous and a multivoltine pest which infests a variety of fruit such as citrus (Rutaceae), avocado, *Persea Americana* (Mill) (Lauraceae), macadamias, *Macadamia* spp. (Proteaceae) and cotton, *Gossypium* spp. (Malvaceae) (Erichsen and Schoeman 1994; Gilligan et al. 2011; Kirkman and Moore 2007; Malan et al. 2018; Mazza et al. 2014; Vennette 2003). It also infests vegetables such as sweet and chili peppers (*Capsicum* spp.), eggplant (*Solanum melongena*) and African eggplant (*Solanum aethiopicum* L. (Mkiga et al. 2019; Giligan et al. 2011). Larval feeding causes pre-harvest fruit drop and post-harvest fruit decay (Kirkman and Moore 2007; Hill and Fullard 2013). Larval incidence as high as 47% was recorded on orange in Kenya (Mkiga et al. 2019). Strict quarantine restrictions are imposed, and a single larva found can result in the rejection of an entire consignment (Moore 2012).

All these losses experienced by producers lead to the use of different control strategies, mostly relying on the application of synthetic insecticides. However, the use of chemical pesticides has several adverse effects on both humans and the environment. The development of pesticide resistance to synthetic pesticides has been reported (Hofmeyr 1998; Khan et al. 2015). There is, therefore, a need to identify alternative control options that are sustainable and environmentally friendly. One of the options being widely explored currently is the use of biopesticides (viruses, bacteria and fungi). Biopesticides, such as, entomopathogenic fungi infect their host through contact (Senthil-Nathan 2015; Agale et al. 2018), while viruses and bacteria must be ingested by the insect pest (Sandhu et al. 2012). Entomopathogenic fungi have shown considerable infectivity to Lepidoptera species, for example, *Plutella xylostella* (Lepidoptera: Yponomeutidae) (Furlong and Pell 2001), *Spoladea recurvalis* (Lepidoptera: Crambidae) (Opisa et al. 2018) and *T. leucotretra* (Coombes et al. 2013).

Entomopathogenic fungi are generally applied through inundative sprays. According to Sookar et al. (2014), the success of EPF as bio-control agents depends on their dispersal efficiency and their ability to cause acute mortality in the host population. The use of autodissemination devices has been demonstrated in several studies (Furlong et al. 1995; Dimbi et al. 2003; Nana et al. 2016). An autodissemination device consists of inoculum applied in a device that contains a pheromone to attract the insect. Once in the device, the insect is contaminated with the fungus to potentially carry the pathogen to the desired location after leaving the device (Vega et al. 1995). However, little is known on the use of dry conidia of the fungus in an autoinoculation device for suppression of *T. leucotreta.* This approach also requires a strong attractant that is compatible with the biocontrol agent.

The compatibility of ICIPE 69 dry conidia and an attractant for *T. leucotreta* moths in an autodissemination device under field conditions could provide an additional control tactic for the pest. The technique is also compatible with several other control tactics for use in an Integrated Pest Management (IPM) program. For example, conidia can be applied in an autodissemination device to target moths while inundative spray can be used on the soil around the canopy of the tree targeting the soil-dwelling stages of fruit infesting pests (Begemann 2008; Goble et al. 2011; Moore et al. 2015). The objectives of the current study were: (1) to test the persistence of fungal conidia when exposed to the *T. leucotreta* sex pheromone overtime in an autodissemination device under field conditions; (2) to determine the quantity of fungal conidia that will be

acquired by *T. leucotreta* moths from an autodissemination device under field conditions; (3) to assess the virulence of the fungus acquired by the moths from the autodissemination device above, over time and (4) to evaluate the suppression of *T. leucotreta* in citrus orchards by means of fungus applied in an autodissemination device and also by combining this method with other management programs.

5.5 Material and Methods

Insects used in this study were obtained from the Mass Rearing Unit at the International Centre of Insect Physiology and Ecology (*icipe*). The larvae of *T. leucotreta* were reared on an artificial diet developed by Moore et al. (2014). Adults were maintained following the procedures described by Opoku-Debrah et al. (2014) with a slight modification reported by Mkiga et al. (2019).

The fungal isolate, *M. anisopliae* ICIPE 69 used in this experiment was obtained from the Arthropod Germplasm Centre of *icipe*. Conidia of the fungus, *Metarhizium anosipliae* isolate ICIPE 69 for use in autoinoculation devices, were mass-produced on a long-rice substrate in Milner bags (60 cm long X 35 cm wide). Rice was autoclaved for 1 h at 121 °C and inoculated with a three-day-old culture of blastopores as described by Jenkins et al. (1998). The inoculated bags were then incubated for 21 days at 20 – 26 °C and 40–70% relative humidity (RH). The rice substrate containing fungal spores could dry for five days at room temperature. Conidia were harvested by sifting the substrate through a 295 µm mesh sieve and then stored in plastic bags in a refrigerator (4 – 6 ºC) until use. The viability of conidia was determined following the procedures described by Goettel and Inglis (1997).

The autoinoculation device used in this study was from Real-IPM®. This device has two chambers. The base of the lower chamber is 9 cm in diameter, 14 cm high with a 12 cm (diameter) ventilated lid. The upper chamber (15.5 cm height) fits onto the ventilated lid of the lower chamber. The upper chamber has three entrance/exit holes (4 cm diameter). The inner walls were wrapped with velvet. An automatic onsets HOBO data logger purchased from Labo-pharma Kenya Ltd was secured in the upper chamber of devices. The screenhouses (260 X 260 X 195 cm) used were constructed at *icipe.*

Last Call FCM and commercialised *Metarhizium anisopliae* campaign

The false codling moth sex pheromone, Insect Science® mixed with insecticide permethrin in grease formulation (Last Call FCM) was also used in the suppression trial. Last Call FCM is the product registered for use in attract and kill technology. According to Moore (2016), the product can be applied three to the four times per season and up to 3000 droplets of the product per hectare per application. The product in the tube containing the pump is applied on the leaf surface by hand or with an applicator pole for the long trees. The entomopathogenic fungus, *Metarhizium anisopliae* isolate ICIPE 69 is commercialized by Real IPM under the trade name Campaign®, for use against thrips, fruit flies and mealybugs. This product contains live conidia as the active ingredient and is mainly applied in the soil targeting soil-dwelling stages of fruit infesting pests or canopy at 200 ml/ ha to control different insect pests and thus reduce excessive use of synthetic chemical pesticides.

Laboratory trials

5.5.1 Compatibility of *Thaumatotibia leucotreta* sex pheromone and *Metarhizium anisopliae* ICIPE 69 under laboratory conditions

The fungal isolate, *M. anisopliae* ICIPE 69 was selected based on its persistence in the soil and virulence to *T. leucotreta* reported by Coombes et al. (2013). A fungal viability test was done before the bioassay by culturing it on Sabouraud Dextrose Agar (SDA) in petri dishes (9 cm in diameter) followed by incubation at 25 ± 2 °C in darkness, according to the procedures by Maniania (1992). Conidia were harvested from 2 to 3-week old cultures using a spatula. Conidia were suspended in 10 ml distilled water containing 0.05% Triton X-100 in universal bottles with glass beads. The conidial suspensions were vortexed to obtain a homogeneous mixture. The fungal spore concentration was quantified with a haemocytometer. Viability of the conidia was determined using the technique described by Goettel and Inglis (1997) whereby 0.1 ml of the suspension was titrated to 3 $\times 10^6$ conidia ml-1 and spread-plated on SDA in petri dishes (9 cm in diameter). The suspension was spread evenly on the SDA plates. Three sterile microscope coverslips were placed randomly on each plate. Plates were incubated at 25 ± 2 °C for 24h and a 12:12 (L:D) h photoperiod. After incubation, percentage germination was determined from spores for each plate by measuring the germ tube length under a light microscope (Leica DMLB) at ×400 magnification. The length of the germ tube must be at least twice the diameter of the conidium as described by Inglis et al. (2012). To facilitate easy counting of spores, lactophenol cotton blue was added to stain the spores and terminate germination.

After determining the germination using procedures as described above and viability being acceptable (>95%), a conidial suspension of entomopathogenic fungus *M. anisopliae* ICIPE 69 was prepared. The suspension was titrated to 1 × 10^7 conidia ml^{-1} and fungal spores for the compatibility were retained with a nitrocellulose filter membrane (diameter 4.7 cm, pore size 0.45 µm, Sigma Chemicals) by pouring 10 ml suspension through a filter holder unit (MFS) under aspirator vacuum (Maniania, 1994). Five nitrocellulose filter membranes were prepared to retain the fungal spores. These membranes were dried for 30 min in a laminar flow cabinet and transferred to a glass desiccator (2.5 L). The dried nitrocellulose filter membranes were individually placed covering each hole of the plate. This ensured exposure to the *T. leucotreta* attractant which was placed on the base at the centre of the desiccator. The nitrocellulose membranes with the fungal spores were exposed to the attractant for periods of 1, 2, 3, 6 and 8 days according to the procedures described by Mfuti et al. (2016). An untreated control without *T. leucotreta* attractant was included. One nitrocellulose filter membrane with fungal conidia was removed from the desiccator on 1, 2, 3, 6 and 8 days after exposure to determine conidial germination. Each nitrocellulose filter was transferred into 10 ml sterile distilled water containing 0.05% Triton X-100 and vortexed for 3 min to dislodge the conidia. From this suspension, 0.1 ml was titrated to 3 × 10^6 conidia ml-1 and spread-plated on SDA plates. Three plates were prepared and incubated at 25 ± 2°C, L12: D12 photoperiod and examined after 18 to 24 h for conidial germination and measuring of germ tube lengths. The experiment was replicated three times.

Field trials

5.5.2 Compatibility of the *Thaumatotibia leucotreta* sex pheromone and *Metarhizium anisopliae* ICIPE 69 under field conditions

An experiment was conducted at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus Kenya (1°13'19.52"S, 36°53'48.13"E; 1616 m above sea level), to determine the persistence of *M. anisopliae* ICIPE 69 under field conditions. The experiment was also done to quantify the number of conidia acquired by *T. leucotreta* moths from the autoinoculation devices following four weeks of exposure under field conditions. There were two treatments, *viz*. autoinoculation devices containing the fungal treatment with the FCM sex pheromone included and the fungal treatment without the FCM sex pheromone included (control). The *T. leucotreta* sex pheromone, containing E3, Z8, Z11-tetradecatrienyl acetate 0.76mg + E3, Z8-tetradecadienyl acetate 0.04 mg used in this study was purchased from Kenya Biologics Ltd. The experiment was replicated three times.

The attractant (pheromone) was placed at the base of the lower chamber of the device and 3 g of dry conidia was spread evenly on the velvet cloth in the upper chamber of each device, which was fixed onto the ventilated lid (Fig. 1). Germination of the conidia was determined immediately after the onset of the experiment (week 0). The daily temperature (°C) and relative humidity (RH%) was recorded by the data logger in the upper chamber. The devices were hung at a height of 150 cm from the ground and at least 500 m apart. Conidial persistence was determined by collecting weekly samples of conidia from each auto-inoculation device with a moist cotton bud. The tip of the cotton bud containing conidia was cut, suspended in 10 ml 0.05% (wt / vol) Triton X-100 and vortexed for 1 min. A sample of 0.1 ml of conidial suspension (titrated to 3 x 10^6 conidia/ ml) was spread-plated on SDA plates and four sterile microscope coverslips were placed randomly on the surface of each plate. The plates were sealed with Parafilm and incubated under complete darkness at 25 ± 2 °C. Conidia germination was assessed after 18 hr. Lactophenol cotton blue was added to terminate germination and to stain the spores. A light microscope (Leica DMLB) at ×400 magnification was used to calculate the percentage germination of 100 randomly selected conidia under each of the coverslips. The conidia were regarded as viable where the germ tube lengths were twice the diameter of the propagule (Goettel and Inglis 1997). There were three replicate plates per treatment.

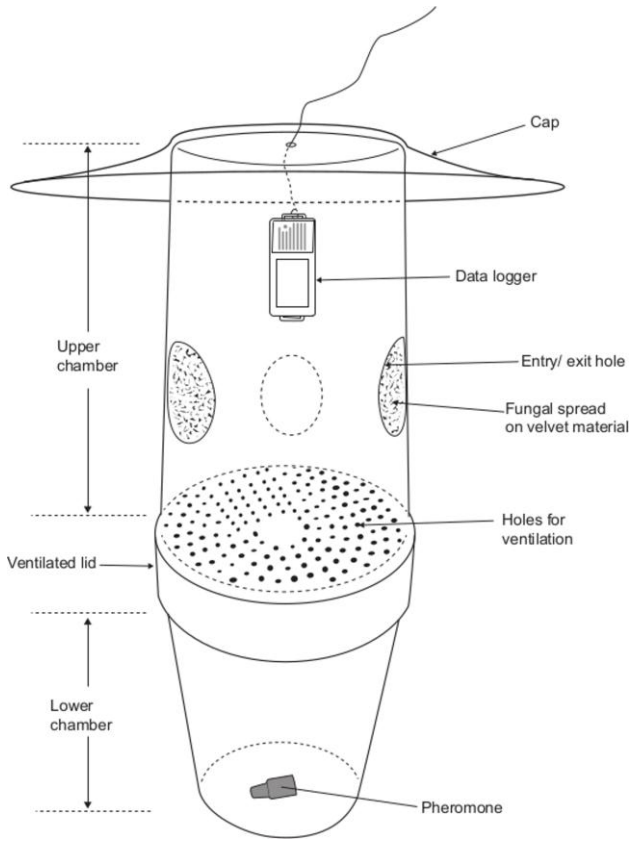

Fig. 1. Autoinoculation device used to test compatibility of ICIPE 69 and FCM pheromone under field conditions.

Source: ***icipe.***

5.5.3 *Metarhizium anisopliae* ICIPE 69 conidia acquisition and virulence to *Thaumatotibia leucotreta* after exposure in screen houses

The experiment described above (see 5.5.2) was replicated in screen houses. Six screen houses were used per week. The autoinoculation devices in three of these screen houses contained the *T. leucotreta* attractant (sex pheromone), while autoinoculation devices in the other three screen houses were without the attractant. The devices were hung in the centre of each screen house (1 per screen house) at a height of 1.5 m above the ground, using a wire. Approximately 500, 1- day old male moths were released into each screen house. After 24 h, 50 moths near the device in each screen house were randomly captured and placed separately into sterile 10 ml glass tubes, and the tubes closed with cotton wool. The moths were transferred individually into 2 ml cryogenic tubes containing 1 ml sterile 0.05% Triton X-100. The tubes were vortexed for 2–3 min to dislodge conidia from the insect and the number of conidia was determined using a haemocytometer. Data from 30 moths which acquired conidia were analysed. Twenty moths were also captured in each screen house after 24 h. The moths were transferred to cages (20 x 10 x15) and a moist cotton ball, as a source of water was provided. The cages were kept in an incubator at 25 ± 2 °C and a 12:12 (L:D) h photoperiod. Mortality was recorded daily for 10 days. Dead moths were surface sterilised with 70% alcohol and rinsed thrice in distilled water. These moths were kept separately in petri dishes lined with sterile moistened filter paper to record mycosis.

5.5.4 Integration of control strategies for *Thaumatotibia leucotreta* management in citrus orchards

5.5.4.1 Assessment of *Thaumatotibia leucotreta* population density at field sites

Sites were identified for field trials in orchards with orange, *Citrus sinensis* (L) Osbeck, var Washington navel, in Makueni (1˚47'11.38" S, 37˚57'51.99" E) and Machakos (1˚16'1.23" S, 37˚19'12.64" E) from May to the end of August 2018 in Kenya. The sites were in citrus producing areas in Kenya with high *T. leucotreta* infestation on orange, previous reported by Mkiga et al. (2019). Three wards/blocks were selected from each county (Fig. 2). Within each ward/block (replicate), four orchards were selected which were at least 1 km apart. Prior to the onset of the trial, the *T. leucotreta* population in each of these experimental sites was monitored for four weeks in April 2018. One trap fitted with a sticky card and baited with a crytrack® lure was installed in each orchard. The attractant is powerful and the orchards were not more than one hectare in size. The sticky cards were removed weekly from the trap and replaced. The cards with *T. leucotreta* catches were put individually in transparent plastic bags. The number of *T. leucotreta* moths on each sticky card was counted and recorded. These traps were removed before commencement of the trial.

The trial consisted of four treatments, *viz*. (1) *Metarhizium anisopliae* campaign + dry *Metarhizium anisopliae* in an autodissemination device, (2) Last Call FCM, (3) *Metarhizium anisopliae* campaign + dry *Metarhizium anisopliae* in autodissemination device + Last Call FCM and (4) Untreated control. The entomopathogenic fungus, *Metarhizium anisopliae* isolate ICIPE 69 (Mazao Campaign Real-IPM®) contains live conidia as the active ingredient and was applied bimonthly in the soil targeting soil-dwelling stages of fruit infesting pest at a rate of 200ml/ha. Dry conidia (3 g) of the potent fungus ICIPE 69 were applied in the autoinoculation devices (see 5.5.2) and replaced monthly. Two months after treatments allocation, the same number of traps were reinstalled, maintained and moth catches recorded in the orchards for post-treatment monitoring. The mean number of moths per trap per day (MTD) was calculated by dividing the mean trap catches by the number of days that the trap was in use.

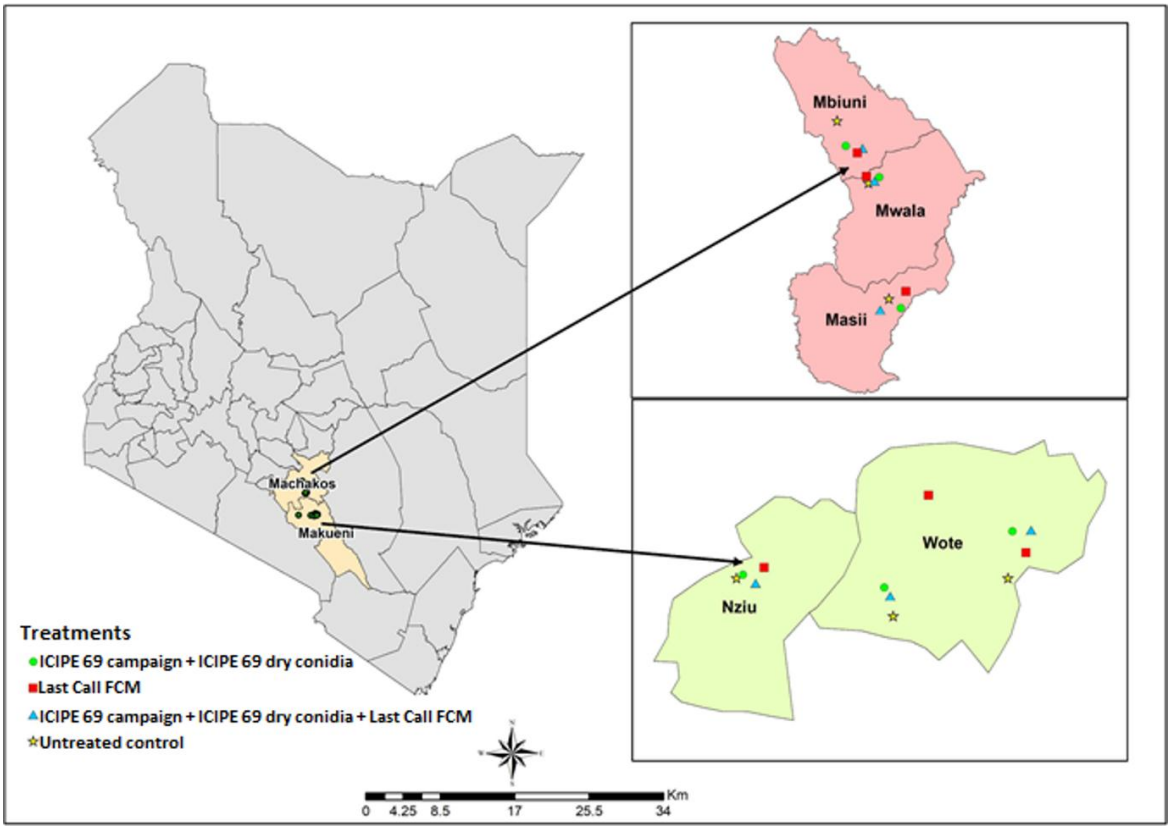

Fig. 2 Study sites and treatments allocation in different orange orchards within Machakos and Makueni counties in Kenya

5.5.4.2 Assessment of fruit infestation by *Thaumatotibia leucotreta*

Bi-weekly fruit sampling was conducted at both sites two months after the onset of the trial in all experimental orchards from July to August 2018. One hundred fruit per orchard were randomly sampled from the trees. Fruit were washed using a non-caustic liquid dish-washing soap and incubated for three weeks. The incubated fruit were dissected and the *T. leucotreta* larvae or pupae were counted. Percentage infested fruit was also calculated. The final instar larvae which were collected, were placed in plastic containers (Kenpoly) (2 L) with a thin layer of sterilized soft sand for pupation. Identification of adult *T. leucotreta* emerging from the pupae was confirmed using standard keys developed by Gilligan et al. (2011).

5.5.4.3 Assessment of marketable yield and economic analysis

Towards the end of the harvesting period, branches containing orange fruit from 25 trees ≈ 1225 m^2 in the experimental orchards, were gently shaken to allow for *T. leucotreta* infested fruit drop (Mkiga et al. 2019). The orange fruit which remained on the trees after shaking were harvested and any infestation was recorded. The marketable orange fruit were counted and weighed on-site and the fruit yield was expressed as kilogram per hectare. Economic analysis was performed based on orange farm-gate prices for 2018 in Kenya. Input costs in terms of pesticides, labor for pesticide application and the cost of a knapsack sprayer (based on its depreciation over five years) were estimated. The cost-benefit ratios for each treatment were calculated by relating input costs with monetary gains from increased marketable orange fruit yields.

5.5.5 Statistical analyses

Data on percentage conidia germination of *M. anisopliae* ICIPE 69 were subjected to logistic linear mixed model with intercept slope using the 'glmer' function from the lme4 package to test the effect of attractant and exposure time as well as the interaction. Likelihood ratio test on a GLM (family: negative binomial link: log) was used to analyse the number of conidia acquired by a single moth from the auto-inoculation devices in the screen house to determine the effect of attractant and exposure time. Pairwise comparison of Least Squares Means (LSMeans; package lsmeans,' function 'lsmeans') of the number of conidia acquired by a single moth in devices with both *M. anisopliae* ICIPE 69 and the attractant were also done between weeks. Mortality data were subjected to Abbott's formula to control for natural mortality (Abbott 1925) before being analysed by using generalised linear models (GLM) assuming binomial distribution and logit link. Lethal time to 50% mortality (LT50) values were estimated by the logit analysis method for correlation data using SPSS version 22. Spearman correlation was used to analyse the relationship between moth mortality, conidia acquisition, conidial persistence in terms of germination and daily temperature and relative humidity (RH). The same analysis was used to determine the association between marketable orange fruit yield, *T. leucotreta* moth abundance and percentage orange fruit infested with *T. leucotreta.*

Field suppression data on *T. leucotreta* temporal abundances were also analysed by using logistic linear mixed model with intercept slope using the 'glmer' function from the lme4 package to test the interaction between treatments and sampling dates. The reduction in the *T. leucotreta* moths collected relative to the control for each treatment was calculated using the formula of Henderson and Tilton (1955), namely {1-([control before x treatment after]/[control after x treatment before])} x 100. The percentage reduction in moth numbers and percentage orange fruit infested with *T. leucotreta* were arcsine transformed and analysed using one-way ANOVA. The estimated marketable orange fruit yield in kg/ha and percentage yield increase as a result of the treatments applied were also analysed using one-way ANOVA. Normality of data was confirmed by Shapiro normality test. All means were separated by Tukey's post-hoc test (P = 0.05). The cost-benefit ratio was calculated based on monetary gains obtained at the farm gate price for marketable orange yields against the total input cost following procedures described by Karel and Ashimogo (1991). All data analyses were performed using R (version 3.2.5) statistical software packages (R Development Core Team, 2016) except for LT50.

5.6 Results

5.6.1 Compatibility of *Thaumatotibia leucotreta* sex pheromone and *Metarhizium anisopliae* ICIPE 69 under laboratory conditions

The viability of conidia used was > 95%. Conidial germination of *M. anisopliae* was significantly affected by exposure time in days (χ^2 = 37.330, df = 4, P < 0.001) (Fig. 3). The lowest percentage conidial germination was recorded eight days after the fungus was exposed to the *T. leucotreta* sex pheromone in desiccators. The attractant did not affect the fungal viability significantly (χ^2 = 0.003, df = 1, P = 0.958). The interaction between the attractant and exposure time in days to the fungus was also not significant (χ^2 = 1.607, df = 4, P = 0.808).

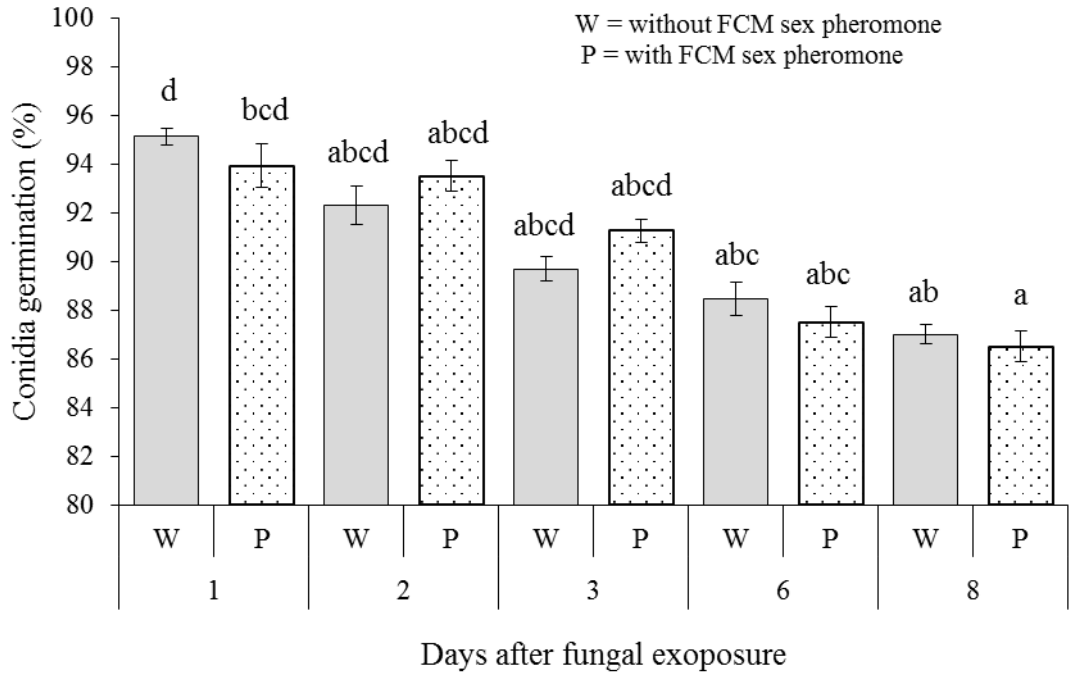

Fig. 3 Mean percentage *Metarhizium anisopliae* ICIPE 69 (±SE) conidial germination over time after exposure to the *Thaumatotibia leucotreta* sex pheromone in a laboratory bioassay. The control was conidia not exposed to the sex pheromone (W) and the treatment consisted of conidia exposed to the sex pheromone (P). Bars caped with the same letter are not significantly different (P = 0.05, Tukey's HSD).

5.6.2 Compatibility of the *Thaumatotibia leucotreta* sex pheromone and *Metarhizium anisopliae* ICIPE 69 under field conditions

Under field conditions, conidial germination of *M. anisopliae* was also significantly affected by exposure time in weeks ($\chi^2 = 122.586$, df = 4, $P < 0.001$) (Fig. 4). However, the fungus was able to persist in the field for several weeks with the viability of up to 70% in the 4th week of exposure. Viability of the conidia, not exposed to the pheromone, was significantly lower from week three after exposure when compared to the initial viability at the onset of the experiment. The attractant did not significantly affect the fungal viability ($\chi^2 = 0.117$, df = 1, $P = 0.732$). The interaction between attractant and time in weeks of fungal exposure was also not significant ($\chi^2 = 0.868$, df = 4, $P = 0.929$).

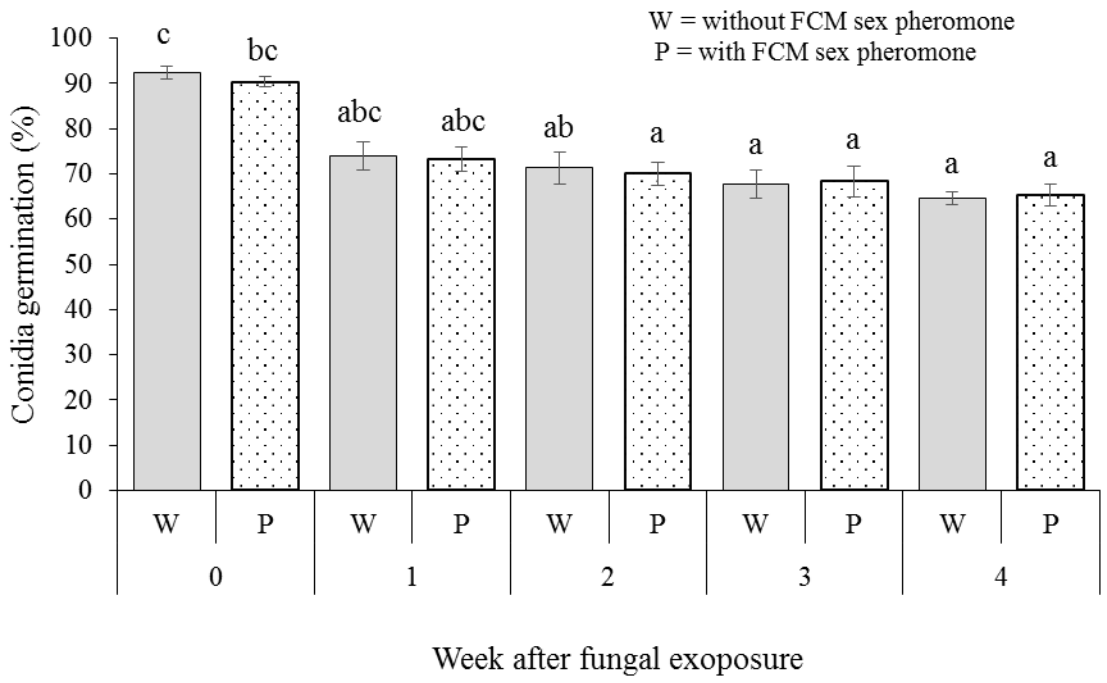

Fig. 4 Mean percentage *Metarhizium anisopliae* ICIPE 69 (±SE) conidial germination over a period of four weeks of field without exposure to the *Thaumatotibia leucotreta* sex pheromone, which served as the control (W) and exposed to the sex pheromone (P). Bars caped with the same letter are not significantly different (P = 0.05, Tukey's HSD).

5.6.3. *Metarhizium anisopliae* ICIPE 69 conidia acquisition and virulence to *Thaumatotibia leucotreta* after exposure in screen houses.

Spore acquisition by moths in the autoinoculation devices with or without (control) the attractant, differed significantly in the screen houses ($\chi^2 = 143.16$, df = 1, $P < 0.001$) (Figure 4). When the pheromone was not included in the device, no conidia was acquired by moths from the *M. anisopliae* treated devices, which confirms that moths did not enter the devices without being attracted by the pheromone. The mean number of conidia acquired by a single moth differed significantly over time of exposure to environmental conditions in the autoinoculation devices ($\chi^2 = 2669.64$, df = 4, $P < 0.001$) (Fig. 5). The number of conidia acquired significantly reduced in the first week of exposure but remained similar for the next three weeks (Fig. 5). Significantly more conidia were, however, acquired during the first week of exposure.

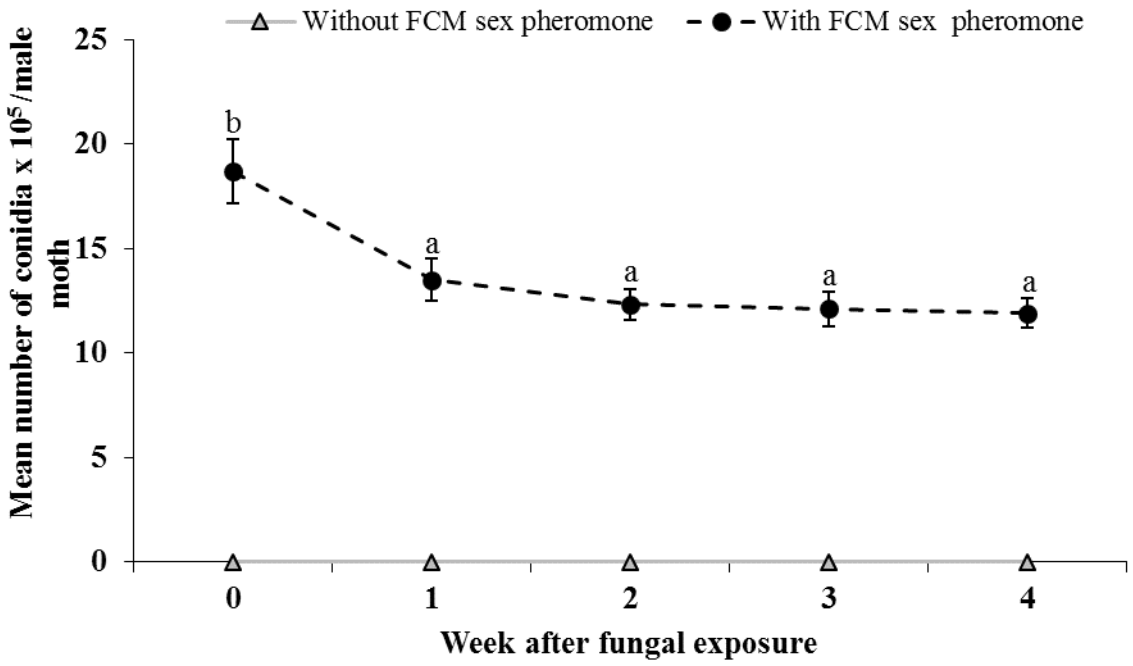

Fig. 5 The mean number of conidia (±SE) acquired by one *Thaumatotibia leucotreta* male moth at the end of each week from immediately after *Metarhizium anisopliae* exposure until 4 weeks after exposure in an autoinoculation devices with and without *Thaumatotibia leucotreta* sex pheromone in screenhouses. Means labelled with the same letter are not significantly different (P = 0.05, Tukey's HSD).

5.6.3.2 Virulence of *Metarhizium anisopliae* ICIPE 69 to *Thaumatotibia leucotreta* after field exposure

Mortality of *T. leucotreta* moths was significantly higher when exposed to the fungus at week zero (χ^2 = 28.013, df = 4, $P < 0.001$) compared to the fungus which was in the device for four weeks (Fig. 6). The shortest LT_{50} values were recorded for the fungus exposed at week zero while the longest was recorded at week four (Table 1). This was also evident from the slopes which were high on week zero compared to week four (Table 1).

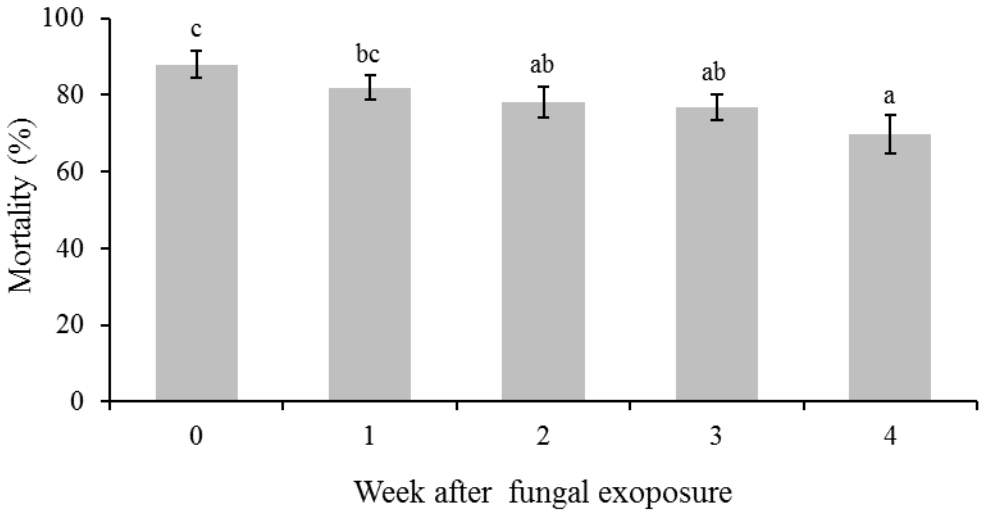

Fig. 6 *Thaumatotibia leucotreta* moth mortality caused by *Metarhizium anisopliae* (ICIPE 69*)* after the fungus conidia had been exposed four weeks in an autoinoculation device with the *Thaumatotibia leucotreta* sex pheromone under field conditions. Bars caped with the same letters are not significantly different (P = 0.05, Tukey's HSD).

Table 1. Lethal time to 50% mortality (LT_{50}) in days and regression line values for *Metarhizium anisopliae* ICIPE 69 after the conidia had been exposed for four weeks in an autoinoculation device with *Thaumatotibia leucotreta* sex pheromone under field conditions. FL represents 95% fiducial limits.

Week after exposure	**LT50 (days) (95% FL)**	**Regression line**	
		Slope	**Intercept**
0	4.86 (4.27-5.42)	0.60	-2.93
1	5.74 (4.79-5.95)	0.54	-2.91
2	5.67 (5.12-6.23)	0.52	-2.92
3	6.13 (5.73-6.54)	0.50	-3.07
4	6.50 (6.04-6.99)	0.48	-3.09

The lowest daily minimum temperature and highest maximum temperatures recorded in the autoinoculation devices were 21.57 and 23.24 ^{o}C, respectively. The lowest minimum RH was 38.17% and the highest maximum RH, 60.67%, respectively. The relationships between *T. leucotreta* mortality, conidial persistence, conidial acquisition, relative humidity and temperature are provided in Table 2. Mortality was positively correlated with conidial persistence and conidial acquisition. The correlation with conidial persistence was, however, not significant. There was a significantly negative correlation between temperature and mortality. Persistence and temperature were also significantly negatively correlated, while there was a significantly positive correlation between persistence and conidial acquisition. Temperature was significantly negatively

correlated with conidial acquisition. There was no significant correlation between relative humidity and any of the parameters, *viz*. mortality, persistence, and temperature and conidia acquisition.

Table 2 Correlation between *Thaumatotibia leucotreta* mortality, *Metarhizium anisopliae* ICIPE 69 conidial persistence, conidial acquisition, relative humidity and temperature exposed in an auto-dissemination device with the FCM pheromone. For each parameter, the top number denotes the Spearman correlation (r_s) and the bottom number the associated P-value.

	Mortality	Persistence	Temperature	Conidial acquisition	Relative humidity
Mortality	-	0.430	-0.560	0.630	0.220
		0.108	**0.031**	**0.013**	0.421
Persistence		-	-0.560	0.910	0.140
			0.030	**0.001**	0.631
Temperature			-	-0.54	-0.190
				0.037	0.502
Conidia acquisition				-	0.060
					0.831

Significant correlations are indicated by bold values (Spearman correlation: $P < 0.05$).

5.6.4 Integration of control strategies for *Thaumatotibia leucotreta* management in citrus orchards

5.6.4.1 Assessment of *Thaumatotibia leucotreta* population density at field sites

The number of *T. leucotreta* moths trapped per day differed significantly between the treatments (χ^2 = 192.46, df = 3, $P < 0.001$) and sampling dates (χ^2 = 18.02, df = 8, P = 0.02) in Machakos (Fig. 7A). However, there was no significant interaction in the number of *T. leucotreta* moths trapped between treatments and sampling dates (χ^2 = 16.12, df = 24, P = 0.88). A similar trend was recorded in Makueni with the number of *T. leucotreta* moths trapped which differed significantly between the treatments (χ^2 = 203.14, df = 3, $P < 0.001$) and sampling dates (χ^2 = 19.285, df = 8, $P < 0.01$). There was, however, also no interaction between treatment and sampling date (χ^2 = 32.22, df = 24, P = 0.12) (Fig. 7B). Generally, at both sites, the numbers of *T. leucotreta* moths trapped with treatments applied in orchards were < *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an auto-inoculation device + Last Call FCM < Last Call FCM only < *M. anisopliae* ICIPE 69 only < *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an auto-inoculation device < untreated orchards.

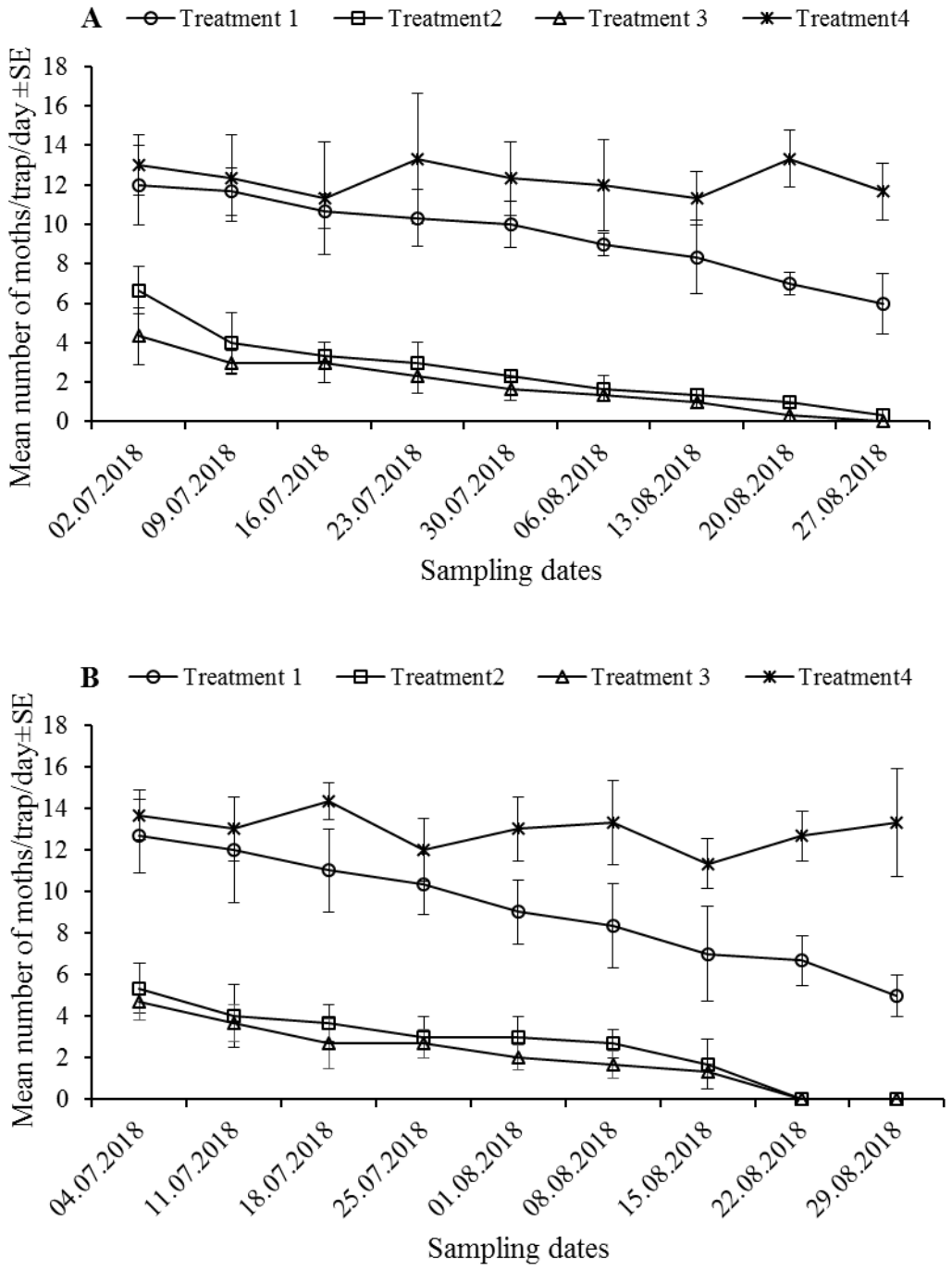

Fig. 7 Temporal trends in number of *Thaumatotibia leucotreta* male moths trapped in citrus orchards where various management treatments were applied in (A) Makueni and (B) Machakos: (1) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device, (2) Last Call FCM, (3) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM and (4) control.

The percentage reduction in *T. leucotreta* moths trapped differed significantly between the treatments at both sites ($F_{(2,\ 12)}$ = 132.82, P < 0.001). The number of moths trapped did, however, not differ significantly between the two sites ($F_{(1,12)}$ = 0.68, P = 0.43). There was also no interaction between treatments and sites in terms of percentage reduction in *T. leucotreta* moths trapped ($F_{(2,12)}$ = 0.01, P = 0.99). The application of *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device reduced the

number of *T. leucotreta* moths significantly less than an application of Last Call FCM and (*Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM). At both sites, there was no significant difference in the reduction of moths trapped in orchards where the latter two treatments were applied (Fig. 8).

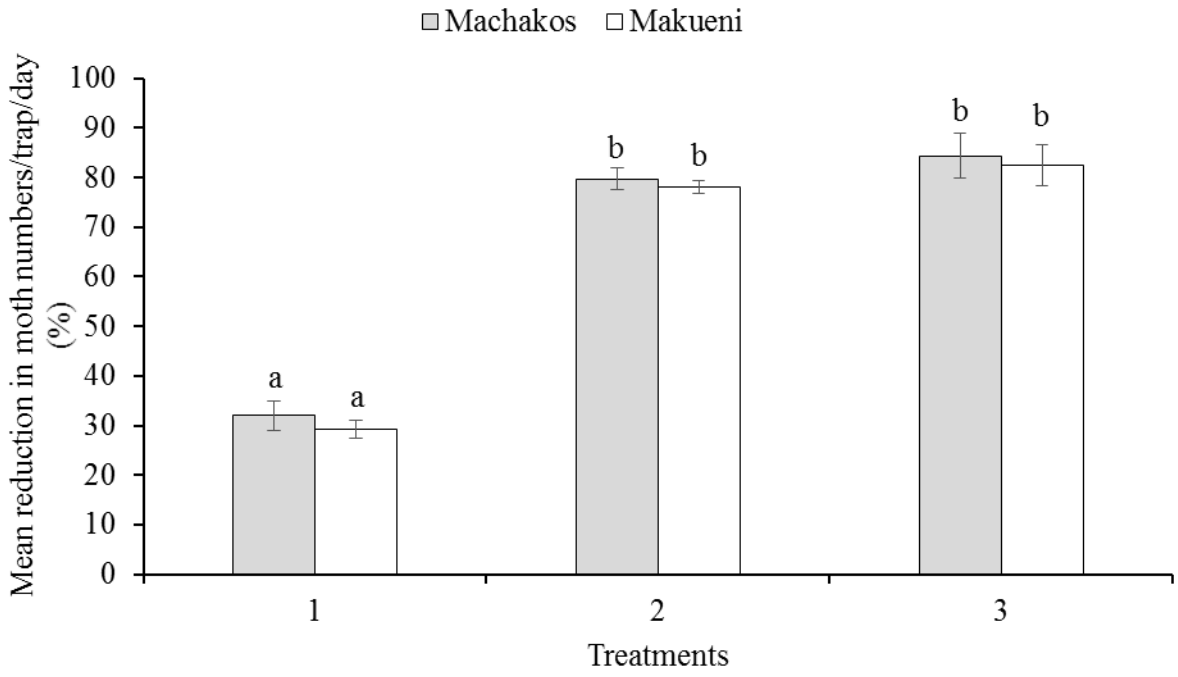

Fig. 8 Percentage reduction in *Thaumatotibia leucotreta* moths trapped in citrus orchards subjected to different management programs at Machakos and Makueni, Kenya: (1) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device (2) Last Call FCM and (3) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM.

5.6.4.2 Assessment of fruit infestation by *Thaumatotibia leucotreta*

The percentage of *T. leucotreta* infested fruit samples from trees where the respective treatments were applied, differed significantly ($F_{(3, 16)} = 65.66$, $P < 0.001$) (Fig. 9). There was, however, no significant difference in fruit infestation between the two sites ($F_{(1,16)} = 3.39$, $P = 0.08$). There was also no interaction effect on percentage *T. leucotreta* infested fruit between the treatments and sites ($F_{(3,16)} = 0.03$, $P = 0.99$). The percentage *T. leucotreta* infested fruit was low in orchards where the treatment, (*Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device) was applied. The percentage infested fruit did not differ significantly where Last Call FCM and the treatment, *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device , were applied at both sites.

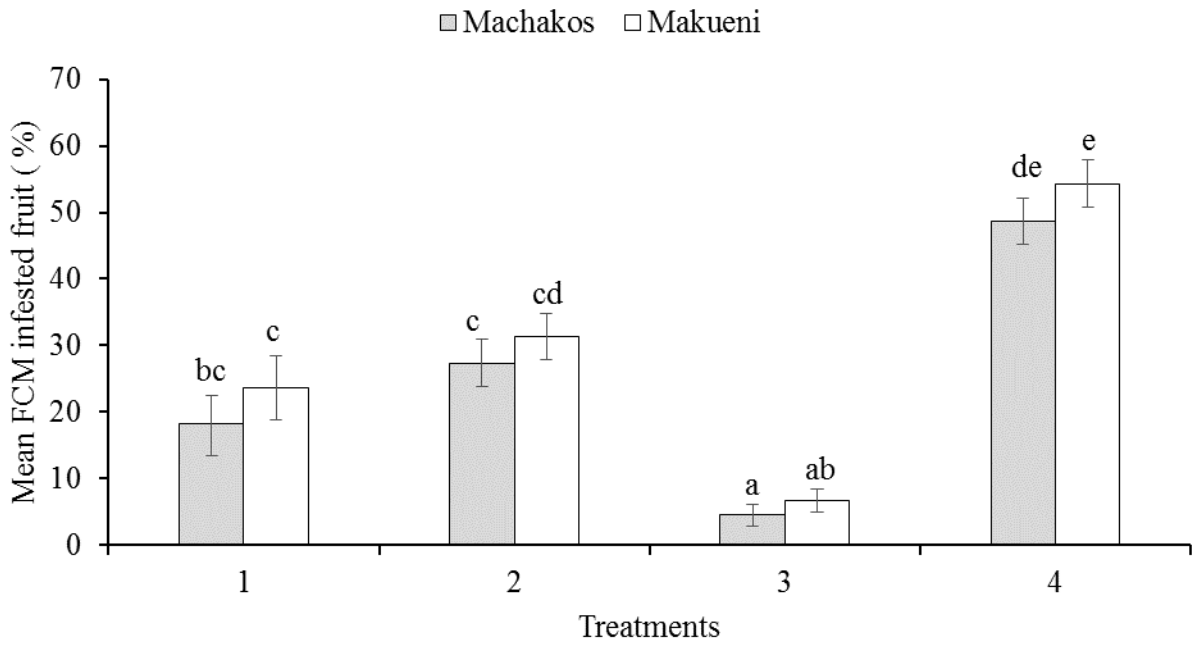

Fig. 9 Mean percentage *Thaumatotibia leucotreta* infested orange fruit in orchards subjected to the respective management programs at Machakos and Makueni counties, Kenya: (1) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device (2) Last Call FCM (3) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM and (4) control.

5.6.4.3 Assessment of marketable yield and economic analysis

Marketable orange fruit yield from the orchards where the respective treatments were applied, differed significantly at both Machakos ($F_{(3, 8)} = 26.85$, $P < 0.001$) and Makueni ($F_{(3, 8)} = 29.43$, $P < 0.001$) (Table 3). The highest marketable orange yield was recorded in orchards where (*Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device) and Last Call FCM was applied together for control of *T. leucotreta*. The lowest marketable orange fruit yield was from the control orchards where no management treatments were applied. At both sites, the yield recorded in orchards where (*Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device) and Last Call FCM, were applied did not differ significantly. However, after calculation of the cost-benefit ratio, suppression of the pest with the use of the fungus only, was estimated to be more beneficial compared to the combined use of entomopathogenic fungi and Last Call FCM or the use of Last Call FCM only (Table 3).

Table 3 Economic analyses of orange yield production based on the respective *Thaumatotibia leucotreta* management options applied at Machakos and Makueni counties Kenya: (1) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device (2) Last Call FCM (3) *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM and (4) control.

Sites/ treatment	Marketable yield (kg/ha) [a]	% yield gain owing to management	Monetary gain (US$/ha) [b]	Cost of treatment application (US$/ha)				Cost-benefit ratio
				Cost of insecticide(s)[c]	Labour cost[d]	Sprayer cost[e]	Total cost	
Machakos								
1	9151 bc	34	3203	87.42	58.37	20	165.79	1:19
2	8122 b	27	2843	165.45	64.95	20	250.40	1:11
3	10881 c	46	3808	212.37	62.74	20	300.61	1:12
4	5944 a	-	-	-	-	-	-	
Makueni								
1	9323 bc	41	3729	87.42	58.37	20	165.79	1:22
2	8300 b	34	3320	165.45	64.95	20	250.40	1:13
3	11192 c	51	4477	212.37	62.74	20	300.61	1:14
4	5459 a	-	-	-	-	-	-	-

a Means within the column followed by the same letter do not differ significantly at P = 0.05 (Tukey's HSD)

b Based on orange farm-gate prices of US$0.35/kg for Machakos and US$0.40/kg for Makueni

c Based on entomopathogenic fungi and Last Call FCM costs in US$

d The labour cost of pesticides application based on wages of US$3.29 per working day (9.50 h).

e Cost of sprayer based on depreciation at US$20.00 for a Green berg Knapsack sprayer over an estimated 5-years life.

The analysis on the relationship between orange fruit yield, moth catches and fruit infestation in Machakos and Makueni showed that orange fruit yield was significantly negatively correlated with the number of *T. leucotreta* moths trapped and fruit infestation by the pest. Furthermore, the number of moths trapped was significantly positively correlated with the fruit infestation.

Table 4 Correlation between fruit yield, *Thaumatotibia leucotreta* moths trapped and fruit infestation in orange orchards at Machakos and Makueni

	Marketable orange fruit yield	*T. leucotreta* moths trapped	*T. leucotreta* orange fruit infestation (%)
Machakos			
Marketable orange fruit yield	-	-0.630	-1.000
		0.028	**< 0.001**
T. leucotreta moths trapped		-	0.650
			0.022
T. leucotreta orange fruit infestation (%)			-
Makueni			
Marketable orange fruit yield	-	-0.68	-1.000
		0.015	**< 0.001**
T. leucotreta moths trapped		-	0.690
			0.013
T. leucotreta orange fruit infestation (%)			-

For each parameter, the top number denotes the Spearman correlation (r_s) and the bottom number, the associated P-value. Significant correlations are indicated by bold values (Spearman correlation: $P < 0.05$).

5.6.5 Discussion

Conidial germination for *M. anisopliae* ICIPE69 exposed to the attractant (pheromone) in the desiccator and an auto-inoculation, device confirmed the compatibility of the fungus with the attractant. The fungi whether exposed to an attractant or not were viable for the duration of the laboratory trial (five days) as well as the field trial (four weeks). Viability did, however, decreased with increasing exposure time. This could be caused by environmental conditions, such as, sunlight, humidity, and temperature (Jaronski 2010). In this study, a negative correlation was found between temperature and conidial germination. The effect of temperature on conidial germination was also studied by Dimbi et al. (2004) who reported 80% conidial germination at 20, 25 and 30 °C of six isolates of *M. anisopliae*, 26 – 67% germination at 35 °C and less than 10% at 15 °C. Mwamburi et al. (2015) reported the optimal temperature for conidial germination of *Beauveria bassiana* (Balsamo) Vuillemin isolates (7320, 7569 and 7771) to be approximately 25 °C, with an upper limit at 30 °C. The decrease in conidial germination with an increase in exposure time was also reported by Maniania (2002) for *M. anisopliae*. Mfuti et al. (2016) also reported a decrease in the viability of *M. anisopliae* ICIPE 69 in traps with and without an attractant within 8 days.

Thaumatotibia leucotreta moths were able to acquire *M. anisopliae* conidia from autoinoculation devices containing an attractant (FCM pheromone) during all four weeks of exposure. No moths released in the screen house where the unbaited device was placed(without the FCM pheromone), acquired any conidia. This confirms the efficacy of the attractant and performance of the autoinoculation device during the exposure period and suggests the potential use of the device in integrated control programs of the pest in citrus orchards and other crops. A high number of conidia acquired by the moth immediately after fungal treatment in the autoinoculation device (week 0) could be attributed to the limited exposure of conidia to environmental factors, such as, wind. Wind could blow away most of the conidia which might be loosely attached to the substrate, but the conidia which are firmly attached may remain over time. The number of conidia acquired by the moths from the devices with conidia exposed for one week can therefore, be similar to those exposed for up to four weeks. The attraction of insects to an autoinoculation device baited with FCM sex pheromone has also been demonstrated for other lepidopterans, such as Diamondback moth,

Plutella xylostella (Linnaeus) (Lepidoptera: Yponomeutidae) (Pell and Macaulay 1993; Furlong et al. 1995). It has also been reported for the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Klein and Lacey 2010) and Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Toledo et al. 2014).

The attractant did also not affect the virulence of *M. anisopliae* ICIPE 69, as proven by the dead and mycosed FCM which were contaminated with the fungus from the autoinoculation device for each week of fungal and attractant exposure. This emphasized how essential the addition of the FCM pheromone is for use of fungi in micro-biocontrol. The decrease in mortality of *T. leucotreta* moths with an increase in the duration of fungal exposure to environmental conditions could be attributed to the decrease in viability as proven in conidial germination tests. This was also reflected in the lethal time to 50% mortality (LT50) in days and the speed of killing (slope) which decreased with an increase in time over weeks of fungal exposure to environmental conditions. The virulence of *M. anisopliae* exposed to field conditions has also been reported for Tsetse flies by Maniania (1998). The mortality due to the fungal infection ranged from 0 to 76 % for *Glossina pallidipes* (Rob-Des.)/*Glossina longipennis* (Westwood) and from 0 to 80% for *Glossina fuscipes* (Newstead) (Maniania, 1998). Navarro-Llopis et al. (2015) reported up to 60% mortality of *C. capitata* after being inoculated with field exposed *M. anisopliae* in a device that also contained an attractant. An added advantage is that the fungus can be mass-produced easily in different media (Latifian et al. 2014; Agale et al. 2018) for use in microbial control. This study proved the compatibility of *M. anisopliae* ICIPE 69 with the FCM attractant (sex pheromone) and can therefore, be used in a suppression program for *T. leucotreta.*

The constant reduction in numbers of *T. leucotreta* moths trapped over time in the areas where the respective management options were applied compared to that in the control orchards, provided evidence for the efficacy of these treatments. The higher numbers of *T. leucotreta* moths recorded in the orchards where the fungus (*M. anisopliae* campaign + dry *M. anisopliae*) were provided in an autoinoculation device, compared to Last Call FCM could be attributed to the slow speed of killing of entomopathogenic fungi (Zimmermann 2007; Vega et al. 2009; Shahid et al. 2012). Last Call FCM contains permethrin (synthetic pyrethroid) which has a quick knockdown effect from the neurotoxin interference of the sodium channels which disrupts the function of neurons ending in paralysis and death of insects (Das and Mukherjee 2003; Drago et al. 2014). Despite the adverse effects of the synthetic pyrethroid, its application in combination with the FCM pheromone (that serves as an attractant) and spot application of this combination as droplets in the orchards, revealed its potential in sustainable pest control (Moore 2012). The pesticide also targets only individual insects by attracting and killing *T. leucotreta* male moths. The low numbers of *T. leucotreta* moths trapped in orchards where both fungus and Last Call FCM were applied, could be the result of the many moths killed by the dry *M. anisopliae* in the autoinoculation device, permethrin in Last Call FCM and infected soil-dwelling stages of the pest due to *Metarhizium anisopliae* campaign applied in the soil. Milner et al. (1997) reported that two entomopathogenic fungal isolates FI-1045, *M. anisopliae* var. anisopliae and FI-147, *M. anisopliae* var. Lepidiotum applied in a sugarcane field, persisted for up to 3.5 years. Two *M. anisopliae* isolates (G 11 3 L6 and FCM Ar 23 B3) were reported by Coombes et al. (2013) to persist in the soil for six months and were able to initiate infection of late fifth instar *T. leucotreta* larvae and subsequent pupae. The lowest number of *T. leucotreta* males trapped was in orchards where treatment combinations of *M.*

anisopliae campaign applied in the soil, dry *M. anisopliae* in an autoinoculation device and Last Call FCM were applied, followed by orchards with applications of Last Call FCM and fungus only.

The highest orange fruit FCM infestation occurred in an orchard where only Last Call FCM was applied compared to orchards where *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device + Last Call FCM and *Metarhizium anisopliae* campaign + *Metarhizium anisopliae* in an autoinoculation device were applied. This is because the Last Call FCM targets only male *T. leoucotetra* moths, leading to more females laying eggs on fruit that cause fruit decay as a result of larval feeding (Mazza et al. 2014). It was expected that *T. leucotreta* females could lay unfertilized eggs due to the attract and killing of the male moths. However, given the polygynic nature of *T. leucotreta* males (Zagatti and Castel 1987), FCM mating could have happened before being attracted and killed by the Last Call FCM. Attract and kill in few/selected orchards may not be effective enough to kill all the male moths and cannot be used as a standalone FCM control tactic (Kirkman and Moore 2007) Although the percentage *T. leucotreta* moths trapped, decreased, a low percentage of fruit was infested in orchards where the fungus was applied when compared to orchards where only Last Call FCM was applied. This could be explained by *M. anisopliae* which killed both male and female moths and the soil applications of *M. anisopliae* campaign which controls the soil-dwelling immature stages. The dry *M. anisopliae* conidia applied in autoinoculation devices are acquired by male moths which could transmit it through horizontal transmission to females. Several studies demonstrated the use of entomopathogenic fungi through autoinoculation techniques for various orders of insects. Examples include Lepidoptera (Furlong and Pell 2001), Blattodea (Quesada-Moraga et al. 2004), Coleoptera (Klein and Lacey 1999) and Diptera (Dimbi et al. 2013; Sookar et al. 2014). The lowest *T. leucotreta* infestation levels were recorded in orchards where the treatment combination *Metarhizium anisopliae* ICIPE 69 in the autoinoculation device + *Metarhizium anisopliae* ICIPE 69 applied as a cover spray around the canopy of trees, were applied, could be attributed to the combined effect of moth and immature control by the respective applications. The benefit of this control method was also evident from the higher estimated yield of marketable fruit per hectare.

5.6.6 Conclusions

Compatibility of dry conidia of *M. anisopliae* ICIPE 69 with the *T. leucotreta* pheromone and persistence of the fungus in the field using an autoinoculation device imply that the package can be used in the integrated control of the pest. High suppression levels of *T. leucotreta* and higher estimated marketable fruit yield in the orchards where Last Call FCM and entomopathogenic fungi were used together, suggest an integration of these control tactics for improvement of the current *T. leucotreta* IPM approach in citrus orchards and other crops. An area-wide, long term application of Last Call FCM is also recommended to suppress male *T. leucotreta* moths. This may lead to a reduction in the number of fertile eggs which will result in fewer larvae which can damage the fruit.

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CHAPTER 6: ARTICLE 4

Bio-ecology of false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), in citrus orchards in Kenya and Tanzania

Prepared for submission to ***Journal of Applied Entomology***

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6.1 Abstract

False codling moth (FCM), *Thaumatotibia leucotreta* is among the key constraints of the horticulture and floriculture industries. Little is known about the population dynamics and genetic diversity of this pest in East Africa. The spatial-temporal population dynamics and genetic diversity of FCM were evaluated in Kenya and Tanzania between May 2017 and August 2018 covering two citrus fruiting seasons. The studies were conducted on citrus orchards in Kilifi, Makueni and Machakos regions in Kenya representing low, mid and high altitudes, respectively, whereas in Tanzania, these altitudes were represented by the Tanga, Morogoro and Mwanza regions respectively. Ten orchards were selected in each region and one delta trap baited with FCM sex pheromone was placed in each orchard. The genetic diversity of FCM sampled in these orchards and from solanaceous hosts, as well as from specimens received from Uganda, Sudan and South Africa were sequenced using the mitochondrial cytochrome oxidase I (mtCOI) gene. A similar spatio-temporal pattern of FCM was found in both Kenya and Tanzania, with the most moths caught in August 2017 and 2018, during the orange fruiting seasons. The lowest FCM catches were recorded in February, during the off-season of orange fruiting. There was no significant difference in the number of moths caught at three altitudes in Kenya. In Tanzania, the number of moths caught at high and mid altitudes did not differ significantly, but it was significantly higher than the number of moths caught at low altitude. A relatively low FCM genetic diversity was recorded at the sites where sampling was done in the respective countries as well as between specimens sampled from different host plants. The same FCM population is therefore present in Africa. A sustainable management strategy is recommended that can be used in both fruit and vegetable cultivation from low to high altitudes of Kenya and Tanzania in all months of the year.

Keywords: FCM, genetic diversity, orange, spatial-temporal, vegetables

6.2 Introduction

Citrus is an internationally important fruit trading commodity (Mazza et al., 2014) produced in both tropical and sub-tropical regions. Orange, *Citrus sinensis* (L) Osbeck is the major citrus crop produced in Kenya and Tanzania. Other citrus species include lime (*Citrus aurantifolia* [Cristm.] Swingle), lemon (*Citrus limon* [L.] Burm. f.), mandarin (*Citrus reticulata* Blanco), and grapefruit (*Citrus paradise* Macfad) (Khamis et al., 2017). Despite the economic importance of orange in Kenya and Tanzania, production of the crop is declining (Kilalo et al., 2009) mainly due to biotic constraints such as diseases and insect pests resulting in loss of fruit yield and quality (Mazza et al., 2014).

False codling moth, *T. leucotreta* (Meyrick) (Lepidoptera Tortricidae) is one of the major pests infesting fruit and vegetables (Erichsen and Schoeman, 1994; Gilligan et al., 2011; Kirkman and Moore, 2007; Malan et al., 2018; Mazza et al., 2014; Venette et al., 2003). The pest is native to Ethiopia and has been reported in many countries within sub-Saharan Africa (Venette et al., 2003). Neonate larvae penetrate the fruit after hatching causing immature fruit to drop and fruit decay after harvesting (Hill and Fullard, 2013; Kirkman and Moore, 2007). Fruit loss of up to 46% for orange and 12% for solanaceous vegetables was reported in Kenya and Tanzania (Mkiga et al., 2019). Odanga et al. (2018) reported losses of more than 25% for avocado fruit produced in Taita hills and Mount Kilimanjaro of Kenya and Tanzania, respectively. Moreover, *T. leucotreta* has been reported in other hosts, up to 24 cultivated and 50 wild plant species (Kirkman and Moore, 2007; Venette et al., 2003) in the different regions of Africa.

Although studies on *T. leucotreta* population dynamics have been conducted in South Africa (Stotter, 2009), very little is known on the spatial and temporal abundance of *T. leucotreta* in orange orchards at different altitudes in Kenya and Tanzania.

In Africa, the genetic diversity of *T. leucotreta* has been studied in South African (Timm et al., 2007) and West African populations (Onah et al., 2016). However, no detailed information is available on the genetic structure of *T. leucotreta* populations in different altitudinal zones of East Africa. Furthermore, previous studies have focused mainly on moths collected with pheromones, while few studies focused on FCM collected from incubated fruit, such as orange, pears (*Pyrus* sp), apples (*Malus domestica* Borkh.), plums (*Prunus salicina* L.) and litchis (*Litchi chinensis* Sonn.) (Onah et al., 2016; Timm, Geertsema and Warnich, 2010). To the best of our knowledge, information on the genetic diversity of the *T. leucotreta* populations infesting solanaceous vegetables is scanty. The

pest was also reported to cause direct losses to African eggplant (*Solanum aethiopicum* L), chili pepper (*Capsicum* spp) and sweet peppers (*Capsicum* spp) (Mkiga et al., 2019) and indirectly through quarantine measures imposed in the East Africa region. For example, there is a self-ban of pepper export to the EU market by Uganda (MAAIF, 2019) leading to market loss of the produce.

Understanding the spatial and temporal population dynamics and effect of solanaceous hosts, especially peppers on the genetic diversity of *T. leucotreta* could provide information for the development of proper control strategies for this pest (Sétamou et al., 2008). According to Deverno et al. (1998) molecular technologies provide ways to study population diversity, as well as to differentiate closely related individuals. The current study therefore aimed at identifying the spatial and temporal abundance of *T. leucotreta* in citrus orchards in Kenya and Tanzania, as well as to determine the genetic structure of *T. leucoterta* populations occurring at different altitudes and on solanaceous host plants in Kenya and Tanzania.

6. 3 Materials and methods

Study sites

Thaumatotibia leucotreta population dynamics were studied and moth samples for molecular studies collected in three citrus-producing areas in Kenya and Tanzania at low (0 – 500 masl), mid (501 – 1200 masl) and high (1201masl and above) altitudes. In Kenya, the studies were conducted in Kilifi (3˚13'6.3" S, 40˚6'54.49" E), Makueni (1˚47'11.38" S, 37˚57'51.99" E) and Machakos (1˚16'1.23" S, 37˚19'12.64" E) representing low, mid and high altitudes, respectively. In Tanzania the studies were carried out in Tanga (5˚5'19.95" S, 39˚6'8.36" E), Morogoro (8˚3'50.31" S, 36˚57'14.79" E) and Mwanza (2˚52'40.81" S, 32˚43'5.3" E) regions representing low, mid and high altitudes, respectively (Figure 1). The rainfall pattern in all study sites except Morogoro is bimodal and occurs between November to December for short rain periods while the long rain periods occur between March and May. Morogoro lies in a transition zone between a monomodal and bimodal rainfall pattern (Mkiga et al., 2019).

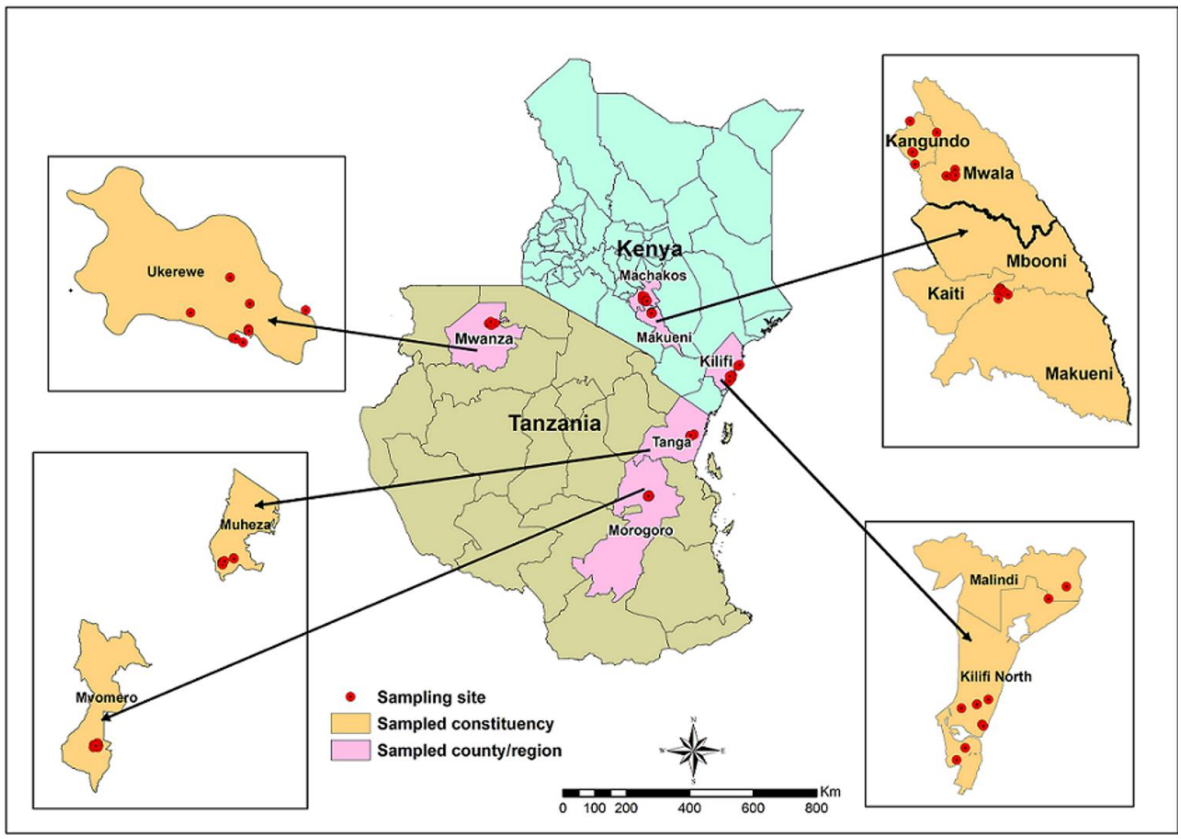

Figure 1. Map of Kenya and Tanzania with sites for *Thaumatotibia leucotreta* population monitoring and moth sampling for molecular studies indicated.

6.3.1 Spatial and temporal abundances of *Thaumatotibia leucotreta* in Kenya and Tanzania

Ten orange orchards with a minimum of 204 trees per orchard were selected at the sites representing a low, mid and high altitude. One delta trap (Kenya Biologics Company), baited with a crytrack lure (E3, Z8, Z11-tetradecatrienyl acetate 0.76mg + E3, Z8-tetradecadienyl acetate 0.04mg) was hung at a height of 2 m from the ground (Odanga et al., 2018) in the middle of each orchard. The orchards were 500 m apart to avoid pheromone interactions. Geographical coordinates were recorded at each trapping point using a Global Positioning System (GPS). Sticky cards in the traps were replaced once a month from May 2017 to August 2019 to trap moths over two orange fruiting seasons. The moths were counted and expressed as a mean number of moths/trap/month. Altitude, time (month) and countries were the sources of variations. Weather data (temperature and relative humidity) were recorded using an Automatic onsets HOBO data logger (Labo-pharma Kenya Ltd).

6.3.2 Molecular analysis of *Thaumatotibia leucotreta* sampled from different regions and host plants

To assess the altitudinal effect on the genetic structure of FCM, moth samples were collected from the delta traps baited with the pheromone, Crytrack®, in citrus orchards representing the three altitudes (Figure 1) during the 2017/18 citrus fruiting season. Moths collected in pheromone traps in Uganda (0°16'17.9"N 32°33'25.63"E), South Africa (33°28'37.0"S 19°39'50.1"E) and Sudan (16°41'33.3"N 33°25'12.6"E) were included for comparison. Samples of FCM infested chili and sweet pepper were also collected to evaluate the genetic diversity of FCM from these solanaceous host plants. The solanaceous fruit sampling and incubation were conducted following the procedure described by Mkiga et al. (2019). The collected infested fruit were washed using a non-caustic liquid dishwashing soap. The fruit were then incubated in ventilated plastic containers (22 x 15 x 8 cm) containing a thin layer of soft sterilized sand for mature larvae to burrow into and pupate. Eclosing adults sampled from each fruit were preserved in 95% ethanol at 4 °C for DNA extraction. Prior to DNA extraction, each insect was surface sterilized in 70% ethanol for 5 minutes followed by three washes in distilled water. DNA was extracted from individual whole insects using the Qiagen DNeasy® Blood and Tissue kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were checked using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific, USA) by measuring optical density at A260 nm and A280nm, respectively. Polymerase chain reaction (PCR) was done to amplify the COI barcode region using the LepF1 5' ATTCAACCAATCATAAAGATATTGG 3'': and LepR1 5' TAAACTTCTGGATGTCCAAAAAATCA 3 (Hajibabaei et al., 2006) primers. PCR was carried out in a total reaction volume of 10 µl containing 5.275 µl PCR$H_2$0, 2 µl of 5× MyTaq reaction buffer (5 mM dNTPs, 25 mM MgCl2, stabilizers, and enhancers) (Bioline), 0.5 µl of each primer, 0.5 µl mM MgCl2 (Thermo Scientific), 0.125 µl MyTaq DNA polymerase (Bioline), and 1 µl of DNA template. These reactions were set up in a Master cycler Nexus gradient (Thermo Scientific) using the following cycling conditions: initial denaturation for 2 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 45 s at annealing temperature of 52 °C and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. The amplified PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining by using KETA GL imaging system trans-illuminator (Wealtec Corp, Meadowvale Way Sparks, Nevada, USA). Amplicons

were purified using the Isolate II PCR and Gel Kit (Bioline, London, UK) following the manufacturer's instructions. The purified samples were sent for bi-directional sequencing to Macrogen Inc Europe Laboratory, the Netherlands.

6.4 Data analysis

Data on *T. leucotreta* spatial and temporal abundances were analysed by means of a logistic linear mixed model with intercept slope using 'glmer' function from lme4 package to test the interaction effect between altitudes and sampling dates. Likelihood ratio test on a GLM (family: negative binomial link: log) was also used to analyse data on overall FCM catches with country (2 levels: Tanzania, Kenya) and altitude (3 levels: high, mid and low) as random factors. There was no significant interaction between country and altitude hence the data were further analysed to test the effect of altitude as the main effect for each country. Pairwise comparison of Least Squares Means (LSMeans; package lsmeans,' function 'lsmeans') of the number of FCM adult catches at the respective altitudes was done with Tukey's HSD ($\alpha = 0.05$). Spearman correlation was used to analyse the association between FCM abundance, temperature and relative humidity. All statistical analyses were performed using R-version (3.5.2) statistical software packages (R Development Core Team, 2016)

Mitochondrion COI sequence reads were checked for quality, assembled and edited using Bioedit 7.2.6 software (Hall, 1999). Homology searches of the obtained mtCOI were done against the non-redundant nucleotide database at the National Center of Biotechnology Information (NCBI) using BLAST (Altschul, Gish, Miller, Myers & Lipman, 1990). Sequence alignments were done using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins, 1997) and phylogenetic reconstruction was done using the Maximum Likelihood method in MEGA X software (Kumar et al., 2018). Support for tree topology was assessed using bootstrap resampling. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms (Gascuel, 1997) to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value (Tamura and Nei, 1993). A sequence of *Thaumatotibia batrachopa* (Meyrick, 1908) (Lepidoptera: Troticidae) retrieved from the GenBank was used as an out-group to root the tree.

6.5 Results

6.5.1 Spartial and temporal abundance of *Thaumatotibia leucotreta* in Kenya and Tanzania

In Kenya, *T. leucotreta* abundance differed significantly between the altitudes (χ^2 = 486.44, df = 2, P < 0.001). The abundance of *T. leucotreta* moth also differed significantly in time between the months (χ^2 = 26047.74, df = 15, P < 0.001). There was also a significant interaction effect of time and altitude on the abundance of the pest (χ^2 = 26047.74, df = 30, P < 0.001) (Figure 2 A).

Similar trends were observed in Tanzania, with *T. leucotreta* moth abundance significantly different between the altitudes (χ^2 = 602.01, df = 2, P < 0.001) and in time (months) (χ^2 = 26879.60, df = 15, P < 0.001). There was also a significant interaction between altitude and time (month) on *T. leucotreta* abundance (χ^2 = 69.88, df = 30, P < 0.001) (Figure 2 B).

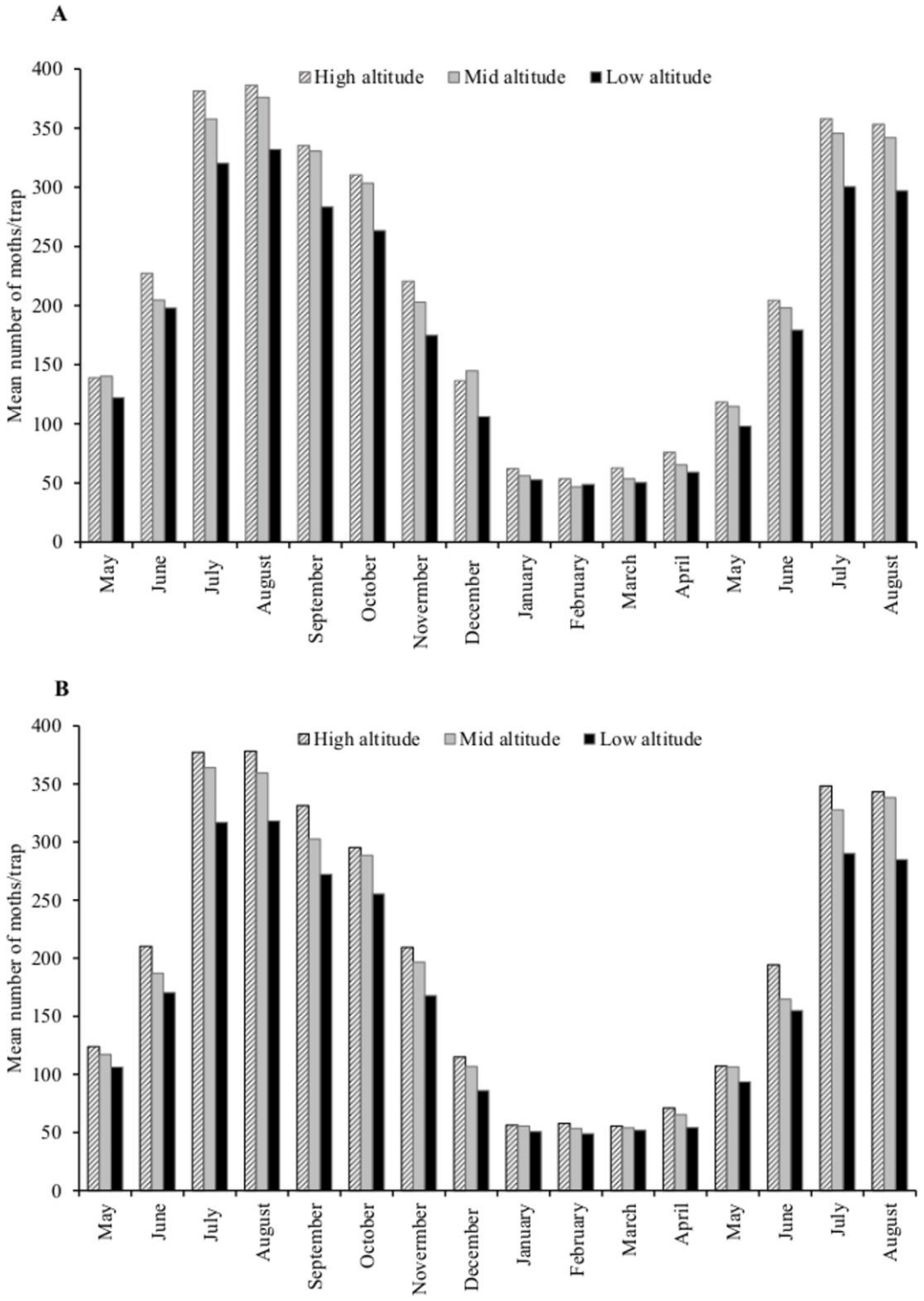

Figure 2: Temporal and altitudinal abundance of *Thaumatotibia leucotreta* in Kenya (A) and Tanzania (B) from May 2017 to August 2018 covering two orange fruiting seasons.

The mean number of moths caught over the total sampling period did not differ significantly between Kenya and Tanzania (χ^2 = 1053.4, df = 1, P = 0.278). However, the mean number of moths caught differed significantly between the altitudes in the countries

(χ^2 = 1042.9, df = 2, P = 0.005). There was no significant interaction effect between country and altitudes (χ^2 = 1042.8, df = 2, P = 0.954). Further analysis showed that, there was a significant main effect of altitude (χ^2 = 520.99, df = 2, P < 0.050) in Tanzania but not in Kenya (χ^2 = 521.83, df = 2, P =1.00) (Figure 3).

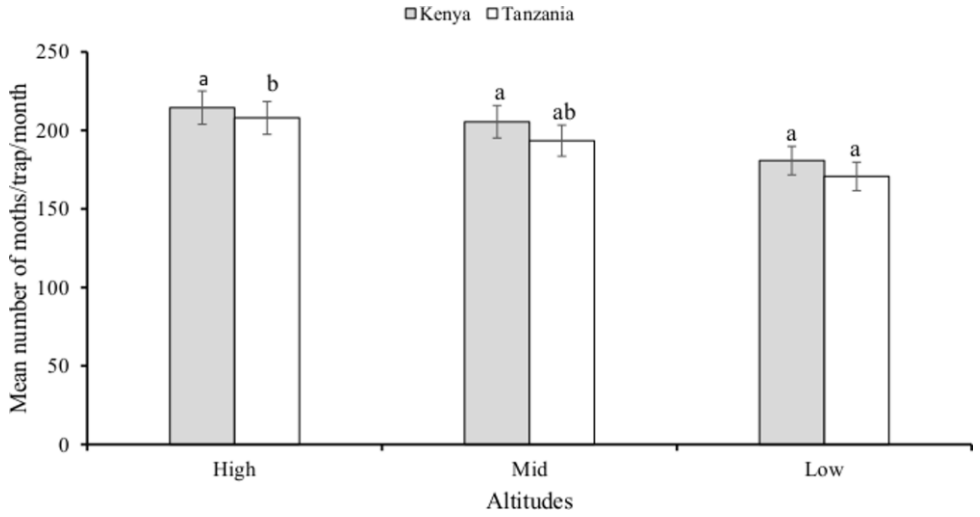

Figure 3: Mean number of *haumatotibia leucotreta* moths caught in pheromone traps at high, mid and low altitude areas of Kenya and Tanzania. Bars for each country, capped with the same letter are not significantly different (α = 0.05, Tukey's HSD).

Temperature and relative humidity (RH) recorded during the study period ranged from 17.59 to 30.12 ºC and from 57.19 to 86.61% RH respectively, for Kenya (Figure 4A). In Tanzania, the temperature ranged from 19.96 to 26.53 ºC and RH from 51.77 to 90.13% (Figure 4B).

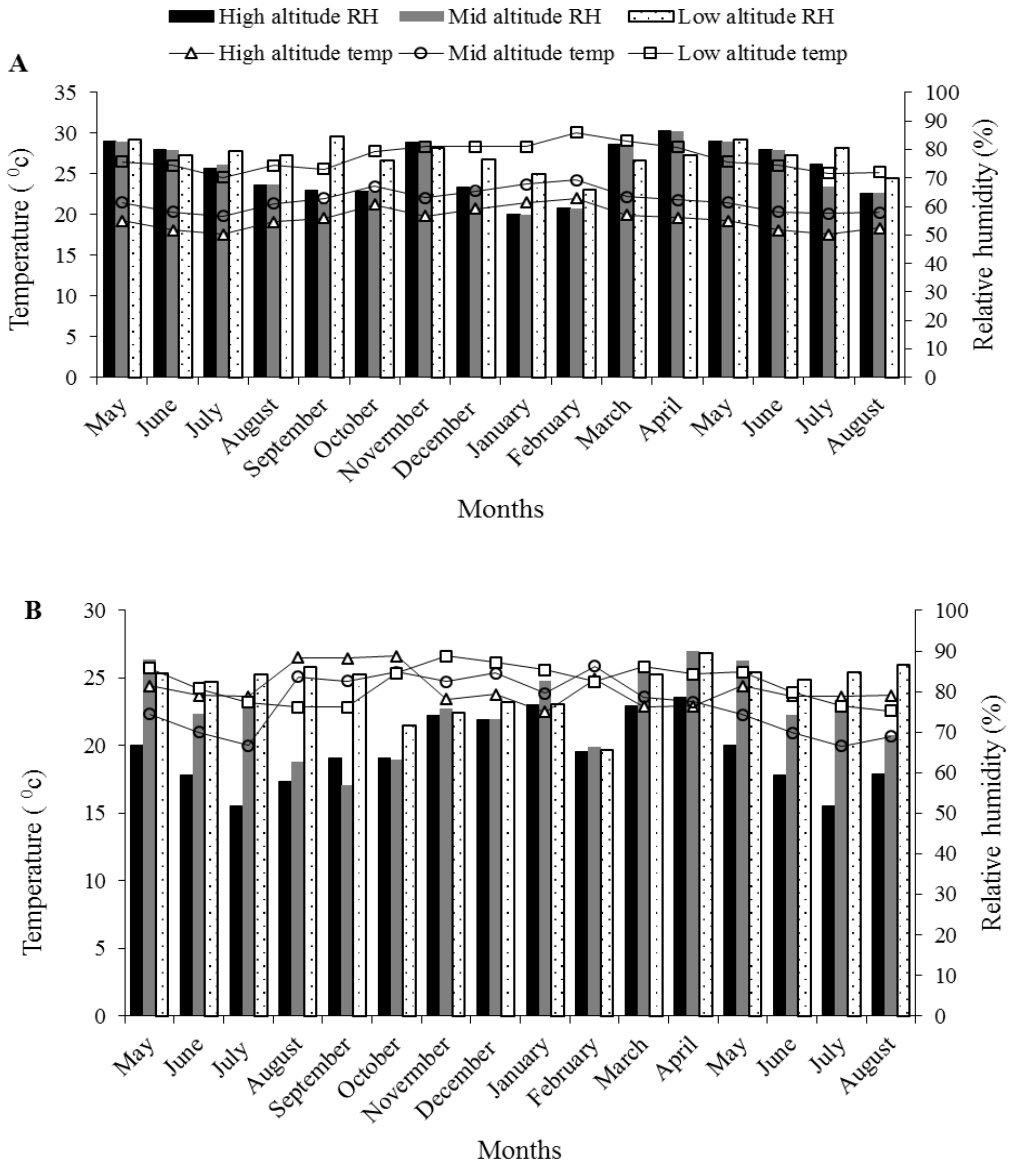

Figure 4. Temperature and relative humidity at different altitudes over time in Kenya (A) and Tanzania (B) from May 2017 to August 2018 covering two orange fruiting seasons.

Abundance, temperature and relative humidity in citrus orchards in Kenya and Tanzania were correlated and are provided in Table 1. The mean abundance was significantly negatively correlated with temperature and relative humidity in both countries. Although the temperature was positively correlated with relative humidity in Kenya, the relationship was not significant. However, in Tanzania, the temperature was significantly negatively correlated with relative humidity.

Table 1. Correlation matrix for *T. leucotreta* abundance, temperature and relative humidity. The top number denotes the Spearman correlation (rs) and the bottom number, the associated P-value.

	FCM abundance	Temperature	Relative humidity
Kenya			
FCM abundance	-	-0.340	-0.14
		< 0.001	**< 0.002**
Temperature		-	0.050
			0.3072
Relative humidity			-
Tanzania			
FCM abundance	-	-0.170	-0.410
		< 0.001	**< 0.001**
Temperature		-	-0.240
			< 0.001
Relative humidity			-

Significant correlations are indicated in bold (Spearman correlation: $\alpha = 0.05$)

6.5.2 Molecular analysis of *Thaumatotibia leucotreta* sampled from different regions and host plants

All sequences from this study clustered closely with those of *T. leucotreta* from the NCBI nucleotide database. Although numerous nucleotide differences were encountered in the majority of the sequences, there was no complete segregation of sequences collected from any country or host plant (Figure 5).

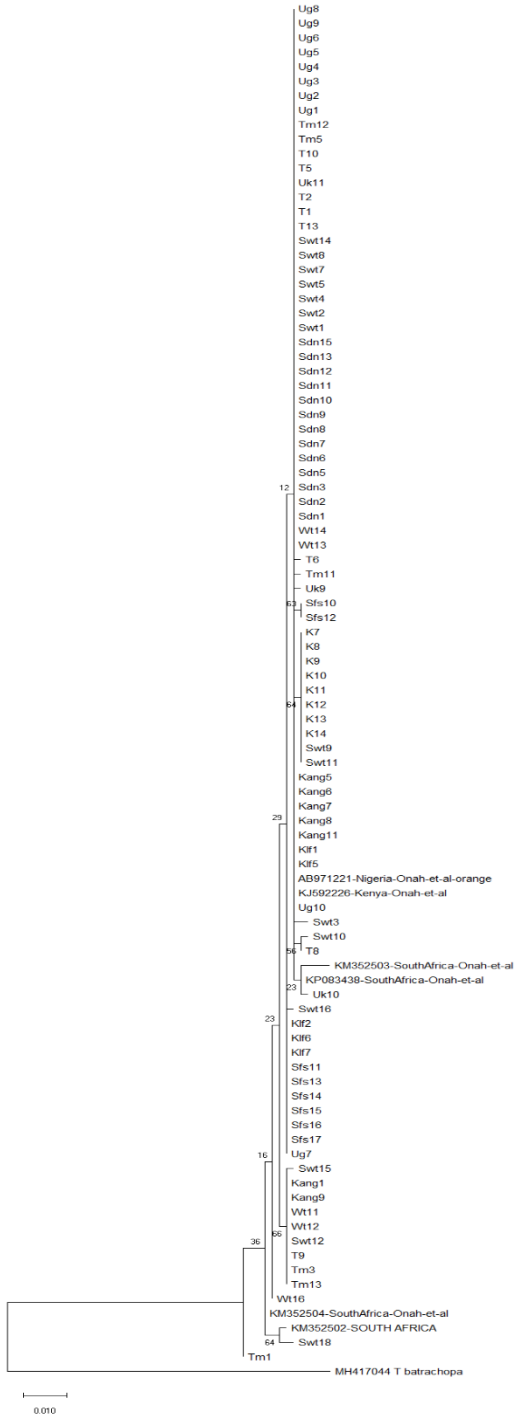

Figure 5: Maximum Likelihood tree of COI gene for *Thaumatotibia leucotreta* showing evolutionary relationships between the moth samples from the study inferred by MEGA X (Kumar, Stecher, Li, Knyaz & Tamura, 2018). K (Kenya) representative samples from chili pepper; Swt (Tanzania) representative samples from sweet pepper; Nigeria AB971221 (representative sample from NCBI for sweet orange); Uk, Tm and T representative samples from high (Ukerewe, Mwanza region), mid (Morogoro region) and low (Tanga region) altitude, respectively, for Tanzania; KANG, Wt, Klf = Representative samples from high (Kangundo, Machakos county) mid (Wote, Makueni county) and low

(Kilifi County) altitude, respectively, for Kenya; Ug (representative sample from Uganda); Sfs (representative sample from the Republic of South Africa); Sdn (representative sample from Sudan); KP083438-SouthAfrica-Onah-et-al, KM352503-SouthAfrica-Onah-et-al, KM352504-SouthAfrica-Onah-et-al and KM352502-SOUTH AFRICA (representative sample from NCBI for South Africa); T batrachopa (MH417044) (*Thaumatotibia batrachopa* sample from NCBI included as an out group).

6.6 Discussion

Knowledge of population dynamics is among the pre-requisites for management planning of insect pests. During this study period, the occurrence of *T. leucotreta* varied between months. High numbers of moths were caught during the orange fruiting season in Kenya and Tanzania. Host plant availability for offspring to develop could be the determining factor since orange is the major host of *T. leucotreta* (Mazza et al., 2014). In the field surveys conducted by Newton (1988), FCM was found to infest orange more than guava, *Psidium guajava* L. fruit in South Africa. Mkiga et al. (2019) reported higher ovipositional preferences and better *T. leucotreta* offspring performance on orange compared to solanaceous vegetable fruit. *Thaumatotibia leucotreta* infestation levels are also higher on orange than on vegetables under field conditions (Mkiga et al., 2019). The abundance of *T. leucotreta* was similar at all altitudes in Kenya and also at the high and mid-altitude areas in Tanzania. This could be explained by the pest's tolerance for a wider range of ambient temperatures, from low to high. Stotter & Terblanche (2009) reported survival of 50% *T. leucotreta* moths after a two-hour exposure to −4.5 °C, while Boardman et al. (2012) found 50% of larvae exposed to −11.5 ± 0.3 °C for two hours, to survive. The lower *T. leucotreta* abundance at low altitude in Tanzania may be attributed to the difference in orange cultivars grown in the country. Matombo sweet is the dominant orange cultivars in the high and mid attitudes and Valencia is the dominant cultivar in the low altitude areas of the country. Valencia orange fruit are less infested by *T. leucotreta* than navel varieties (Newton, 1988) and may, therefore, explain the low *T. leucotreta* occurrence in this area.

Thaumatotibia leucotreta abundance was negatively correlated with temperature and relative humidity during the study period in Kenya and Tanzania. The study sites were at an altitude ranging between 0 – 1500 masl and a temperature range of 17.59 to 30.12 °C in the citrus orchards. According to Daiber (1980) 25 °C is the optimal temperature for *T. leucotreta* reproduction in terms of female fecundity. Temperature and relative humidity were positively correlated in Kenya while in Tanzania these parameters were negatively correlated. This difference in correlation could be attributed to topographical features.

These features include water-bodies and mountains present in the areas where the study sites were located (Richardson et al., 2003; Seidel et al., 2007).

For example, most of the study sites were situated near the Uluguru Mountains in Morogoro Tanzania, the Indian Ocean in Tanga and Kilifi in Tanzania and Kenya, respectively, as well as Lake Victoria in Mwanza, Tanzania. *Thaumatotibia leucotreta* is generally spatially distributed in low, mid and high altitudes in Kenya and Tanzania. The pest is also abundant in all months of the year with a peak in population during the fruiting seasons for rainfed oranges in the countries. This implies that control of the pest should start early in the season during fruit set.

Understanding the classification of a pest is among the key factors for management strategies and phytosanitary measures (Khamis et al., 2017; Timm et al., 2010). Molecular characterization was done from *T. leucotreta* specimens sampled at different altitudes in Kenya and Tanzania as well as from selected countries in Africa and from infested solanaceous vegetables. DNA barcoding, a commonly used method for species delineation (Ratnasingham and Hebert, 2007) was used. No variability occurred between the *T. leucotreta* populations from different altitudes in Kenya and Tanzania as well as those from other countries. This could possibly be explained by the nativeness of *T. leucotreta* to Africa (Venette et al., 2003). It is in agreement with the reported genetic homogeneity among population of *T. leucotreta* sampled from different zones of Nigeria by Onah et al. (2016). Low genetic diversity among *T. leucotreta* specimens sampled from infested solanaceous host plants compared to those from infested sweet orange obtained from GenBank, was also found. Timm et al. (2010) also reported a similar population genetic structure of *T. leucotreta* sampled from *C. scinensis*, *Pyrus* sp, *M. domestica*, *P. salicina* and *L. chinensis*. Cognisance should be taken of the low genetic diversity among different populations of the Afro-tropical species, *T. leucotreta*. The same management strategy can therefore be used across Africa for both fruit and vegetable crops.

6.7 References

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CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

The false codling moth (FCM), *Thaumatotibia leucotreta* is amongst the constraints of the horticulture industry. The pest is native to Ethiopia (Kirkman and Moore, 2007) and present in many countries in Africa. It is also an economically important pest in East Africa and causes direct losses through immature fruit drop and harvestable fruit decay (Kirkman and Moore, 2007; Mazza *et al.*, 2014). Extensive indirect losses are also incurred due to quarantine measures (Mazza *et al.*, 2014). Detection of even a single larva of the pest in a batch can result in rejection of the whole consignment (Moore, 2012). Injuriousness of the pest to crops results in the intensive use of synthetic insecticides for its control in East Africa. Many growers prefer chemical pesticides for the management of pests because they are effective, easily accessible and perform consistently (Ruberson *et al.*, 1998). However, the development of pesticide resistance through continued use of synthetic pesticides has been reported (Hofmeyr, 1998; Pérez *et al.*, 2000; Roush and Tabashnik, 2012: Khan *et al.*, 2015).

Little is known on the biology, ecology and sustainable control of *T. leucotreta* in East African citrus-producing areas. The aims of the study were to 1) determine the field and laboratory performance of *T. leucotreta* on orange and selected vegetables, 2) determine the pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against *T. leucotreta*, their horizontal transmission between adults and effects on reproductive potential, 3) develop an Integrated Pest Management (IPM) approach for control of *T. leucotreta* (Lepidoptera: Tortricidae) in citrus orchards in Kenya and to 4) determine *T. leucotreta* population dynamics and genetic diversity of *T. leucotreta* specimens collected from Kenya and Tanzania from orange and vegetables. This study was successful in achieving these aims.

Field surveys were carried out to assess the damage caused by *T. leucotreta* on orange and selected vegetables in low (0 – 500 masl), mid (500 – 1000 m asl) and high (1200 m asl and above) altitudes of Kenya and Tanzania. High fruit infestation was recorded in the mid and high altitudes of Kenya. Orange fruit were sampled on the ground around the canopy of citrus trees, as well as from the trees. At a harvestable stage, higher *T. leucotreta* larval incidence was recorded in the fruit sampled from trees compared to fruit sampled from the ground. The incidence of *T. leucotreta* at the harvestable stage of orange

fruit has implications on post-harvest losses through fruit decay, as well as indirect loss due to quarantine measures. *Thaumatotibia leucotreta* prefers both orange and solanaceous vegetables for oviposition and the hosts are suitable for the development of this pest. The possible reason could be the phytophagous nature of the pest which has been reported to infest approximately 24 cultivated and 50 wild plant species (Venette *et al.*, 2003: Kirkman and Moore, 2007). Fruit infestation, oviposition preference and offspring performance recorded on both orange and solanaceous vegetables proved that control strategies of the pest should also include vegetables being planted near or in mixed cropping with orange trees.

All the entomopathogenic fungi evaluated for control of *T. leucotreta,* which included 17 *Metarhizium anisopliae* and five *Beauveria bassiana* isolates, were pathogenic to *T. leucotreta* moths. The virulence of isolates varied between the fungus species. The moths were also able to transmit the conidia of the potent *M. anisopliae* ICIPE 69 isolate between male and female moths through horizontal transmission. The horizontal transmission of the fungus caused a sub-lethal effect on the reproductive potential of the infected *T. leucotreta* females which consequently laid significantly fewer eggs compared to uninfected moths. These results are in agreement with other studies where variation in pathogenicity between the isolates of the same fungal species, as well as between fungal species were reported on different life stages of insects. For example, Coombes *et al*. (2013) reported variation in virulence of two *M. anisolpliae* fungal isolates as well as a *B. bassiana* isolate on last instar larvae and pupae of *T. leucotreta.* Differences in virulence by *Beauveria bassiana* isolates ICIPE 725, ICIPE 284 and USDA 2729, as well as *M. anisopliae* isolates ICIPE 18, ICIPE 69 and ICIPE 30, to *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae) were reported by Furlong and Pell (2001). Quesada-Moraga *et al.* (2006) also reported differences in virulence of *B. bassina* EABb 04/01-Tip, EABb 01/33-Su, 01/110-Su, Bb-1333 and *M. anisopliae* EAMa 01/58-Su isolate against pupae and adults of *Ceratitis capitata* (Weidemann) (Diptera: Tephritidae). The ability of a fungus to infest different developmental stages and species of insects positively impacts on the persistence of the inoculum in an ecosystem, as well as sustainable control of the pest. The potential of EPF for controlling agricultural pests has been reported (Mfuti *et al.*, 2016; Opisa *et al.*, 2018). The reduction in fecundity of fungal infected moths recorded in this study provides more evidence of the potential of entomopathogenic fungi for the control of *T. leucotreta* which can be included in IPM strategies of this pest. Several other studies have also reported a reduction in oviposition of fungal infected insects.

Oviposition by the German cockroach, *Blatella germanica* (L.) (Blattodea: Blattellidae), reduced to between 58.4 and 72.1% after being infested by *M. anisopliae* strain EAMa 01/121-Su (Quesada-Moraga *et al.*, 2004). Sookar *et al.* (2014) also reported low egg-laying by *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) after being infected with *M. anisopliae.*

Studies on the compatibility of *M. anisopliae* ICIPE 69 with the FCM sex pheromone showed that the fungus remained virulent to *T. leucotreta* after being exposed to the pheromone in an autoinoculation device for up to four weeks. Therefore, the fungus and pheromone can be used together in a control strategy for the pest. The moths were also able to acquire the spores in the autoinoculation device. The dry fungal spores can, therefore, be used together with the pheromone in an autodissemination device for the control of *T. leucotreta* in citrus orchards, as well as in other crops including solanaceous vegetables. The effective use of pheromone traps for the dissemination of *Zoophthora radicans* Brefeld (Zygomycetes: Entomophthorales) has been reported by Pell *et al.* (1993) for the control of Diamondback moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). Klein and Lacey (1999) provided evidence for successful autodissemination of *M. anisopliae* with an attractant trap for the control of the Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae). An autodissemination device containing *B. bassiana* for the control of *C. capitata* was documented in Toledo *et al*. (2017).

Different IPM packages for control of *T. leucotreta* were evaluated at mid-altitude in Kenya because more *T. leucotreta* infested orange orchards, as well as higher infestation rates were recorded at these localities. The most effective IPM package for control of *T. leucotreta* in citrus orchards was determined as the entomopathogenic fungus, *M. anisopliae* ICIPE 69 combined in an autoinoculation device with the FCM sex pheromone and integrated with *M. anisopliae* campaign and Last Call FCM. This combination of treatments significantly reduced *T. leucotreta,* as well as fruit infestation in citrus orchards when compared with untreated orchards. More marketable oranges were produced in orchards where this combination of treatments was applied, compared with orchards where fungus only or Last Call FCM only was applied. The use of this integrated control approach against *T. leucotreta* targeted all stages of the pest. This is in contrast to the use of only dry conidia of ICIPE 69 in an autoinoculation device that targets the adult males of the pest only, which can transmit the inoculum to females during mating. *Metarhizium anisopliae* campaign targeted soil-dwelling stages of the pest while the Last Call FCM is

an attract and kill technology targeting adult males. Good control of the fruit fly, *B. dorsalis,* was also reported in mango orchards where IPM was applied resulting in lower infestation rates and fewer larvae per fruit compared to the untreated control (Verghese *et al.,* 2004). The combined use of different control measures compensates for the shortcomings of a single tactic on suppression of the pest. For example, the use of Last call FCM "Attract and kill control tactic" is effective only when FCM pressure is low (Kirkman and Moore, 2007).

The abundance of *T. leucotreta* in citrus orchards was similar in Kenya and Tanzania, as well as between altitudes. Studies associated with the spatial and temporal population dynamics of *T. leucotreta* on citrus orchards showed a similar occurrence of the pest between the countries and altitudes of the countries. There was also low genetic diversity in the *T. leucotreta* populations sampled from the different regions and host plants. The proposed IPM package can therefore be used in different regions and with different host plants for the suppression of *T. leucotreta*. The similarity in *T. leucotreta* populations at different altitudes has also been reported by Odanga *et al.* (2018), with no variation in pest abundance across elevations for both Taita Hill and Mount Kilimanjaro in avocado orchards in Kenya and Tanzania, respectively. The low genetic diversity between the *T. leucotreta* populations of the two regions and amongst the host plants recorded in this study is an added advantage for the use of common control tactics such as the IPM package demonstrated above. Similar genetic structures of different *T. leucotreta* populations from different zones were reported by Onah *et al.*(2016). There were also no differences in genetic structures for *T. leucotreta* sampled from sweet orange, *Citrus sinensis* (L) Osbeck, pear, *Pyrus* sp, apple, *Malus* × *domestica* Borkh., plum, *Prunus salicina* Lindell and litich, *Litchi chinensis* Sonn (Timm *et al.,* 2010).

7.2 Conclusions

- There are higher FCM infestations on orange in high and mid compared to low altitudes of Kenya and Tanzania. Higher larval incidence was recorded in oranges still on the trees than in fruit that was found on the ground at the ripe/harvestable stage.
- Okra, African eggplant, chili and sweet peppers act as reservoirs for *T. leucotreta* when planted near or intercropped with oranges.
- Orange is strongly preferred for oviposition and more suitable for *T. leucotreta* larval development and survival than African eggplant, chili, and sweet peppers. Amongst the solanaceous vegetables, sweet pepper was more preferred for

oviposition than chili pepper and African eggplant, but it is less suitable for larval survival.

- Dry conidia of entomopathogenic fungi *M. anisopliae* and *B. bassiana* are pathogenic to *T. leucotreta* moths. The *M. anisopliae* ICIPE 69 isolate was the most virulent with a low lethal time to 50% mortality (LT_{50}).
- Both male and female *T. leucotreta* were able to acquire dry *M. anisopliae* ICIPE 69 conidia from a treated substrate and were able to transmit the inoculum between each other during mating. Female *T. leucotreta* moths which were horizontally infected with *M. anisopliae* ICIPE 69 laid significantly fewer eggs compared to uninfected females.
- *Thaumatotibia leucotreta* numbers were suppressed by an IPM strategy which consisted of dry *Metarhizium anisopliae* ICIPE 69 presented with the pheromone in an autoinoculation device, *Metarhizium anisopliae* campaign and Last Call FCM and resulted in more marketable orange fruit.
- There was a low variation in *T. leucotreta* population from different altitudes in Kenya and Tanzania. There is also low *T. leucotreta* genetic diversity between specimens sampled from different altitudes of Kenya and Tanzania, from Uganda, Sudan and South Africa, as well as between specimens from incubated orange, chili, and sweet peppers.

7.3 Recommendations

- Orange fruit should be checked for FCM infestation during harvesting and packing. Gently shaking of branches allows for infested fruit to drop. This practice is recommended.
- Control strategies of *T. leucotreta* in citrus orchards should also include okra, African eggplant, chili and sweet peppers grown near or within orange orchards.
- The strong preference of *T. leucotreta* for chili and sweet peppers for oviposition and relatively higher mortality of the larvae on these crops should be investigated further for suppression of this pest in field trials. Although there is currently a very potent male moth attractant commercially available, further research to identify semiochemicals that can attract female *T. leucotreta* moths for both monitoring and suppression of the pest is recommended.
- Training on integrated management of FCM in citrus and other crops across Africa is recommended.

- The control strategy which includes dry *M. anisopliae* ICIPE 69 presented with the pheromone in an autoinoculation device, *Metarhizium anisopliae* campaign and Last Call FCM for control of *T. leucotreta* should be used across the regions/countries regardless of the host plant since there is low genetic diversity among the populations of the pest.

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APPENDICES

APPENDIX A

NWU Havard Reference styleof the faculty of Law APA. Library Services, Potchefstroom Campus.

Text references

A text reference consists of the author's surname, the date and page number(s) of the original source of information. A complete reference must appear in the reference list. Text references can be made in either one of two ways:

As part of the sentence:

According to Salatin (2009:195) the most unnatural characteristic of the food industry is the notion that all food items should be available everywhere at all times.

At the end of the sentence:

Text:

The notion that all food items should be available everywhere at all times, is the most unnatural characteristic of the food industry (Salatin, 2009:195).

Direct quotations

Direct quotations should be used functionally. Do not use quotations in order to avoid difficulties with translation.

If the quotation forms part of a comprehensive argument, the punctuation is part of that argument and is placed outside the brackets or inverted commas.

Visser (1983:12) contends that "there must be a conclusion for all sentences".

They pass the remark that "few such cases exist" (De Beer, 1986:3).

When a sentence between brackets or inverted commas starts with a capital letter, it indicates that this sentence is used independently and therefore requires a full stop inside the bracket or inverted comma.

It was anciently believed that a baby born with teeth already formed would 'bite the world' and was made for villainy." (Smith, 1977:70). Visser (1983:12) asserts: "There must be a conclusion for all sentences."

Indirect quotations / paraphrasing

When you give an author's ideas in your own words, you have to take care that the spirit and argumentation of the original are retained. Do not use and twist a citation to your own purposes. Also, vary the style of citation in your argument.

By comparing the view of Jones (1986:15) with that of Smith (1994:147), it … This argument is supported by Cogan (1989:156).

According to King (1995:21) it is … Mayer (1990:10) maintains that …

It is preferable to place the reference at the end of the sentence if the author is an institution.

Secondary sources

A secondary source is, for example, when an author writes about Freud's view of psychoanalysis without reading Freud's own work. Making use of the work of other authors like Smith, Jones or White who wrote about Freud, asks for a secondary citation. It is, however, possible that these authors interpreted Freud incorrectly, causing you to work unscientifically and unethically. Keep secondary citations therefore to the minimum as it is "second-hand" information.

If you are forced to use secondary information because the primary source is unavailable or written in a foreign language, it can be cited in the text as follows:

Text:

According to Freud (cited by Williams, 1996:10), dreams are …

Reference list:

Williams, L. 1996. Freud's theories about dreams. London: Batsford.

Only sources you have handled are indicated in the source list. In this case it is Williams.

Text references to more than one source

When referring to more than one source when a statement is made, arrange the references alphabetically according to the first author, in the same order they will appear in the reference list. Use a semi-colon after each reference.

A recession is expected (Crade, 1995:89; Leeds & Smith, 1996:8; Vance, 1994:6).

Authors

The author is the person (or institution) responsible for the intellectual content of a source.

An author can also be an institution, for example in the case of a yearbook of a university or a report by a state department.

One author

In the text, give the author's surname, date of publication and page number.

Text:

South Africans feel they have been cut off from their past and that their identity is that of fragmentation and substitution (Duvenhage, 2009:23).

Reference list:

Duvenhage, P. 2009. The ambivalent face of globalization. Discourse: unforeseen consequences of globalization, 37(1):18-23.

Two authors

Text:

Gardner and Shelton (1967:40) refer to the problem of communication with paralysed patients who also suffer …

Text:

All procedures must be explained to the patient (Gardner & Shelton, 1967:74).

Note the difference between the two examples in the text: & is used in the brackets but and is used in the full sentence.

Reference list:

Gardner, E.K. & Shelton, B. 1967. The intensive therapy unit and the nurse. London: Faber and Faber

More than two authors

When a source has three or more authors the first author is mentioned in the text, followed by et al. in italics (note the full stop only at the end as it is an abbreviation for et alii, meaning and others). This is followed by the date and page number. Mention all the authors or contributors in the source list.

Text:

According to Meyer et al. (1973:74) photosynthesis is …

Reference list:

Meyer, B.S., Anderson, D.P., Bohning, R.H. & Fratanna, D.G. 1973. Introduction to plant physiology. New York: Van Nostrand.

Exception: when two different sources by the same first author and year are used and they look similar in the text, give the second author (or more authors if necessary to ensure distinction) followed by et al.

Bruning, McGrew & Cooper, 2006, as well as Bruning, DeMiglio & Embry, 2006, will both abbreviate to Bruning et al., 2006.

Text:

(Bruning, McGrew et al., 2006:26) and (Bruning, DeMiglio et al., 2006:35)

Reference list:

Bruning, S.D., DeMiglio, P.A. & Embry, K. 2006. Mutual benefits as outcome indicator: factors influencing perception of benefit in organization-public relationships. Public relations review, 32(1):33-40.

Bruning, S.D., McGrew, S. & Cooper, M. 2006. Town-gown relationships: exploring university- community engagement from the perspective of community members. Public relations review, 32(2):125-130.

More than one reference to the same author(s) in the same year

When using more than one publication by the same author(s) published in the same year, distinguish it by adding a, b or c after the date in the text, as well as in the reference list.

Text:

Packaging serves a dual role from a logistics perspective (Pienaar, 2010a:230).

Text:

Because of their fixed right of way, access to pipelines is limited (Pienaar, 2010b:109).

Reference list:

Pienaar, W.J. 2010a. Logistics aspects of petroleum pipeline operations. Journal of transport and supply chain management, 4(1):224-242.

Pienaar, W.J. 2010b. Efficiency and effectiveness aspects of petroleum pipeline operations: pipes, pumps and valves. IMIESA, 35(10):105-106, 109, 111.

Text:

Packaging serves a dual role from a logistics perspective (Pienaar, 2010a:230).

Text:

Because of their fixed right of way, access to pipelines is limited (Pienaar, 2010b:109).

Reference list:

Pienaar, W.J. 2010a. Logistics aspects of petroleum pipeline operations. Journal of transport and supply chain management, 4(1):224-242.

Pienaar, W.J. 2010b. Efficiency and effectiveness aspects of petroleum pipeline operations: pipes, pumps and valves. IMIESA, 35(10):105-106, 109, 111.

THE REFERENCE LIST

General principles

- All sources referred to in the text must be included in one alphabetical list (according to first authors).
- Use the term "reference list" to refer to sources which were directly used. A "bibliography" refers to a more substantial list covering the subject.
- Complete bibliographical information for every source is essential so that the source can be traced easily.
- The language of the source and not that of the document (research paper or thesis), is used in the reference list.
- Start the reference list on a new page.
- Do not number entries or use "bullets" in the reference list.
- Use 1.5 spacing and leave open a line between entries or use 18 pt paragraph spacing after each entry.
- The reference list must be left aligned and not justified ("justify" creates block format and this leaves unnecessary open spaces especially when typing internet addresses).
- Leave two spaces after each element of the entry. Compare the following examples where ** represents two spaces.

Book:

Bester, H.**2006.**How to cope with AD/HD: a South African guide for parents, teachers & therapists.**Cape Town: Human & Rousseau.

Journal:

Malan, C.W.**1998.**Development communication as part of culture.**Communicare,

17(1):49-78.

Alphabetical arrangement

Arrange the entries in alphabetical order according to the first author. Remember the principle of “nothing before something”. A surname such as Le Roux is placed before Leaky.

Arrange sources by the same author as follows:

• Chronologically from old to new

• Sources with one author

• Sources with co-authors

Compare the entry for Deci, E.L. in the example of a reference list in this guide.

Arrange works by different authors with the same surname alphabetically according to the first initial. Surnames starting with “Mc” or “Mac” are arranged alphabetically according to the word.

Mac Lean, W.

Mc Donald, B.

McArthur, K.

APPENDIX B

Instructions to authors-*insects* (MDPI**)**

insects

IMPACT FACTOR 2.139

Instructions for Authors

Shortcuts

- **Manuscript Submission Overview**
- **Manuscript Preparation**
- **Preparing Figures, Schemes and Tables**
- **Supplementary Materials, Data Deposit and Software Source Code**
- **Research and Publication Ethics**
- **Reviewer Suggestions**
- **(/) English**
- **Corrections**
- **Preprints and**
- **Conference**
- **Papers**

Authorship

Editorial Procedures and Peer-Review

Clinical Trials Registration

SUBMISSION CHECKLIST

Please.

1. read the **Aims & Scope (/journal/insects/about)** to gain an overview and assess if your manuscript is suitable for this journal;
2. use the **Microsoft Word template (/files/word-templates/insects-template.dot)** or **LaTeX template (https://www.mdpi.com/authors/latex)** to prepare your manuscript;
3. make sure that issues about **publication ethics**, **research ethics**, **copyright (https://www.mdpi.com/authors/rights)**, **authorship**, **figure formats**, **data** and **references format** have been appropriately considered;
4. ensure that all authors have approved the content of the submitted manuscript.

MANUSCRIPT SUBMISSION OVERVIEW

Types of Publications

Insects has no restrictions on the length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *Insects* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

Manuscripts submitted to *Insects* should neither been published before nor be under consideration for publication in another journal. The main article types are as follows:

- *Articles:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although Short *Communications* of preliminary, but significant, results will be considered. Quality and impact of the study will be considered during peer review.

- *Reviews:* These provide concise and precise updates on the latest progress made in a given area of research. Systematic reviews should follow the PRISMA **guidelines. (https://www.mdpi.com/editorial_process#standards)**

Accepted File Formats

Authors must use the **Microsoft Word template (../../../files/word-templates/insects-template.dot)** or **LaTeX template (https://www.mdpi.com/authors/latex)** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the editorial office insects@mdpi.com. Accepted file formats are:

- *Microsoft Word:* Manuscripts prepared in Microsoft Word must be converted into a single file before submission. When preparing manuscripts in Microsoft Word, the ***Insects*** **Microsoft Word template file (https://www.mdpi.com/files/word-templates/insects-template.dot)** must be used. Please insert your graphics (schemes, figures, *etc.*) in the main text after the paragraph of its first citation.

- *LaTeX:* Manuscripts prepared in LaTeX must be collated into one ZIP folder (include all source files and images, so that the Editorial Office can recompile the submitted PDF). When preparing manuscripts in LaTeX, please use the ***Insects*** **LaTeX template files (https://www.mdpi.com/authors/latex)**. You can now also use the online application **writeLaTeX (https://www.writelatex.com)** to submit articles directly to *Insects*. The MDPI LaTeX template file should be selected from the **writeLaTeX template gallery (https://www.writelatex.com/templates/mdpi-article-template/fvjngfxymnbr)**.

- *Supplementary files:* May be any format, but it is recommended that you use common, nonproprietary formats where possible (see **below** for further details).

Cover Letter

A cover letter must be included with each manuscript submission. It should be concise and explain why the content of the paper is significant, placing the findings in the context of existing work and why it fits the scope of the journal. Confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. Any prior submissions of the manuscript to MDPI journals must be acknowledged. The names of proposed and excluded reviewers should be provided in the submission system, not in the cover letter.

Note for Authors Funded by the National Institutes of Health (NIH)

This journal automatically deposits papers to PubMed Central after publication of an issue. Authors do not need to separately submit their papers through the NIH Manuscript Submission System (NIHMS, **http://nihms.nih.gov/ (https://www.nihms.nih.gov/)**).

Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
  - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords
  - **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
  - **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.

- **Review manuscripts** should comprise the **front matter**, literature review sections and the **back matter**. The template file can also be used to prepare the front and back matter of your review manuscript. It is not necessary to follow the remaining structure. Structured reviews and metaanalyses should use the same structure as research articles and ensure they conform to the **PRISMA (https://www.mdpi.com/editorial_process)** guidelines.

- **Graphical abstract:** Authors are encouraged to provide a graphical abstract as a self-explanatory image to appear alongside with the text abstract in the Table of Contents. Figures should be a high quality image in any common image format. Note that images displayed online will be up to 11 by 9 cm on screen and the figure should be clear at this size.

- **Abbreviations** should be defined in parentheses the first time they appear in the abstract, main text, and in figure or table captions and used consistently thereafter.

- **SI Units** (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible

- **Accession numbers** of RNA, DNA and protein sequences used in the manuscript should be provided in the Materials and Methods section. Also see the section on **Deposition of Sequences and of Expression Data**.

Equations: If you are using Word, please use either the Microsoft Equation Editor or the MathType add-on. Equations should be editable by the editorial office and not appear in a picture format.

- **Research Data and supplementary materials:** Note that publication of your manuscript implies that you must make all materials, data, and protocols associated with the publication available to readers. Disclose at the submission stage any restrictions on the availability of materials or information. Read the information about **Supplementary Materials** and Data Deposit for additional guidelines.

- **Preregistration:** Where authors have preregistered studies or analysis plans, links to the preregistration must be provided in the manuscript. **Guidelines and standards:** MDPI follows standards and guidelines for certain types of research. See **https://www.mdpi.com/editorial_process**

(https://www.mdpi.com/editorial_process#standards) for further information.

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.
- **Author List and Affiliations:** Authors' full first and last names must be provided. The initials of any middle names can be added. The PubMed/MEDLINE standard format is used for affiliations: complete address information including city, zip code, state/province, and country. At least one author should be designated as corresponding author, and his or her email address and other details should be included at the end of the affiliation section. Please read the **criteria to qualify for authorship**.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings: 1) Background: Place the question addressed in a broad context and highlight the purpose of the study; 2) Methods: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. 3) Results: Summarize the article's main findings; and 4) Conclusion: Indicate the main conclusions or interpretations. The abstract should be an objective representation of the article: it must not contain results which are not presented and substantiated in the main text and should not exaggerate the main conclusions.
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Research Manuscript Sections

- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested. The current state of the research field should be reviewed carefully and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the main conclusions. Keep the introduction comprehensible to scientists working outside the topic of the paper.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while wellestablished methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available. Include any preregistration codes.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their

implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned. This section may be combined with Results.

- **Conclusions:** This section is mandatory, and should provide readers with a brief summary of the main achievements/results of your work.
- **Patents:** This section is not mandatory, but may be added if there are patents resulting from the work reported in this manuscript.

[Return to top]

Back Matter

- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed. Clearly indicate grants that you have received in support of your research work and if you received funds to cover publication costs. Note that some funders will not refund article processing charges (APC) if the funder and grant number are not clearly and correctly identified in the paper. Funding information can be entered separately into the submission system by the authors during submission of their manuscript. Such funding information, if available, will be deposited to **FundRef (https://www.crossref.org/fundref/)** if the manuscript is finally published.
- **Author Contributions:** Each author is expected to have made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work; or have drafted the work or substantively revised it; AND has approved the submitted version (and version substantially edited by journal staff that involves the author's contribution to the study); AND agrees to be personally accountable for the author's own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

  For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, X.X. and Y.Y.;
  Methodology, X.X.; Software, X.X.; Validation, X.X., Y.Y. and Z.Z.; Formal Analysis, X.X.;

  Investigation, X.X.; Resources, X.X.; Data Curation, X.X.; Writing – Original Draft Preparation, X.X.;

  Writing – Review & Editing, X.X.; Visualization, X.X.; Supervision, X.X.; Project Administration, X.X.; Funding Acquisition, Y.Y.", please turn to the **CRediT taxonomy (https://www.mdpi.com/data/contributor-role-instruction.pdf)** for the term explanation. For more
  background on CRediT, see **here (http://docs.casrai.org/CRediT)**. "**Authorship must include and be limited to those who have contributed substantially to the work. Please read the section concerning the criteria to qualify for authorship carefully**
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or

interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the choice of research project; design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results must be declared in this section. *Insects* does not publish studies funded by the tobacco industry. Any projects funded by pharmaceutical or food industries must pay special attention to the full declaration of funder involvement. If there is no role, please state "The sponsors had no role in the design, execution, interpretation, or writing of the study".

- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as **EndNote (http://endnote.com/)**, **ReferenceManager (http://www.refman.com/)** or **Zotero (https://www.zotero.org/)** to avoid typing mistakes and duplicated references. We encourage citations to data, computer code and other citable research material. If available online, you may use reference style 9. below.
- Citations and References in Supplementary files are permitted provided that they also appear in the main text and in the reference list.

In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

The reference list should include the full title, as recommended by the ACS style guide. Style files for

Endnote (http://endnote.com/downloads/style/mdpi) and **Zotero (https://www.zotero.org/styles/?q=id%3Amultidisciplinary-digital-publishing-institute)** are available.

References should be described as follows, depending on the type of work:

- Journal Articles:

1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range.

- Books and Book Chapters:

2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154– 196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196. ●Unpublished work, submitted work, personal communication:
4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript inpreparation).
5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).

6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country)

Personal communication, Year.

- Conference Proceedings:

7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

- Thesis:

8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University,Date of Completion.

- Websites:

9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as **WebCite (https://www.webcitation.org/archive)**.

Archived websites should be cited using the link provided as follows:

10. Title of Site. URL (archived on Day Month Year).

See the **Reference List and Citations Guide (https://www.mdpi.com/authors/references)** for more detailed information.

PREPARING FIGURES, SCHEMES AND TABLES

- File for Figures and Schemes must be provided during submission in a single zip archive and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.
- *Insects* can publish multimedia files in articles or as supplementary materials. Please contact the editorial office for further information.

All Figures, Schemes and Tables should be inserted into the main text close to their first citation and must be numbered following their number of appearance (Figure 1, Scheme I, Figure 2, Scheme II, Table 1, *etc.*).

- All Figures, Schemes and Tables should have a short explanatory title and caption.
- All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Authors should use the Table option of Microsoft Word to create tables.
- Authors are encouraged to prepare figures and schemes in color (RGB at 8-bit per channel). There is no additional cost for publishing full color graphics.

SUPPLEMENTARY MATERIALS, DATA DEPOSIT AND SOFTWARE SOURCE CODE

Data Availability

In order to maintain the integrity, transparency and reproducibility of research records, authors must make their experimental and research data openly available either by depositing into data repositories or by publishing the data and files as supplementary information in this journal.

Computer Code and Software

For work where novel computer code was developed, authors should release the code either by depositing in a recognized, public repository or uploading as supplementary information to the publication. The name and version of all software used should be clearly indicated.

Supplementary Material

Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable, however we recommend that common, non-proprietary formats are used where possible.

Unpublished Data

Restrictions on data availability should be noted during submission and in the manuscript. "Data not shown" should be avoided: authors are encouraged to publish all observations related to the submitted manuscript as Supplementary Material. "Unpublished data" intended for publication in a manuscript that is either planned, "in preparation" or "submitted" but not yet accepted, should be cited in the text and a reference should be added in the References section. "Personal Communication" should also be cited in the text and reference added in the References section. (see also the MDPI reference list and citations style guide).

Remote Hosting and Large Data Sets

Data may be deposited with specialized service providers or institutional/subject repositories, preferably those that use the DataCite mechanism. Large data sets and files greater than 60 MB must be deposited in this way. For a list of other repositories specialized in scientific and experimental data, please consult databib.org or re3data.org. The data repository name, link to the data set (URL) and accession number, doi or handle number of the data set must be provided in the paper. The journal **Data (https://www.mdpi.com/journal/data)** also accepts submissions of data set papers.

Deposition of Sequences and of Expression Data

New sequence information must be deposited to the appropriate database prior to submission of the manuscript. Accession numbers provided by the database should be included in the submitted manuscript. Manuscripts will not be published until the accession number is provided.

- *New nucleic acid sequences* must be deposited in one of the following databases: GenBank, EMBL, **(/)** or DDBJ. Sequences should be submitted to only one database.
- *New high throughput sequencing (HTS) datasets* (RNA-seq, ChIP-Seq, degradome analysis) must be deposited either in the GEO database or in the NCBI's Sequence Read Archive.
- *New microarray data* must be deposited either in the GEO or the ArrayExpress databases.The "Minimal Information About a Microarray Experiment" (MIAME)

guidelines published by the Microarray Gene Expression Data Society must be followed.

- *New protein sequences* obtained by protein sequencing must be submitted to UniProt (submission tool SPIN).

All sequence names and the accession numbers provided by the databases should be provided in the Materials and Methods section of the article.

References in Supplementary Files

Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

RESEARCH AND PUBLICATION ETHICS

Research Ethics

Research Involving Human Subjects

When reporting on research that involves human subjects, human material, human tissues, or human data, authors must declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (**https://www.wma.net/what-we-do/medical-ethics/declaration-ofhelsinki/ (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki//)**), revised in 2013. According to point 23 of this declaration, an approval from an ethics committee should have been obtained before undertaking the research. At a minimum, a statement including the project identification code, date of approval, and name of the ethics committee or institutional review board should be cited in the Methods Section of the article. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Editors reserve the right to reject any submission that does not meet these requirements.

Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

A written informed consent for publication must be obtained from participating patients who can beidentified (including by the patients themselves). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or
images of patients, authors must obtain signed informed consent from patients (or their **r**elatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. **A template permission form (https://res.mdpi.com/data/mdpi-patient-consentform.docx)** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission.

You may refer to our **sample form (https://www.mdpi.com/data/mdpi-consent-form.docx)** and provide an appropriate form after consulting with your affiliated institution. Alternatively, you may provide a detailed justification of why informed

consent is not necessary. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Any experimental work must also have been conducted in accordance with relevant national legislation on the use of animals for research. For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [1].

Manuscripts containing original descriptions of research conducted in experimental animals must contain details of approval by a properly constituted research ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be cited in the Methods section.

Insects endorses the ARRIVE guidelines (**www.nc3rs.org.uk/ARRIVE**

(http://www.nc3rs.org.uk/ARRIVE)) for reporting experiments using live animals. Authors and reviewers can use the ARRIVE guidelines as a checklist, which can be found at **www.nc3rs.org.uk/ARRIVEchecklist (http://www.nc3rs.org.uk/ARRIVEchecklist)**.

1. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care
of Animals Used in Scientific Procedures. Available online: **http://www.officialdocuments.gov.uk/document/hc8889/hc01/0107/0107.pdf (http://www.officialdocuments.gov.uk/document/hc8889/hc01/0107/0107.pdf)**.

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity (http://www.cbd.int/convention/)** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora (http://www.cites.org/)**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oriza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from 'Crown Mix' (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX , institute, city, country).

Publication Ethics Statement

Insects is a member of the Committee on Publication Ethics (**COPE (http://publicationethics.org/)**).

We fully adhere to its **Code of Conduct (http://publicationethics. (/toggorleg/resou_desktorp_lacesy/code-conout_cookie) duct)** and to its **Best Practice Guidelines (http://publicationethics.org/resources/guidelines)**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *Insects* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *Insects* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.

- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication. Simultaneous submission of manuscripts to more than one journal is not tolerated.
- Republishing content that is not novel is not tolerated (for example, an English translation of a paper that is already published in another language will not be accepted).
- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding publication of publishing addenda and corrections (https://www.mdpi.com/files/authors/20130308-MDPI-Policy- Regarding-Corrections-and Retractions.pdf)**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions (https://www.mdpi.com/authors/rights)** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *Insects* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source
      must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.
      If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, we may publish a correction or retract the paper.

Image files must not be manipulated or adjusted in any way that could lead to misinterpretation of the information provided by the original image.
Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

REVIEWER SUGGESTIONS

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last five years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper. English Corrections

To facilitate proper peer-reviewing of your manuscript, it is essential that it is submitted in grammatically correct English. Advice on some specific language points can be found **here (https://www.mdpi.com/authors/english-editing)**.

If you are not a native English speaker, we recommend that you have your manuscript professionally edited before submission or read by a native English-speaking colleague. This can be carried out by
MDPI's **English editing service (https://www.mdpi.com/authors/english)**. Professional editing will enable reviewers and future readers to more easily read and assess the content of submitted manuscripts. All accepted manuscripts undergo language editing, however **an additional fee will be charged** to authors if very extensive English corrections must be made by the Editorial Office: pricing is according to the service **here (https://www.mdpi.com/authors/english)**.

PREPRINTS AND CONFERENCE PAPERS

Insects accepts articles that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints*** **(https://www.preprints.org)**, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors (https://www.preprints.org/instructions_for_authors)** for further information.

Expanded and high quality conference papers can be considered as articles if they fulfil the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *Insects* does not publish pilot studies or studies with inadequate statistical power.

AUTHORSHIP

MDPI follows the International Committee of Medical Journal Editors (**ICMJE (http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-ofauthors-and-contributors.html)**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or revising it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgements. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE) (http://www.icmje.org/recommendations/browse/roles-andresponsibilities/defining-the-role-of-authors-and-contributors.html)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

REVIEWERS RECOMMENDATION

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflict of interests before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

EDITORS AND JOURNAL STAFF AS AUTHORS

Editorial independence is extremely important and MDPI does not interfere with editorial decisions.

Editorial staff or editors shall not be involved in the processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers.

Decisions will be made by other editorial board members who do not have conflict of interests with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

CONFLICT OF INTERESTS

According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest." Any role of the funding sponsors in the design of the study; in the collection, analyses or interpretation of data; in the writing ofthe manuscript; or in the decision to publish the results must be declared in this section. If there is no role, please state “The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results”.

EDITORIAL PROCEDURES AND PEER-REVIEW

Initial Checks

All submitted manuscripts received by the Editorial Office will be checked by a professional in-house *Managing Editor* to determine whether they are properly prepared and whether they follow the ethical policies of the journal. Manuscripts that do not fit the journal's ethics policy or do not meet the standards of the journal will be rejected before peer-review. Manuscripts that are not properly prepared will be returned to the authors for revision and resubmission. After these checks, the *Managing Editor* will consult the journals’ *Editor-in-Chief* or *Associate Editors* to determine whether the manuscript fits the scope of the journal and whether it is scientifically sound. No judgment on the potential impact of the work will be made at this stage. Reject decisions at this stage will be verified by the *Editor-in-Chief.*

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past five years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, however once the paper has been published changes will only be made at the discretion

of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee an impartial refereeing the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review

process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:

  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.

- *Reconsider after Major Revisions*:

  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. Usually, only one round of major revisions is allowed. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments.

Reject and Encourage Resubmission:

If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.

- *Reject*:

  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments. The *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the Editor-in-Chief, Associate Editor, or Editorial Board member. The academic Editor being consulted will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. A reject decision at this stage is final and cannot be reversed.

In the case of a special issue, the *Managing Editor* of the journal will forward the manuscript and related information (including the identities of the referees) to the *Editor-in-Chief* who will be asked to give an advisory recommendation on the manuscript and may recommend acceptance, further peerreview, or uphold the original rejection decision. A reject decision at this stage will be final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading bythe authors, final corrections, pagination, and, publication on the **www.mdpi.com (https://www.mdpi.com)** website.

CLINICAL TRIALS REGISTRATION

Registration

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register or and to cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov (https://clinicaltrials.gov/)**, **the EU Clinical Trials Register (https://www.clinicaltrialsregister.eu)** and those listed by the World Health Organisation

International Clinical Trials Registry Platform

(http://www.who.int/ictrp/network/primary/en/index.html).

CONSORT Statement

Insects requires a completed CONSORT 2010 **checklist (https://www.mdpi.com/data/consort-2010checklist.doc)** and **flow diagram (https://www.mdpi.com/data/consort-2010-flow-diagram.doc)** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org (http://www.consortstatement.org)**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist. Meeting these basic reporting requirements will greatly improve the value of your trial report and may enhance its chances for eventual publication.

APPENDIX C

Instructions to authors-Journal of economic entomology (Oxford Academic)

Manuscript Preparation

NEW SUBMISSIONS

Formatting

For new submissions, our formatting requirements are simple—just make sure your paper has the following items:

- Continuous line numbers

Skip to Main Content

- Double-spaced lines
- A title page and abstract in the main document
- A main document in a doc, docx, tex (converted to PDF for review), or rtf le type
- Tables in a Word document (we cannot accept Excel files, unless they are supplementary les)
- Figure and table A main document in a doc, docx, tex (converted to PDF for review), or rtf le type

- Tables in a Word document (we cannot accept Excel files, unless they are supplementary les)
- Figure and table legends in the main document
- All coauthors entered into the online review system (email addresses required)

Please note there are more formatting guidelines for revised versions, as those are closer to being accepted (see the Revised Versions section of these author instructions).

- References listed in alphabetical order, cited by author and year in the text (not numbered)
- Figures and tables at the end of the main document after the references, or uploaded as separate les. Figure legends should be included at the end of the main text after the references, and table legends should be next to their corresponding tables •Text is single-column

Article types and lengths

•

Research article: No limit (under 7500 words recommended)

•

Review: No limit

•
•

Forum: No limit

•

Short Communication: 2,000 words or less (not including abstract, references, or figure and table legends)

Letter to the Editor: 2,000 words or less

For explanations of the diferent article types, please see the Article Types section of these author instructions.
•

Language

•

English. A second abstract in a second language is permitted. Authors are responsible for the accuracy of non-English abstracts. Skip to Main Content

Manuscripts with poor English that would not be understandable for reviewers will be withdrawn before review. Those authors are encouraged to pursue English-language assistance from a native speaker or editing service before resubmitting their paper. Having a paper in good English makes it easier for editors and reviewers to focus on the scientic merits of the paper. For more information on language editing, please see the Language Editing section of these author instructions.

legends in the main document

All coauthors entered into the online review system (email addresses required)

Please note there are more formatting guidelines for revised versions, as those are closer to being accepted (see the Revised Versions section of these author instructions).

References listed in alphabetical order, cited by author and year in the text (not numbered)

Figures and tables at the end of the main document after the references, or uploaded as separate les. Figure legends should be included at the end of the main text after the references, and table legends should be next to their corresponding tables Text is single-column

Article types and lengths

Research article: No limit (under 7500 words recommended)

Review: No limit

Forum: No limit

Short Communication: 2,000 words or less (not including abstract, references, or figure and table legends)

Letter to the Editor: 2,000 words or less

For explanations of the diferent article types, please see the Article Types section of these author instructions.

Language

- English. A second abstract in a second language is permitted. Authors are responsible for the accuracy of non-English abstracts. Skip to Main Content

Manuscripts with poor English that would not be understandable for reviewers will be withdrawn before review. Those authors are encouraged to pursue English-language assistance from a native speaker or editing service before resubmitting their paper. Having a paper in good English makes it easier for editors and reviewers to focus on the scientic merits of the paper. For more information on language editing, please see the Language Editing section of these author instructions.

Acceptable file types

- Main document: doc, docx, rtf

-

Tables: Editable tables at the end of the main document. xls and xlsx files are not accepted (except as supplementary files)

-

Figures: tif (preferred), eps (preferred), rtf, doc/docx, ppt/pptx, pdf, ps, psd, ai, gif, png

-

Supplementary les: Most file types accessible to users. Extremely large files should be uploaded in a third-party repository.

Page charges

In order to publish in JEE, authors are required to pay page charges or an Open Access fee. ESA members are exempt from page charges and receive a discount on the Open Access fee. For more information, including pricing, please visit the Charges and Licensing page.

Plagiarism and text recycling

All submitted papers are evaluated for excessive direct copying through CrossRef's Ithenticate service. Papers should be written in the authors' own words. Direct copying of sentences or paragraphs, even if the original source is cited or if it is your own previous work, is unacceptable (although some overlap is expected in materials and methods). For more information, please see the Publication Ethics section of these author instructions.

Theses, dissertations, and pre-prints

If your paper (or a previous version of it) was posted on a pre-print server or is part of a thesis or dissertation that has been published online or in an institutional repository, please note this in your cover letter so that it won't be agged for plagiarism.

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CrossRef Funding Data Registry

In order to meet funder requirements, authors are required to name their funding sources, or state if there are none, during the submission process. For further information on this process or to nd out more about CHORUS, visit the CHORUS initiative.

Previous rejections

Papers that have been rejected from one ESA journal cannot be resubmitted to any other ESA journal. Papers that have been withdrawn can be resubmitted to the same journal or another ESA journal. Authors whose papers have been rejected are entitled to appeal their rejection to the journal's editorial board. More information on the appeals process can be found on the Journal Policies page.

Statistics and sample size

Statistics should be fully reported (i.e., F-value, both degrees of freedom [treatments and replicates], and exact P-value [unless it's less than 0.001]). Furthermore, the paper will be withdrawn if Duncan's Multiple Range Test is used for papers that do not deal with plant

resistance. For more information on statistics, please see the Statistics section of these author instructions.

Papers that have insu cient sample sizes (e.g., only a single year of data collected at one location for either insect surveys, pesticide studies, or other eld data) are immediately withdrawn. The duration and size of trials/sampling must be biologically signi cant.

Plant extract papers

For papers that test the efficacy of plant extracts or other compounds on control or behavior modi cation of insects, the concentrations of the chemical constituents must be listed.

Personal communications

Personal communication citations should be accompanied by a letter from the person being cited giving permission to use him or her as a citation and verifying the claim being cited. This letter should be uploaded as a supplementary le.

Abbreviations

Abbreviations should be used sparingly. Standard abbreviations for measurements according to Scienti c Style and Format, 8th edition, are acceptable, as well as common abbreviations that improve the readability of a manuscript (e.g., DNA, PCR). All other abbreviations used Skip to Main Content should be de ned at the rst use.

PUBLICATION ETHICS

JEE is committed to ethical behavior in all aspects of scholarly publishing. Please ensure your paper meets the following ethical criteria:

•

The author list is complete and correct. Please see the "Journal Policies" page for authorship information and policies.

No portions of text are directly copied from other sources, including one's own previous papers (although some overlap is tolerated in materials and methods). Direct quotes should be placed in quotation marks. All manuscripts undergo a plagiarism test before they are sent out for review.

•

The data have not been published elsewhere. Data published in another paper, including in a paper in another language, may not be published again. If portions of data published previously are being used, the author must provide explicit written consent from the publisher of the previous paper to reuse the data.

EXPERIMENTAL ETHICS

Research published in JEE must adhere to minimal ethical and compliance requirements for medical, veterinary, and wildlife conservation research. Medical entomology research may include human subjects and/or domestic and wild animals and therefore requires that authors reference compliance protocols to indicate adherence to federal, state, and local regulations, permits, and

authorizations. International authors should reference similar compliance documents from their government and/or institution.

Listed below is a minimal series of basic requirements requested from USA and international researchers to be included, as needed, within an ethical section positioned in the Materials and Methods section of each manuscript:

1.

Biological Use Authorization (BUA). Surveillance, epidemiological, and experimental infection studies with pathogens require containment for diagnostics and culture depending upon the virulence of the organism and the risk of vectorborne, contact, and/or aerosol transmission. Descriptions of appropriate containment for di erent biosafety levels. A search engine and database to determine the Risk Group of the organism[s] concerned can

Researchers should report their institution's required compliance review and approved containment level BUA protocol for the pathogen(s) or arthropods used.

2.

Institutional Review Board (IRB) compliance. Use of human subjects in research must be approved by IRB committees adhering to US Department of Health and Human Services guidelines. Each organization may have di erent interpretations of guidelines required for human subject activities which may range from surveys, house entry for arthropod collection, use of humans to feed arthropods, use of humans as bait for sampling hostseeking insects, test subjects for candidate repellents, etc.[2]

[1] Aultman, K. S., E. D. Walker, F. Gi ord, D. W. Severson, C. B. Beard, and T. W. Scott. 2000. Managing risks of arthropod vector research. Science 288: 2321-2322.

Conflicts of interest

Institutional Animal Care and Use Committee (IACUC) compliance. Use of animals in research must adhere to protocols meeting minimal ethical requirements for collection, maintenance, and experimental procedures. Research done within the USA or funded by USA agencies must adhere to requirements described by the US National Institutes of Health, and these protocols should be appropriately referenced.

4.

Sampling wildlife. Most countries and states/provinces/districts require permits to collect vertebrate animals and some insects for research purposes. This is especially true for migratory species, such as birds, where international agreements are in place; for example, see permitting requirements for collecting and banding birds in North America.

An example of state permits required for taking or trapping and release of wildlife within California can be found at on this page.

Additional permits may be required for sampling on wildlife refuges or nature conservatory properties. Reference to these permits should be required to ensure sampling was done in compliance with regional oversight, especially for endangered or threatened species.

5.

[2] Aultman, K. S., E. D. Walker, F. Gi ord, D. W. Severson, C. B. Beard, and T. W. Scott. 2000. Managing risks of arthropod vector research. Science 288: 2321-2322.

Transport and release of organisms. With the increasing use of genetically modi ed arthropods for population or pathogen control, medical entomologists must adhere to correct oversight governing production and release. The US Department of Agriculture has strict requirements for the transport and/or release of organisms as well as experimental use permitting for applications of experimental compounds for arthropod control.

The Biotechnology Quality Management System (BQMS) Program within the USDA helps organizations, including small businesses and academic researchers, analyze the critical control points within their management systems to better maintain compliance with the APHIS regulations (7 CFR part 340) for the import, interstate movement, and eld release of regulated genetically engineered (GE) organisms.

Potential con icts of interest include any relationships of a nancial or personal nature between an author or coauthor and individuals or organizations which, in theory, could a ect or bias an author's scienti c judgment, or limit an author's freedom to publish, analyze, discuss, or interpret relevant data.

Sources of nancial support originating outside the coauthors' home institution(s) for any aspect of a study must be indicated in the Acknowledgments section of the paper. Financial support includes not only funding, but gratis provision of materials, services, or equipment. Any additional potential con icts of interest, not covered in the acknowledgments of nancial support, must be revealed to the editor at submission, and disclosed in a statement immediately following the Acknowledgments.

If an author or coauthor has entered into an agreement with any entity outside that authors' home institution, including the home institution of another coauthor, giving that entity veto power over publication of the study or over presentation, analysis, discussion, or interpretation of any results of the study, whether or not such veto power was exercised, this information must be disclosed in a statement immediately following the Acknowledgments.

ARTICLE TYPES

Research

Research articles report original observations and experiments, the results of the experiment, and a discussion of the signi cance of the results. There is no word limit for research articles.

Review

Review articles review and synthesize current information on a topic. Review articles can also contain historical threads of important ideas (i.e., are not con ned to recent citations). There is no word limit for review articles.

Forum

Forum articles are authored by acknowledged leaders in the eld, review a research area, and include a stimulating, thought-provoking discussion that focuses on important, and sometimes controversial, issues. They should provide an innovative approach to current thought and speculate about future research directions.

Short Communications

Skip to Main Content

Short communications should be similar to a research article, but with briefer Materials and Methods and Discussion. Total length should be 2,000 words or less.

Letter to the Editor

JEE will consider submissions in the form of a letter to the editor in which the authors express their viewpoint on scienti c issues. Appropriate content can include comments or criticisms in reference to a published paper, whether or not in an ESA journal, or comments and opinions unrelated to a speci c published paper. A letter will be limited to 2,000 words, 10 references, and one table or gure.

The Editor-in-Chief (EIC) will judge whether a submitted letter merits consideration for potential publication based on relevance, inherent interest, and coherence of the submission, but with a view to allowing a range of opinions to be expressed. If the EIC considers the submission to be suitable in principle, he/she will send it to at least one anonymous reviewer for comments and will edit it for style and appropriate language before returning it to the corresponding author for revisions. More information on letters to the editor can be found on our Journal Policies page.

TITLE PAGE

The title page should include:

1. Corresponding author: Include full name, mailing address, telephone number, and email address.
2. Title: Should be as short as possible. Only include common names that are listed in the ESA Common Names of Insects & Related Organisms. Do not include authors of scienti c names. Insert "([Order]: [Family])" immediately after the name of the organism.
3. Author list: Include all authors in the order the names should be published.
4. A liation line: Include full addresses of all authors. If there are multiple a liations, designate through numbered footnotes.
5. Abstract
    a. 250 words or less.
    b. Give scienti c name and authority at first mention of each organism.
    c. Do not cite references, gures, tables, probability levels, or results.
    d. Refer to results only in the general sense.
    e. A second abstract in a second language is permitted.
6. Keywords
    a. Below the abstract, provide three to ve keywords, separated by commas. Skip to Main Content
    b. Do not use abbreviations, combined keywords, or species names.

Sample title page.

BODY

Introduction

Clearly state the basis of your study along with background information and a statement of purpose.

Materials and Methods

Include a clear and concise description of the study design, experiment, materials, and method of statistical analysis.

Results

Clearly present the results. Do not include interpretation of results or interpretation of statistical analysis—simply present the results of the experiment and the results of the statistical analysis. Data listed in tables should not be listed in the results; instead, refer to the table.

Discussion

Interpret and discuss results of the study and their implications. Include suggestions for direction of future studies, if appropriate.

Acknowledgments

Place the acknowledgments after the text. Organize acknowledgments in paragraph form in the following order: persons, groups, granting institutions, grant numbers, and serial publication number.

Following the Acknowledgments, you may include a statement of author contribution outlining the speci c contributions of each author to the article. A statement of author contribution is welcomed but not required.

REFERENCES

- EndNote style is “Environmental Entomology,” and Reference Manager style is “Journal of Skip to Main Content

  Medical Entomology.”

- Only cite published or formally accepted (in press) articles, not submitted articles.

- References should be in alphabetical order. If multiple references from the same author are cited, those references should be in chronological order.

- Abbreviate journal titles according to the most recent issue of BIOSIS Serial Sources.
- For non-English titled journals that are cited in the references, the title of the journal should be spelled out.
- Systematics-related articles may specify that all serial titles be spelled out for nal publication.

Sample reference styles

Journal Articles

Evans, M. A. 2000. Article title: subtitle (begin with lowercase after colon or dash unless first word is a proper noun). J. Abbr. 00:000–000.

Evans, M. A. 2001a. Article title. J. Abbr. 00: 000–000.

Evans, M. A., and R. Burns. 2001. Article title. J. Abbr. 00: 000–000.

Evans, M. A., and A. Tyler. 2001. Article title. J. Abbr. 00: 000–000.

Evans, M. A., A. Tyler, and H. H. Munro. 2000. Article title. J. Abbr. 00: 000–000.
Evans, M. A., R. Burns, and A. A. Dunn. 2001. Article title. J. Abbr. 00: 000–000.

In Press

Evans, M. A. 2002. Article title. J. Econ. Entomol. (in press).

Books

Burns, R. 2001. Title (initial cap only): subtitle (no initial cap after colon). Publisher, city, state abbreviation or country.

Evans, M. A. 2001. Colorado potato beetle, 2nd ed. Publisher, city, state abbreviation or country.

Tyler, A. 2001. Western corn rootworm, vol. 2. Publisher, city, state abbreviation or country.

Article/Chapter in Book

Tyler, A. 2001. Article or chapter title, pp. 000–000. In T.A.J. Royer and R. B. Burns (eds.), Book Skip to Main Content title. Publisher, city, state abbreviation or country.

Tyler, A., R.S.T. Smith, and H. Brown. 2001. Onion thrips control, pp. 178–195. In R. S. Green and P. W. White (eds.), Book title, vol. 13. Entomological Society of America, Lanham, MD.

No Author Given

(USDA) U.S. Department of Agriculture. 2001. Title. USDA, Beltsville, MD.

(IRRI) International Rice Research Institute. 2001. Title. IRRI, City, State or Country.

Patents

Harred, J. F., A. R. Knight, and J. S. McIntyre, inventors; Dow Chemical Company, assignee. 1972 Apr 4. Epoxidation process. U.S. patent 3,654,317.

Proceedings

Martin, P. D., J. Kuhlman, and S. Moore. 2001. Yield e ects of European corn borer (Lepidoptera: Pyralidae) feeding, pp. 345–356. In Proceedings, 19th Illinois Cooperative Extension Service Spray School, 24–27 June 1985, Chicago, IL. Publisher, City, State.

Rossignol, P. A. 2001. Parasite modi cation of mosquito probing behavior, pp. 25–28. In T. W. Scott and J. Grumstrup-Scott (eds.), Proceedings, Symposium: the Role of Vector-Host

Interactions in Disease Transmission. National Conference of the Entomological Society of America,10 December 1985, Hollywood, FL. Miscellaneous Publication 68. Entomological Society of America, Lanham, MD.

Thesis/dessertation

James, H. 2001. Thesis or dissertation title. M.S. thesis or Ph.D. dissertation, University of Pennsylvania, Philadelphia.

Software

SAS Institute. 2001. PROC user's manual, version 6th ed. SAS Institute, Cary, NC.

Online Citations

Reisen, W. 2001. Title. Complete URL (protocol://host.name/path/ le.name) and/or DOI (Digital Object Identi er)

TABLES

Tables should be editable tables in a Word document.

- If a table continues on more than one page, repeat column headings on subsequent page(s). Skip to Main Content

All columns must have headings.

- Leave no space between lowercase letters and their preceding values (e.g., 731.2ab).

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- Use approved abbreviations or abbreviations already de ned in the text and de ne others in the general footnote.

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(versus), vol (volume), wt (weight) Jan (January), Feb (February), Mar (March), April, May, June, July, Aug (August), Sept (September), Oct (October), Nov (November), and Dec (December). Sample table

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Figures should be at least 300 dpi, or 1200 dpi for line graphs.

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Supplementary Material may be submitted in the form of one or more les to accompany the online version of an article. Such material often consists of large tables, data sets, or videos that are not possible or convenient to present in print media. Supplementary Material represents substantive information to be posted on the ESA journal website that enhances and enriches the information presented in the main body of a paper; however, the paper must stand on its own without the need for the reader to access the supplementary information to understand and judge the merits of the paper. All Supplementary Material must be provided at the time of manuscript submission and will be distributed to reviewers as part of the normal peer-review process. It will not be edited during production and will be published as submitted.

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SPECIES AUTHORITY, ORDER, FAMILY, AND COMMON NAMES

•Authors should provide the authority, order, and family for all organisms that are central to the paper (including plants, bacteria, and other non-arthropod organisms) at the rst mention of the organism. It is the author's responsibility to provide accurate authority, order, and family information. Organisms mentioned in passing or whose importance to the paper is limited do not need to have full authority, order, and family listed, nor do mentions of common names of groups (e.g., mosquitoes, beetles, ticks, etc.).

•If a species name is included in the title of a paper, either the ESA-approved common name or the Latin name, the order and family should also be provided. If the order and family of an organism is provided in the title of the paper, it does not need to be provided again in the abstract and main text.

•If an organism is not listed in the title but is central to the paper, the order and family should be provided at first mention of the organism in both the abstract and the main text.

•The taxonomic authority of an organism that is central to the paper should be given the rst time the organism is mentioned in both the abstract and the main text, but not in the title. For tables that include lists of species, authority should be given for each species if it is the rst time it is being mentioned in the paper.

•If multiple organisms in the same genus are central to the paper, order and family only need to be provided for the first species mentioned in the genus. If multiple organisms are central to the paper, are in di erent genera, but are in the same order and family, order and family should be provided at first mention of each organism.

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STATISTICS

All data reported (except for descriptive biology) must be subjected to statistical analysis. Results of statistical tests may be presented in the text, in tables, and in figures. Statistical methods should be described in Materials and Methods with appropriate references. Descriptions should include information such as sample sizes and number of replications.

Only t-tests, Chi square, and analyses of variance require no citation. Cite the computerSkip to Main Content program user's manual in the References Cited.

Probit/logit

When presenting results of probit/logit analysis, the following columns should be included in tables in the following order (left to right); n, slope + SE, LD (or LC) (95% CL), and chi-square. When a ratio of one LD versus another is given, it should be given with its 95% CI. Statistical tests to show what model best fits data intended to estimate the 99.9986% level of effectiveness should be presented to justify use of any model, including the probit model. Thus, we do not recommend use of the Probit 9 without tests to show that the probit model fits the data.

Analysis of Variance or t-test

When presenting the results of analysis of variance or a t-test, specify F (or t) values, degrees of freedom, and P values. This information should be placed in parentheses in the text. Example: ($F = 9.26$; $df = 4, 26$; $P < 0.001$). If readability of the text is afected by the presence of repeated parenthetical statistical statements, place them in a table instead.

Regression

In regressions, specify the model, define all variables, and provide estimates of variances for parameters and the residual mean-square error. Italicize variables in equations and text.

Variance and sample size

Include an estimate of the variance (or standard error) and sample size for each mean regardless of the method chosen for unplanned multiple comparisons. The use of Duncan's Multiple Range Test (DMRT) is not acceptable as a mean separation test as it was designed to be a very liberal test intended to find even minor differences in resistance between plant lines used for breeding.

MODEL ANALYSIS, GUIDELINES, EQUATIONS, AND COMPUTER CODE

Model Analysis

At the beginning of the manuscript, authors should state clearly the goals of their model construction and analysis. Evaluation by reviewers depends upon these goals and the type of model. Authors should attempt to describe the main conclusions, limitations, and sensitivity of results to assumptions. For stochastic models, describe the variability in the results. Skip to Main Content

Modeling Guidelines

- The following guidelines pertain to any mathematical model calculated for purposes other than statistical analysis.
- Authors must adequately describe both model structure and model analysis.
- Authors must explain and justify original equations and computer programs or justify the selection of a published software package used in the computation of models.
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- Without presenting extensive computer code, the text must permit an understanding of the model that would allow most mathematically inclined scientists to duplicate the work.
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- Unless their derivation is self-evident, show how the equations were derived and mention the underlying assumptions.
- Express how the equations are solved over time and space.
- Provide references for standard techniques (e.g., matrix manipulation, integration).
- De ne all variables and parameters in each equation and describe their units (e.g., time, space, and mass).
- In the Materials and Methods or Results section, present the range of parameter values included in the model, and describe the uncertainty in or range of validity of these values.

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Consult *Mathematics into Type* for correct formatting of equations and mathematical variables. Italicize all mathematical variables.

Validation or Testing of Model Results

Data used for validation must be independent of data used to build or calibrate the model. Authors must state why the model did not require testing (e.g., theoretical study), why it cannot be tested (e.g., lack of data), or how it was tested.

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For models solved or simulated by computers, mention the programming language and computer used. Describe the important numerical methods used in calculating the model (e.g., integration and random number generation). Mention how the program's logic and

algorithms were tested and veri ed. When published software is computed, provide a reference and state which procedures were used. Discuss in any section of the manuscript the limitations of the published software. Original computer programs should be made available at the request of reviewers and readers.

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- Inclusion of a GenBank/EMBL accession number for primary nucleotide and amino acid sequence data is required.
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- The accession number may be included in the original manuscript or the sequence may be provided for review and an accession number provided when the manuscript is revised.

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In order to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN) with regard to nomenclatural works, ALL articles published by ESA's journals, regardless of whether they include nomenclatural information, will be immutable; this means that no changes will be allowed to any article without the publication of an erratum clearly stating the changes that have been made. Therefore, it is the responsibility of the authors to carefully check their proofs for accuracy, and to notify the publisher of any changes that are necessary prior to Advance Access publication.

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- Maximum width (one-column gure): 82 mm.
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- The quality in which gures are submitted is the quality in which they will print—please ensure gures are high quality.
- Each gure should be submitted as an individual le. Please do not copy and paste them into the main document.
- The following le types of gures are accepted: tif, eps, rtf, doc/.docx, ppt/pptx, pdf, ps, psd, ai, gif, png.
- Figures should be prepared in CMYK format (not RGB).
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ESA journals use “Environmental Entomology” style in EndNote, or “Journal of Economic Entomology” style in Reference Manager. Please see the References section in these author instructions for examples.

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Use metric units. English units may follow within parentheses if needed.

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- Give full scienti c name and authority at rst mention of each organism (including plants and non-insect animals) in the abstract and again in the text.
- Only use common names listed in the current ESA Common Names of Insects & Related Organisms online database. Common names should be lowercase, except for proper nouns and their derivations.
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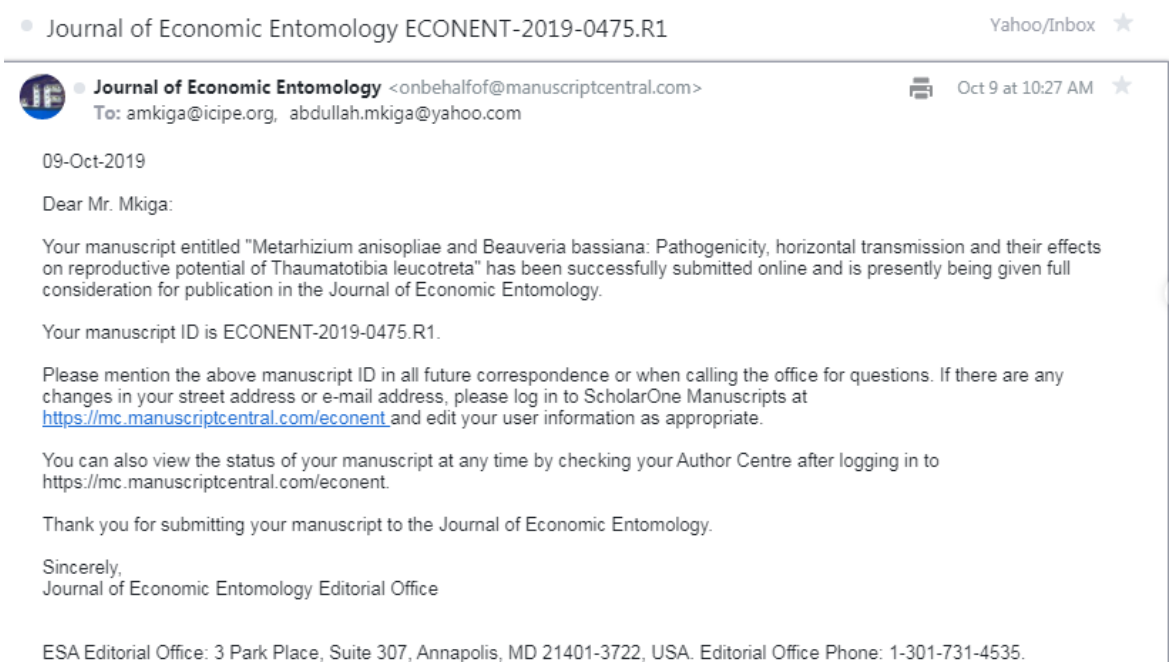

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APPENDIX D

Instructions to authors -Journal of Pest Science (Springer)

Journal of Pest Science

- Journal home›
- Submission guidelines

Submission guidelines

CONTENTS

Manuscript Submission

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

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- Visiting the English language tutorial which covers the common mistakes when writing in English.
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The title page should include:

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- A clear indication and an active e-mail address of the corresponding author
- If available, the 16-digit ORCID of the author(s)
- If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

Key Message

(limit 80 words): Please summarize the main achievement of your work, above and beyond what may have been conveyed in the manuscript title in bullet point style. In brief, the Key Message should state why the work was conducted (knowledge gap(s) as well as key question(s) and/or hypotheses tested) and highlight the main finding(s) and the conclusions derived from this study. The latter should address the wider implications of the work and the relevance for pest control. All text should be generic, seminal and understandable to nonspecialists. The Key Message should be part of your submitted manuscript and will be published in front of the Abstract.

Author Contribution Statement

Authors must provide a short description of the contributions made by each listed author (please use initials). This will be published in a separate section in front of the Acknowledgments.

For example: AM and DB conceived and designed research. AM and BB conducted experiments. GR contributed new reagents and/or analytical tools. AM, BB and GR analyzed data. AM wrote the manuscript. All authors read and approved the manuscript.

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Manuscripts should be submitted in Word.

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Abbreviations should be defined at first mention and used consistently thereafter.

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- reason for your study, the context and the hypotheses being tested.- Materials and MethodsInclude sufficient details for the work to be repeated.- ResultsState the results, drawing attention to important details in tables and figures.DiscussionPoint out the importance of the results and place them in the context of previous studies and in relation to the application of the work. Within a conclusion at the end of the discussion synthesize the paper's general contribution to pest sciences and its importance to sustainable pest control. Where appropriate, set out recommendations for management or policy.
- Genus and species names should be in italics.
- Units and symbols: Temperatures may be expressed in degrees Celsius, time in seconds (s), minutes (min), hours (h), days, etc. Otherwise, the International System of Units (Sl, Système
- International d'Unités) should be used wherever possible. [Consult, e.g., U.S. Department of Commerce, National Bureau of Standards, Special Publication 330, The International System of Units, latest edition].
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Citation

Cite references in the text by name and year in parentheses. Some examples:

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  This result was later contradicted by Becker and Seligman (1996).

- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work. Order multiauthor publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically.

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Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

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- ISSN LTWA

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- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and Illustrations Guidelines

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- Supply all figures electronically.
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- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
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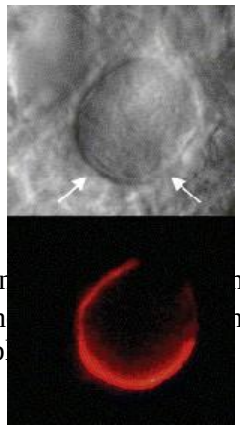

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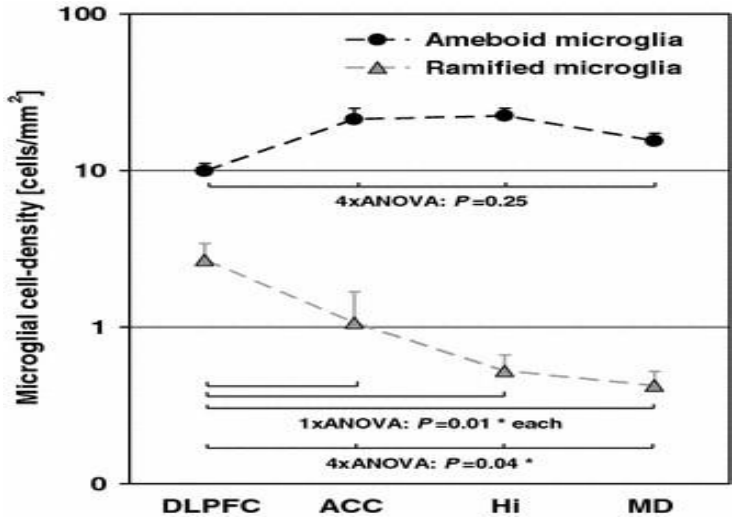

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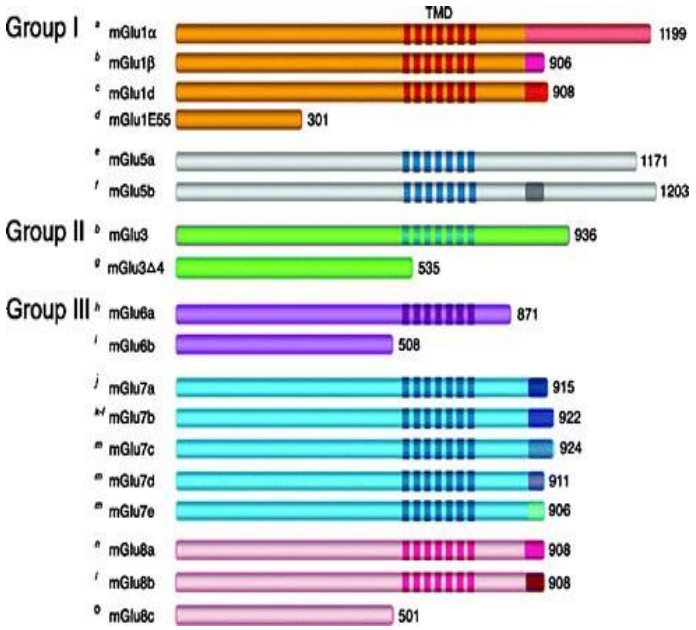

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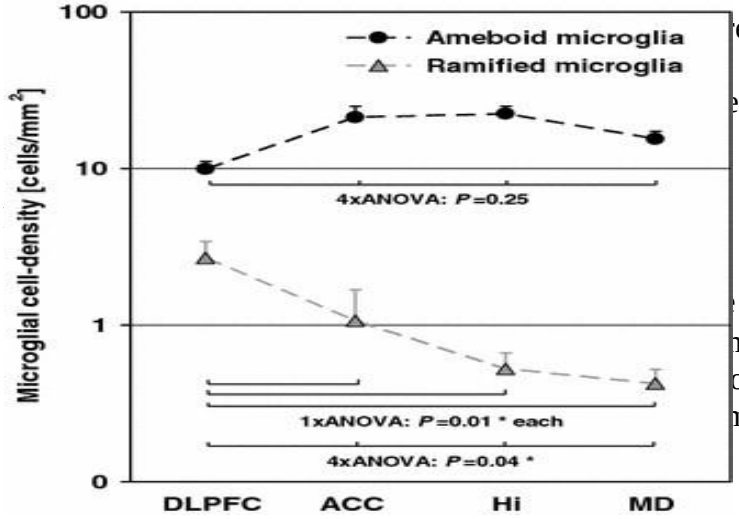

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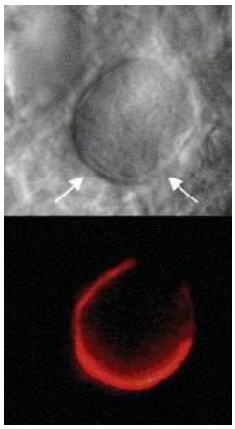

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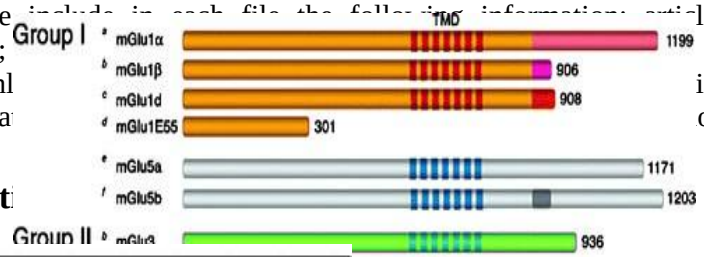

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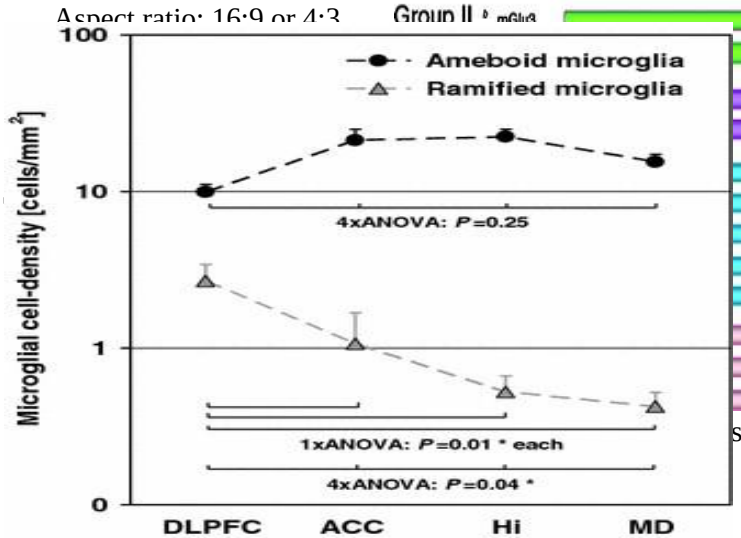

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(*Diabrotica rgifera virgifera LeConte*) amid crop rotation systems in Europe. In S. Vidal, U. Kuhlmann & R. Edwards (Eds.), *Western corn rootworm: ecology and management* (pp. 189–220). Wallingford, United Kingdom: CABI Publisher.

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