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SEXUAL ATTRACTION AND MATING COMPATIBILITY BETWEEN *THAUMATOTIBIA LEUCOTRETA* POPULATIONS AND IMPLICATIONS FOR SEMIOCHEMICAL DEPENDENT TECHNOLOGIES

A thesis submitted in fulfilment of the requirements for the degree of

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Of

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By

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Declaration

I, Jennifer Kate Upfold (g14u0893) hereby declare that the thesis submitted is my own work. It is being submitted for the degree of Masters of Science at Rhodes University. It has not been previously submitted for assessment of any degree at any other university or other body, organisation outside of the university.

02/12/19

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Abstract

False codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick), is the most important pest for the cultivation of citrus in South Africa. False codling moth is indigenous to southern Africa and is a regulated pest of many international markets for phytosanitary concerns. Considerable research efforts have been invested in the past decades to develop semiochemcial technologies, such as monitoring with sex pheromones, attract-and-kill, mating disruption and the sterile insect technique. One of the potential obstacles identified with semiochemical control is the differences in the ratio of the compounds comprising the sex pheromone at different geographical locations, resulting in what is known as regional attraction. This has been identified in FCM populations from three different countries, however, regional attraction within South African FCM populations was unknown. Therefore, the study assessed the genetic integrity of five laboratory-reared FCM populations originating from geographically isolated populations in South Africa using the AFLP technique in order to assess regional attractiveness within the country. The results found isolated populations from Addo, Citrusdal, Marble Hall, Nelspruit and a fifth group found to be closely related to Addo and Citrusdal called the 'Old' colony. These five genetically isolated populations as well as a population from Xsit (Pty) Ltd, used for the sterile insect technique (SIT), were used in regional attractiveness trials. Males were significantly (P = <0.05) more attracted to females originating from the same population. No significant attraction could be determined from the sterile males, as the recapture rates in the trap were too low. Furthermore, regional attractiveness was assessed through choice/ no-choice mating compatibility trials. Significant sexual isolation (ISI) occurred between mating combinations Addo × Nelspruit (ISI = 0,13; t_2 = 6.23; p = 0.02), Addo × Marble Hall (ISI = 0,11; t_2 = 4.72; p = 0.04), Citrusdal × Nelspruit (ISI = 0,11; t_2 = 4.95; p = 0.04), and Citrusdal × Marble Hall (ISI = 0,12; t_2 = 4.31; p = 0.04). In these combinations, Addo and Citrusdal males were found to have outcompeted Nelspruit and Marble Hall males for more mating events. Significant sexual isolation was also recorded for Sterile × Marble Hall (ISI = 0.12; t_2 = 4.98; p =0.01) and Sterile × Citrusdal (ISI = 0.13; t_2 = 3.96; p = 0.01) populations. The male relative performance index was significant in both combinations, indicating that non-sterile laboratory males outcompeted the sterile males in these two combinations. When given no choice, evaluated as spermatophore transfer/ female/ 48h, all males (including sterile) were successful in transferring spermatophores to all FCM populations, with no significant differences. These results indicate that there may be incipient pre-isolation mechanisms

affected by local natural selection, resulting in localised sexual attraction via differences in the sex pheromone ratios. These findings provide important information for semiochemical technologies and the implication of these results with regard to monitoring with sex pheromones, attract-and-kill, mating disruption and sterile insect technique are discussed.

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Table of Contents

List of Figures

Chapter 3

Chapter 4

Chapter 5

List of Tables

List of Abbreviations

ATP……..... Adenosine Triphosphate

AFLP……. Amplified Fragment Length Polymorphism

AMOVA……..... Analysis of molecular variance

ANOSIM……..... Analysis of Similarities

CGA……... Citrus Growers' Assosciation

CMVC……..... Citrus Market Value Chain

COI…… Cytochrome Oxidase I

CrleGV…….. Cryptopphlebia leucotreta granulovirus

CTAB…… Cetyl trimethylammonium bromide

DNA…….. Deoxyribonucleic acid

ddH_2O…… Distilled water

EPPO……. European and Mediterranean Plant Protection Organisation

et al..... et alia (and others)

EU…….. European Union

F1……... First filial generation

FAO/IAEA/USDA……. Food and Agriculture Organization/ International Atomic Energy Agency/United States Department of Agriculture

FCM …….. False Codling Moth

FRPI……... Female Relative Performance Index

GC-MS……... Gas chromatography- mass spectrometry

Gy…….. Gray

Ha……... Hectares

i.e……..... Id est (namely)

IPM……..... Integrated Pest Management

ISI…….. Index of Sexual Isolation

Lab……. Laboratory

Ltd…….. Limited

MCMC……... Markov Chain Monte Carlo

*mt*DNA…….. Mitochondrial Deoxyribonucleic acid

MRPI……. Male Relative Performance Index

PBAN……. pheromone biosynthesis activating neuropeptides

PBANr……... pheromone biosynthesis activating neuropeptides receptors

PCR……..... Polymerase Chain Reaction

PCoA……. Principal Coordinate Analysis

PM……. Participation in Mating

PPB…… Percentage polymorphic bands

Pty…….. Party

RAPD……. Random Amplification of Polymorphic DNA

RE…….. Restriction Enzyme

RL…….. Restriction/Ligation

RSA……..... South Africa

RSI……. Relative Sterility Index

RTA……... Relative Trade Advantage

SA…….. South Africa

SD...……... Standard deviation

SEP…… Sample Enrichment Probes

SIT……. Sterile Insect Technique

SSR…… Simple Sequence Repeats

UPGMA……. Unweighted pair group method with arithmetic mean

USA……... United States of America

Xsit…….. X Sterile Insect Technique Pty. (Ltd)

ZAR……... Rand

&……..... and

×……. cross

°C……..... Degree Celsius

Mg…….. Milligrams

M…… Molar

µl……..... Micro litre

µM……. Micro Moles

Ng…….. Nanograms

%..... Percentage

±……. Standard deviation

U…… Units

V…… Volts

Chapter 1

Review of Literature

1.1. Citrus in South Africa: Brief Overview

The agricultural sector plays an important role in South Africa's economy, especially the production of citrus (Rutaceae). The citrus industry is the third largest horticultural industry within the sector and contributed a record ZAR 20 billion to the total gross value of the South African agricultural production in the 2017/18 season (Citrus Growers' Association (CGA) 2019). A large proportion of citrus, especially orange, lemon, mandarin and grapefruit cultivars, are exported providing a steady stream of foreign income (Citrus Market Value Chain (CMVC 2017). Since 2000, Western Europe and the United Kingdom have been the largest export markets, however, significant effort has been put into developing Asian markets which has subsequently grown from 16% in 2000 to 25% in 2017 (CGA 2018). In contrast to the decline of other agricultural industries, citrus exports are expected to reach record numbers (approx. 137 million cartons) in 2019, (CGA 2018; Business insider 2019). As the citrus industry exports most of its produce, the fruit needs to be kept at an international standard free of insect pests, diseases and reduced residues of pesticides on produce (Grout & Moore 2015; CGA 2017).

Keeping citrus pest-free can be a formidable task due to the wide range of pests that attack the plant (Smith & Peña 2002). Due to the favourable climate, over 100 pest species have been recorded in South Africa, with a general trend of increased pests in warmer, more humid areas (Bedford 1998; Urquhart 1999; Smith & Peña 2002). Approximately 30-60% of these pests are Hemiptera (scale, mealybug, whitefly and leafhoppers) followed by Lepidoptera (fruit boring, leaf rolling and miners), and lastly some damage is caused by mites, beetles, flies and thrips (Moore 2002; Smith & Pena 2002). A key pest of citrus in South Africa is the false codling moth (hereafter FCM), *Thaumatotibia leucotreta* Meyrick (Lepidoptera: Torticidae). False codling moth is a problem for South Africa as it is a native cryptic pest, involved in both pre- and post-harvest crop damage and it does not occur in many of South Africa's trading partner countries (Moore 2002; Venette *et al.* 2003; Stibick 2010; CGA 2017). For this reason, there are phytosanitary regulations in place to prevent the spread of the moth outside southern Africa, especially to the EU and the Americas (Moore 2002; Moore & Hattingh 2012). This puts

pressure on farmers to provide fruit for export that is grown in an environmentally conscious manner as well as free from FCM. If South Africa wants to maintain current trade relationships (Table 1.1), especially given the current economic structures and trade regimes, then it is a priority that export fruit is grade 1 (top class) and pest- and disease-free (Moore 2002; Dikilili & van Rooyen 2018).

The South African citrus industry has maintained a largely positive Relative Trade Advantage (a product's degree of superiority and attractiveness to customers over similar existing products) (RTA) growing from an RTA of 4.6 in the 1960s to an RTA of 18.6 in 2016 (Dikilili & van Rooyen 2018). On a global scale this places South Africa as the most globally competitive southern hemisphere producing region; however, this status is constantly threatened. Thus, it is important that FCM is properly controlled and not present in export produce, as these consignments can be rejected, resulting in substantial monetary loss to farmers and penalties against South Africa. This can also lead to a reduced competitive climate, which could threaten the future growth and development of the citrus industry, impacting jobs, livelihoods and South Africa's economy (Dikilili & van Rooyen 2018).

Table 1.1. Strategic markets, defined as countries with a relatively large demand in which South Africa can potentially grow its citrus exports. Compiled from Kapuya *et al.* (2014).

Non-EU markets	**EU markets**
Russia	Netherlands
Saudi Arabia	Sweden
Iran	Germany
China	France
United States of America	Italy
United Arab Emirates	United Kingdom
Ukraine	Lithuania
Japan	Finland
Canada	Ireland
Malaysia	Portugal
Philippines	
Singapore	
Hong Kong	
Kuwait	

1.2. False Codling Moth

1.2.1. Taxonomy and Classification

FCM is a native pest to southern Africa. It is of phytosanitary concern according to the European and Mediterranean Plant Protection Organization (EPPO 2013). FCM was originally detected on citrus crops in the early 1900s in the KwaZulu-Natal Province of South Africa (Fuller 1901). The species has had a few taxonomic changes, originally classified as *Carpocapsa* sp. by Fuller (1901) and named the Natal codling moth. It was later re-described as *Argyroploce leucotreta* (Eucosmidae, Olethreutinae) after a reported sighting of an "Orange codling moth" (Meyrick 1912). It was then transferred by Clarke (1958) into the genus *Cryptophlebia,* but finally transferred to the genus *Thaumatotibia* by Komai (1999).

1.2.2. Host Range and Distribution

FCM is endemic to, and has a wide distribution across, sub-Saharan Africa. Much of the moth's host range falls in South Africa due to the production of citrus, a preferred host. After an accidental introduction of the pest into Israel more than 30 years ago, pest populations were considered negligible, believed to be in part due to the Mediterranean climate. However due to climate change, population numbers have been growing and outbreaks have been recorded on pomegranate, macadamias, cotton and castor bean in that region (Wysoki 1986; Levi-Zada 2019). FCM is known to have an extensive host range affecting over 70 plant species in more than 25 families both with and without economic value (Table 1.2). In tropical Africa, FCM is becoming a serious pest of cotton and maize (EPPO 2013). In South Africa, all citrus growing regions are affected by FCM (Moore 2002). The citrus growing regions span across the country in all provinces barring the Free State and Gauteng, with Limpopo, Eastern and Western Cape provinces comprising >85% of the growing area (Figure 1.1) (CMVC 2018).

Table 1.2. Hosts that can support completion of the life cycle (EPPO 2013; Moore 2015)

Plant family	**Species**	**Common name**
Annonaceae	*Annona muricate*	Soursop
Fabaceae	*Quercus robur*	Common oak
Juglandaceae	*Juglans sieboldiana*	Walnut
Lythraceae	*Punica granatum*	Pomegranate
Lauraceae	*Persea Americana*	Avocado
Malvaceae	*Gossypium hirsutum*	Cotton
Malvaceae	*Abelmoschus esculentus*	Okra
Myrtaceae	*Psidium guajava*	Guava
Oxalidaceae	*Averrhoa carambola*	Carambola
Poaceae	*Zea mays*	Maize
Poaceae	*Sorghum halepense*	Sorghum
Proteaceae	*Macadamia ternifolia*	Macadamia
Rosaceae	*Prunus persica variety nectarina*	Nectarine
Rubiaceae	*Coffea Arabica*	Coffee
Rutaceae	*Citrus sinensis*	Orange
Rutaceae	*Citrus paradisi*	Grapefruit
Sapindaceae	*Litchi chinensis*	Litchi
Solanaceae	*Capsicum spp.*	Peppers
Theaceae	*Camellia sinensis*	Tea
Vitaceae	*Vitis spp.*	Grape
Ebenaceae **	*Diospyros lycioides*	Jakkalsbessie
Euphorbiaceae **	*Ricinus communis*	Castor bean

** denotes wild hosts

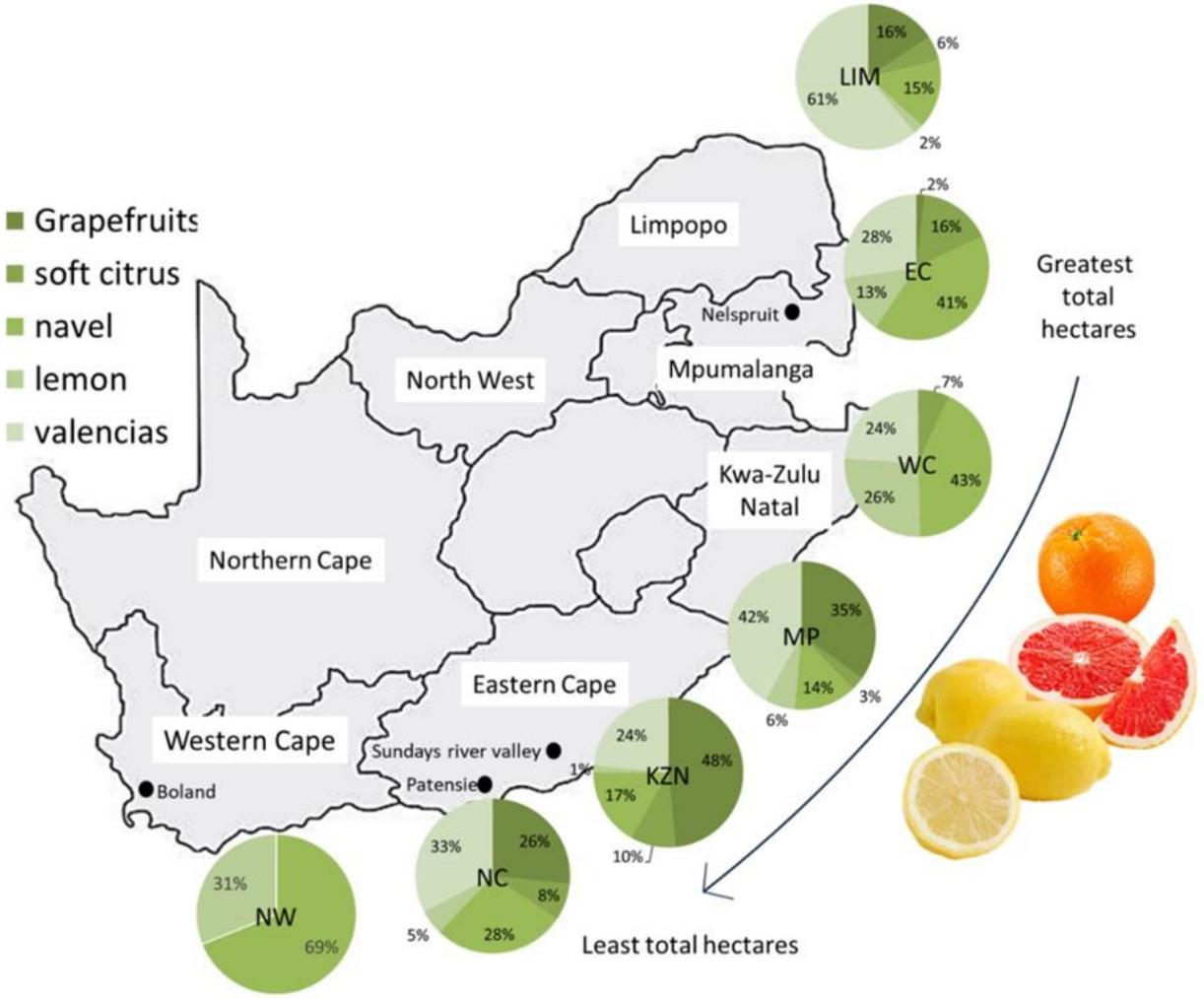


Figure 1. 1. Citrus growing regions of South Africa. Pie charts indicate the percentage of hectares of each citrus type grown per province, with Limpopo as the largest production region and North West as the smallest (compiled using data from CMVC 2017).

1.2.3. Biology and Life History on Citrus

FCM is multivoltine and polyphagous with a life cycle ranging between 30 to 120 days depending on temperature and food quality (Bloem *et al.* 2003; Terblanche *et al.* 2014). This can lead to six overlapping generations in one year in South Africa, with the possibility of more (Terblanche *et al.* 2014). The length of the life stage is temperature dependent, with warmer temperatures resulting in faster growth rate and cooler temperatures resulting in slower growth rates (Daiber 1989). The sex ratio of FCM in the wild is relatively even of 1:1 between wild males and females. The sex ratio is independent of temperature and the size of moth may change with laboratory culturing (Daiber 1989).

Mature females can lay between 100 and 250 eggs individually on the fruit or foliage (Hofmeyer *et al.* 2016). The eggs are cream, and disk shaped with a granulated surface (Figure 1.2a). Daiber (1979a) has recorded the average length of the egg as 0.77 mm and the width as 0.6 mm. FCM eggs turn red a few days after oviposition and black before hatching. This is due to the black head capsule forming, which is visible through the egg shell. The neonate larvae burrow into the fruit shortly after hatching and stay close to the surface. Cannibalism may also occur at this stage, leading to fruit usually being infested with only a single larva (Daiber 1979a). The larva develops through five larval instars and may cause premature fruit drop (Daiber 1979b). First to fourth instar larvae are spotted and creamy white before turning pink at maturity (Daiber 1979b; Dreistatd 2012) (Figure 1.2b). Daiber (1979b) recorded the length of each larval stage at varying temperatures and found that larvae grew at regular intervals every three days, every two days and every day at 15°C, 20°C, and 25°C respectively.

The larvae drop to the ground on silken threads and pupate in the upper soil surface (Love *et al.* 2014). A prepupa begins to form after a period of two to three days. The pupal stage can last between 21-80 days in the field, but in a laboratory setting eclosion can be as early as 16 days (Daiber 1979c, Malan *et al.* 2011). FCM sex can be determined at the pupal life stage via two notches on the second last segment present in males and absent in females. The pupae are initially soft and pale, gradually becoming harder and darker with age. Following eclosion, adult FCM have mottled brown-grey colouration on their forewings. Their hindwings are fringed and paler than the forewings but more evenly toned. Sexual dimorphism between males and females occurs with the females appearing larger with a wingspan of 19-20 mm and males with a wingspan of 15-16 mm (Daiber 1980). Males have heavily tufted hind tibia which is

absent in females (Figure 1.2c). Depending on temperatures, the females have a slightly longer lifespan to males, which varies between 15 and 48 days and 13 to 34 days, respectively (Daiber 1980).

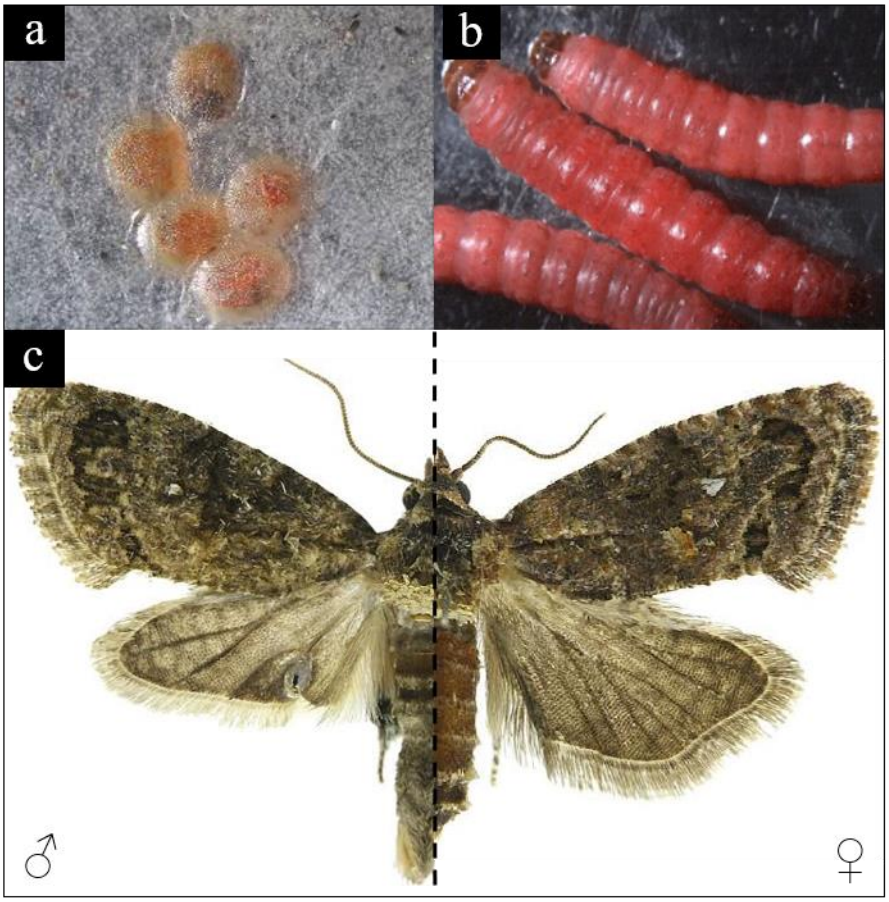


Figure 1.2. (a) FCM eggs, (b) mature FCM larva, (c) adult FCM male with fur on hindleg and last abdominal section (left) and female without tufts on hindleg and abdomen (right) (Pest Fact Sheet 2019; invasive.org; greenlife.co.ke).

1.2.4. Pest Status and Economic Importance

As discussed, FCM can become a pest on over 25 cultivated plants, leading to serious economic complications if not controlled properly (EPPO 2013; Moore 2015). For example, FCM has caused devastation in Uganda when between 42-90% of cotton was lost (Reed 1974) and in South Africa when up to 28% crop loss was recorded in a late maturing peach cultivar. If left uncontrolled, or inadequate control is implemented, FCM can devastate crops. FCM's favoured

host in South Africa, citrus, has also experienced losses of up to 20% in some years (Glas 1991). Begemann & Schoeman (1999) correctly predicted that the most susceptible citrus cultivar, Navel oranges, experienced up to 1.6% crop losses annually, directly due to FCM.

A set of criteria must be assessed for an insect to gain pest status. These criteria are a combination of ecological suitability; host suitability/availability; survey methodology; taxonomic recognition; entry potential into a country; the destination of already infested material; and the potential economic impact that goes hand in hand with establishment potential (Venette *et al.* 2013). FCM became a regulated pest in the European Union (EU) in October 2014 after finding that the moth could establish in some member states if it were to be accidentally introduced (EPPO 2013). Citrus is a valuable commodity in South Africa because of the large volume that is exported and the income this generates. Therefore, the citrus industry plays an important role in the agricultural sector and needs to be protected from damage by FCM. A plethora of control strategies have been developed to combat FCM infestations such as employing a high standard of orchard sanitation, biological control, microbial control and chemical control (Moore & Hattingh 2016; Malan *et al.* 2018). Other control strategies rely on the sex-pheromone due to its highly targeted approach, either through monitoring, attract-and-kill, mating disruption or the sterile insect technique.

1.3. Lepidopteran Sex Pheromone

1.3.1. Sex Pheromones in Lepidoptera: Brief Overview

The lepidopteran evolutionary success in terms of species numbers can be attributed to the role of sex pheromones (Ando *et al.* 2004). It is the second largest insect Order, in part due to the specialised communication involved in mating. The sex pheromone is released for the benefit of a specific partner and plays an important role in reproductive isolation, which can lead to speciation (Campion & Nesbitt 1981). Therefore, thousands of variations in the chemical structures of the pheromone have been found, not only at a species-specific level but also at the population level. Consequently, due to the highly targeted control strategies that can be implemented using the sex pheromone, enormous efforts have been expended on research into lepidopteran sex pheromones (Ando *et al.* 2004). For an adult moth to find a mate, the female 'calls' for a male to reproduce with her, through the release of the sex pheromones (Campion & Nesbitt 1981). Volatiles are typically released by the female from a gland located at the end

of their abdomen (Cardé *et al.* 1977). Within the Lepidoptera, it is usually the females releasing the sex pheromone but, in some species, it may be released by males (Holdcraft *et al.* 2016). Even rarer, a dual system is used in which both male and female-emitted chemicals cause aggregation (Cardé *et al.* 1977). The pheromone released can be comprised of innumerable blends of compounds, however, subtle differences in ratios, even when the same chemical structures are utilised, can result in reproductive isolation between species (Campion & Nesbitt 1981; Ando *et al.* 2004; Ando & Yamakawa 2011).

Even within a species, different populations may have pheromone ratio variants of the same compounds. This demonstrates the narrow chemical communication system that maintains a tight control over the production of each unique isomeric ratio (Roelofs & Bjostad 1984). Lepidoptera use pheromones as a form of intraspecific sexual communication that in turn strengthens interspecific isolation (Groot *et al.* 2016). The divergence of sex pheromones from one population to another has confused scientists due to the intricate relationship between signal production from the females and the response from the males. This system is expected to result in evolutionary stasis, not diversification (Gould *et al.* 2010). Nevertheless, this process of speciation has occurred in many moth species that are geographically distinct, where pheromones are quantitatively different despite sharing the same components of the secretions (Khannoon *et al.* 2013).

1.3.2. Inter-population pheromone variation

Pheromone ratio variants have previously been identified in FCM, where three compounds (trans-8-dodecenyl acetate, cis-8-dodecenyl acetate and dodecyl acetate) were found in ratios of 76%, 10% and 14% respectively in South Africa, 32%, 52% and 16% in Malawi and 69%, 23% and 8% in the Ivory Coast (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986; Hofmeyr & Calitz 1991). Due to the newer techniques of sequential solid-phase microextraction gas chromatography–mass spectrometry analysis used by the Israeli researchers, clearer results in the sex pheromone of female FCM found 11 components comprising the sex pheromone. However, several of these components comprised only less than 5% of the total pheromone, with four components described as the major compounds (trans-8-dodecenyl acetate (64 ± 10.5%), trans-8-dodecenyl alcohol (11 ± 2.1%), dodecyl acetate (11 ± 2.2%), and cis-8-dodecenyl acetate (6 ± 1.1%) (Levi-Zada *et al.* 2019).

All the different ratios of the sex pheromone led to uncertainty whether these differences were as a result of extraction methods or due to geographically distinct populations. Timm (2010) found significantly genetically differentiated populations of FCM in South Africa, leading to one hypothesis that the differing pheromone blends could be a result of genotypic differences leading to differences in sexual attractiveness amongst populations (Timm 2010). Changes in the composition of sex pheromones at different geographic locations has also been discovered in the Japanese rice leaffolder moth, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). The leaffolder moth populations in India, the Philippine's and Japan all had a pheromone blend consisting of four components, but the percentage of each component within the blend changed geographically (Kawazu *et al.* 2000). Sex pheromone composition changes have also been discovered in fall armyworm, *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae). Unbehend *et al.* (2014) discovered that geographic differentiation between populations may cause regional sexual communication differences when two different commercial pheromone blends were tested on fall armyworm.

1.3.3. Chemical structure of sex pheromones

Structurally, lepidopteran sex pheromones are simple, but diverse (Ando *et al.* 2004). Lepidopteran sex pheromones comprise two main groups, Type I and Type II, as well as a third smaller novel group. FCM pheromones are Type I as they are comprised of long straight chains (C_{10}-C_{18}) with primary alcohols and their derivates (mainly acetates and aldehydes) (Ando *et al.* 2004). Type I forms the predominant group with 75% of lepidopteran species utilising these compounds. Type I pheromones also have a mixture of three fatty acids with both single and double bonds, and the hydrocarbon chains all have acetate ester functional groups. The three different compounds which constitute the FCM pheromone blend, are often challenging to measure as the content of pheromone typically released by the insect is quite low (Ando *et al.* 2004).

The cellular process of sex pheromones occurs in the hemolymph when chemical signals, known as pheromone biosynthesis activating neuropeptides (PBAN) are released. The PBANs released in the haemolymph activates a G-protein-coupled receptor known as PBANr, within the pheromone gland. Calcium channels are opened when PBAN binds to the PBANr (receptor), allowing an influx of Ca^{2+} which triggers pheromone production via secondary messengers (Groot *et al.* 2016). The female moths then release the chemical signals which are

unimodal and readily quantified by the males. Methods of measuring the pheromones includes extracting the entire pheromone gland, however, this measures every compound present and not necessarily only those that are expressed and are important in calling. Gas chromatography coupled to mass spectrometry (GC-MS) is more readily used to reveal the chemical structure (Burger 1985; Ando & Yamakawa 2011).

1.3.4. Role of sex pheromones in mating

Although adult male lepidoptera often show secondary sexual characters as glands over certain places in the body, termed androconia, it is the female moth behaviour that plays an important role in the dispersal of the sex pheromone in most species (Gemeno *et al.* 2000; Ando *et al.* 2004; Levi-Zada *et al.* 2019). When the female moth calls for a mate, she will cling to a surface and release the pheromones from the abdominal tip between segments eight and nine at the dorsal side (Attygalle *et al.* 1986). As the pheromone is released, the wings become rigid and vibrate. The maximum calling time has been reported as 4-5 hours after scotophase, as well as between 4:00 and 6:00 am with the maximum calling age reported at three days post-eclosion (Atygalle *et al.* 1986; Levi-Zada *et al.* 2019). The release of these pheromones elicits a sexual response in conspecific males (Greenfield 1981). The sex pheromone released by the female travels downwind, where males can pick up the pheromone molecules and orientate themselves towards the female (Hirooka 1986). The female sex pheromone, once released, creates a 'plume' or tunnel-like formation towards the male, aiding the male moth in his search for a mate. To remain in the plume and not drift away, the male moth experiences a number of programmed turnings when exiting the plume to remain on the correct path (Figure 1.3) (Svensson 2008).

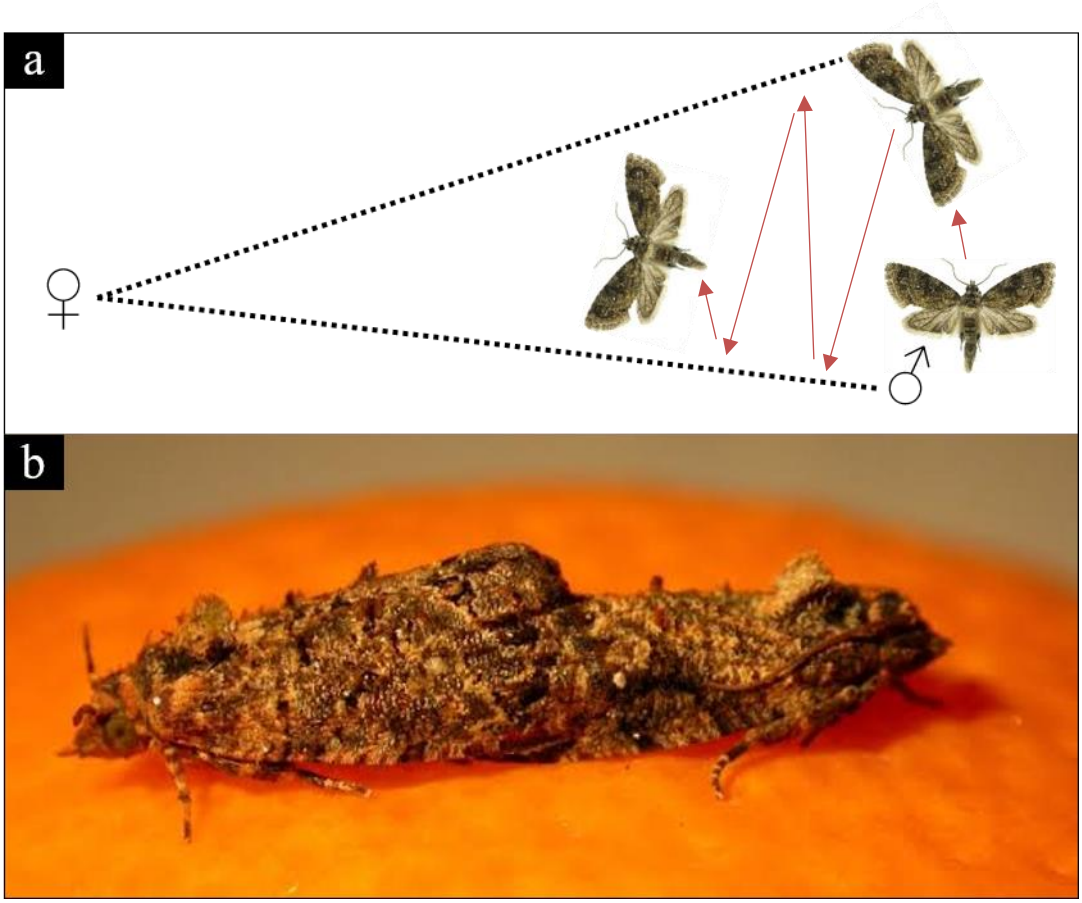


Figure 1.3. (a) Diagram derived from Hirooka (1986) demonstrating the structure of the sex pheromone plume and a track of a male flying through it, (b) resulting in a mating couple (invasives.org).

1.3.5. The exploitation of pheromones for integrated pest management (IPM)

The use of synthetic pheromones to manage lepidopteran pests has been around for more than 50 years (Bellas *et al.* 1983). In the early days of such pheromone use, countries with advanced technology could readily utilise these methods (Campion & Nesbitt 1981). However, due to the success with using pheromones to help monitor pests, a need for this expanded in the mid-70's in south eastern Africa. At this time, this region of Africa was facing immense crop damage by African armyworm, *Spodoptera exempta* Walker (Lepidoptera: Noctuidae), and red bollworm, *Diparopsis castanea* Hampson (Lepidoptera: Noctuidae) (Campion & Nesbitt 1981). The Scientific Units of the British Overseas Development Administration helped local research institutions develop pheromone traps for these pests in order to help slow down the

spread of the pest through appropriate spraying determined from the capture in pheromone traps. Pheromone traps made a significant difference to agriculture helping farmers to monitor if the pests are present, and in what numbers. This allowed farmers to calculate how much and how often they would need to spray, resulting in the success of the Shire Valley cotton region of Malawi which was able to save 100% of their yield from bollworm damage in 1977 (Campion & Nesbitt 1981). At present, there are four categories of control utilising synthetic pheromones namely, monitoring, attract-and-kill, mating disruption, and the sterile insect technique (Moore & Hattingh 2016). These are discussed with respect to FCM management below.

1.3.5.1. Monitoring

Monitoring is mostly used to identify and alert the presence of FCM in orchards, when further management strategies can be implemented. Monitoring as a management tool can aid in determining the correct timing of control application, prioritising control of orchards and data from the trap can be used in comparisons of FCM numbers between seasons (Moore 2011). A product called F.C.M PheroLure® (Insect Science, Tzaneen, RSA) is comprised of the female FCM sex pheromone (E7-dodecenyl acetate (12.5 mg); E8-dodecenyl acetate (10.4 mg); Z8-dodecenyl acetate (2.1 mg)) and is hung in a yellow delta trap with a sticky floor, at the top of citrus trees upwind of the orchard in order for the wind to carry the pheromone downwind towards any males. In FCM-susceptible citrus, a single trap is placed every 4 to 6 hectares and as high up in the tree as possible (Hofmeyr & Burger 1995; Moore 2019). Monitoring must be used in conjunction with control methods and must be used with weekly orchard inspections of fruit infestation to maintain orchard pest numbers at an acceptable level (Moore 2011).

1.3.5.2. Attract-and-Kill

This method involves a synthetic pheromone source and an insecticide (Krupke *et al.* 2012). The deployment of the pheromone source lures moths (usually males) where they then come into contact with the insecticide and die. This reduces the number of males in the orchard available to mate with females (Campos & Phillips 2014). Attract-and-kill is different to mating disruption in that a lower concentration of synthetic pheromone is used along with a toxicant in order to attract and kill the male moth, instead of flooding the target area with large amounts of synthetic pheromone. A registered product called Last Call™ FCM (Insect Science,

Tzaneen, RSA) can be used for attract-and-kill. The Last Call product consists of a synthetic pheromone, a pyrethroid and a protective gel which is applied to the trees. The males are then attracted to the droplets and are killed shortly after contact with the droplet (Moore & Hattingh 2012). This control method is only effective when there are low FCM population numbers. Therefore, it should be used early in the citrus growing season (Moore 2017). This is because the trap is in direct competition with females releasing the pheromone (Poullot *et al.* 2001) and because the amount of synthetic pheromone released is usually not enough to outcompete wild females. For pests other than FCM, attract-and-kill can be conducted both in the field or in a warehouse where food is stored to reduce infestations post-harvest (Campos & Phillips 2014).

1.3.5.3. Mating disruption

The premise behind mating disruption is to confuse males when they are trying to find a mate by placing pheromone dispensers and flooding the area with sex pheromones. By preventing mating, the number of viable eggs oviposited onto the fruit is reduced (Moore 2019). Therefore, mating disruption cannot be used in conjunction with attract-and-kill as the increased pheromones will render the trap redundant. Mating disruption has some limitations as it needs to cover a large area to reduce edge effects as well as preventing moths from mating and moving into the control area. This control also needs to be employed early in the season before large FCM populations establish. Mating disruptors need to be hung relatively high in the trees as the synthetic pheromones are denser than the surrounding air and will sink (Moore & Hattingh 2012). Therefore, if they are hung too low, the flying moths may be unaffected. Orchard hygiene and FCM population numbers needs to be properly managed for mating disruption to be used as part of an integrated pest management scheme. There are currently four registered commercial products used for mating disruption: Isomate® (Bioglobal Limited, Australia), Checkmate® FCM-F (Suterra, USA), Splat-FCM (River Bioscience, Port Elizabeth, RSA) and X-Mate™ FCM (Insect Science, Tzaneen, RSA) (Moore 2017). The Isomate product comes in the form of a polyethylene tube dispenser that is hung in the citrus tree and releases synthetic pheromone ((E)-7-dodecenyl acetate, (E)-8-dodecenyl acetate and (Z)-8-dodecenyl acetate (Moore & Hattingh 2012). Isomate has been shown to reduce FCM infestation by 55% in an orchard with high FCM abundance and by 75% in an orchard with low FCM abundance (Moore & Hattingh 2012). Checkmate is a formulated spray, which is applied to the top third of the tree every 21-28 days (Moore & Hattingh 2012; Moore 2016). The amount of synthetic

pheromone that is released into the orchards using these products may potentially mask the monitoring traps synthetic pheromone lure and cause trap shutdown.

1.3.5.4. Sterile Insect Technique (SIT)

The sterile insect technique (SIT) was introduced in South Africa in the 1990s after its success in eradicating New World screwworm flies in North America, with research into extending SIT to control FCM beginning in 2002 (Hofmeyr *et al.* 2016). Sterile insect technique has also been reported as successfully controlling a large number of agricultural pests including tsetse fly, bollworm, melon fly and Queensland fruit fly (Hendrichs & Robinson 2009). For FCM, sterilisation occurs via irradiation of the reproductive cells, resulting in premature death of zygotes due to breaks in chromosomes, leaving the moth sterile (Robinson 2005). Irradiation is conducted at a frequency of 150 Gy, produced from cobalt-60 and caesium-137 point source radiators (Bloem *et al.* 2003; Robinson 2005; Hofmeyr *et al.* 2015). Mixed-sex sterilisation is performed, as the irradiation process does not discriminate between males and females. At this level of radiation, females are 100% sterile while males have residual fertility, i.e. partially sterile, in the event that these males produce progeny, the offspring will be predominantly male and 100% sterile (F1 sterility) (Bloem *et al.* 2003). Reducing radiation to the lowest possible level is important to maintain competitive mating behaviour in the sterile individuals (Bloem *et al.* 2003). Sterile FCM are released into a target area at an overflooding ratio of 10:1, sterile:wild (Bloem *et al*. 2003; Hofmeyr *et al.* 2015).

This control method is conducted on a commercial scale by a company, Xsit (Pty) Ltd, which has been able to reduce FCM population numbers in orchards by 95% since it was registered in 2007 (Hofmeyr *et al*. 2015; Nepgen *et al.* 2015). In Navel oranges in the Eastern Cape, 80% reduction in FCM was achieved and in the Citrusdal region up to 93% decrease in pre-harvest crop loss from 2007 to 2010 was recorded (Hofmeyr *et al.* 2015). Xsit treats over 13 000 ha of orchards and are able to produce up to 12 million sterile moths per week (Hofmeyr *et al.* 2015). The sterile moths are transported to orchards via cooled vehicles to reduce activity and possible damage to moths that may reduce overall fitness (Hofmeyr *et al.* 2015). Upon arrival at orchards, sterile moths are dropped aerially by R22 helicopters, gyrocopters, all-terrain vehicles and small crop spraying aircrafts into the orchards where they compete with wild moths to mate. Sterile insect technique extends over the entire growing region and air drops need to happen twice a week, for over 10 months (Hofmeyr *et al.* 2015). This is to reduce the

potential of already mated females moving into the area under SIT, which could then lead to the unaffected females laying their eggs onto the fruit (Hendrichs 2002). Xsit (Pty) Ltd currently release sterile moths in orchards in the Western Cape Province, Eastern Cape Province and the Northern Cape Province, and in future will extend to all citrus growing regions.

1.4. Geographically Distinct Populations

Insect population genetic diversity and population structure are shaped by intrinsic and extrinsic traits. Intrinsic traits identify migration behaviour and dispersal ability as opposed to extrinsic features, which includes host plant use, and the distribution of populations (Kirk *et al.* 2013). Population genetics is a useful tool in pest management, especially information on gene flow among populations, identification of invasive species, and characterisation of host races (Rollins *et al.* 2006; Groot *et al.* 2011, Medina *et al.* 2012*;* Silva-Brandao *et al.* 2012; Kirk *et al.* 2013). Genetic variation resulting in speciation can occur in geographically isolated populations. As discussed, the idea of geographically distinct populations of FCM was first tested by Attygalle *et al*. (1986) who concluded that there are likely to be sub-populations of the moth, due to different ratios in the compounds comprising the sex pheromone in moths from different regions. On a local scale, Timm (2005) investigated if geographically distinct populations occurred within South Africa, using microsatellite molecular techniques. High levels of polymorphism amongst FCM populations were found in the Western Cape, Eastern Cape and Mpumalanga provinces. Polymorphisms are regions of DNA that differ between individuals, with greater polymorphism resulting in greater differences between individuals. FCM from the Western Cape yielded the greatest genetic variation from other South African populations, however, FCM sampled within the same province were generally more closely related than FCM from different provinces. Timm (2005) concluded that FCM had more or less locally adapted populations, which could create challenges when implementing control strategies, speculating that FCM may vary in its response to insecticide resistance or microbial susceptibility.

Recently the species status of FCM was tested by Mgocheki & Addison (2016), using *mt*DNA. Their results found that all individuals tested from three provinces (Western Cape, Eastern Cape, Mpumalanga) formed one clade and therefore comprise no more than one species. However, in orchards, where only short distance flights are required for FCM to reach another

host plant, and where host plants are long-lived, the most successful ecological strategy for the moth would be to stay within the habitat. This would allow individuals to avoid the risks associated with long-range dispersal, which include the likelihood of not locating alternate resources and the increased probability of predation (Hardie *et al.* 2001; Weisser 2001). Therefore, in a fragmented ecosystem, such as an agricultural environment, it is suspected that genetically differentiated populations would arise.

Given this high level of genetic variation amongst FCM populations, coupled with the knowledge that sex-pheromone blends may vary amongst geographically isolated populations, it is possible that FCM within an area are more attracted to one another than those from elsewhere, as a result of probable differences in isomer ratios within the pheromone. Preliminary field trials conducted by Joubert (2017) showed that male FCM from a population originating from Citrusdal preferred females from their own population when offered the choice of virgin females from other citrus-producing regions. In addition, cross-mating trials with moths from four different regions and a mixed laboratory population (Addo, Citrusdal, Marble Hall, Nelspruit, Old) found females produced more eggs when mated with males from the same population; however, this was only significantly different for Marble Hall moths Joubert (2017).

Comparative biological performance of several false codling moth populations was conducted by Opoku-Debrah (2008) where it was found that the Citrusdal population had a low comparative biological performance. Opoku-Debrah (2008) attributed this to the fact that FCM is not indigenous to this area and is considered to have been accidentally introduced (Hofmeyr 2016). However, Timm (2005) assessed the genetic diversity of moths from the Western Cape and found that the diversity was still high. The population did not appear to have suffered from a genetic bottleneck, resulting from founder effects, which usually decrease genetic diversity. This high level of genetic diversity may partly explain the success of FCM in a variety of habitats. Further evidence of regionally distinct populations can be shown through the FCM specific Cryptophlebia leucotreta Granulovirus (CrleGV-SA). Opoku-Debrah *et al*. (2013) found that there were five CrleGV-SA isolates latent within the insect populations.

1.5. Motivation and Aims

FCM threatens the citrus industry, which is worth over twenty billion ZAR (CGA 2017). International markets expect high quality fruit, free of insect pests and diseases but more

importantly the phytosanitary pest, FCM. Many of our current export partners have a zero tolerance for FCM, as the pest is not present in those countries. Therefore, if the pest is detected in an export consignment, the entire consignment can be rejected, resulting in substantial monetary loss. Multiple management options have thus been developed to protect the industry. Many of these rely on pheromones, namely monitoring, mating disruption, attract and kill and the sterile insect technique (SIT). As previously mentioned, the most recent analysis indicates the female FCM pheromone as being comprised of four different major isomers: (E)-8-dodecenyl acetate, (E)-7-dodecenyl acetate, (Z)-8-dodecenyl acetate and dodecyl acetate (Levi-Zada *et al.* 2019). However, the relative composition of these isomers may change regionally (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986).

Joubert's (2017) study is important as it showed clear favouring by males for females from the same region. Another example of this phenomenon is displayed by male FCM from Mpumalanga, South Africa, that are not readily attracted by the commercial synthetic pheromone used in delta traps to monitor FCM population in the Western Cape Province (Mgocheki & Addison 2016). This could be problematic for control strategies that rely on the sex pheromone, such as SIT, where the sterile moths already have a disadvantage in reduced fitness over wild males. Reduced fitness from irradiation and transport, coupled with reduced sexual communication, may result in a considerable decrease in SIT efficacy. However, due to its success to date, SIT has been rolled out elsewhere in the country, including the Eastern Cape (Sundays River Valley and Gamtoos River Valley) and the Northern Cape, with potential to expand into Mpumalanga and Limpopo.

Due to the current control technologies utilising the sex pheromone, which offer unprecedented reduction in infestation, with no negative secondary environmental effects, it is important to identify if regional compatibility between geographically distinct populations of FCM occurs in South Africa. Additionally, the regional suitability of sterile moths must be determined, to avoid potential loss in efficacy of SIT if used in other locations across the country. If regional attractiveness is found to be an issue, strategies to overcome its effect need to be devised. Therefore, this research aimed to establish if genetic integrity of regional FCM populations in laboratory-reared cultures has been maintained (Chapter 2), analyse regional attractiveness between males and females from different geographic regions (Chapter 3), and determine whether these translate into differences in mating compatibility between populations (Chapter 4).

Chapter 2

Regional population structure of laboratory-reared FCM using amplified fragment length polymorphism (AFLP)

2.1. Introduction

Molecular genetic analysis can be a powerful tool offering insights into population dispersal and gene flow (Haymer 2016). Understanding the genetic diversity of a pest species is important as this knowledge can increase the efficacy of its control. FCM is a pest across South Africa in all citrus growing regions (CMVC 2017). The need to maintain and implement pest control strategies with reduced non-target environmental impacts has never been more pressing (Kirk *et al.* 2013; Nicolouli *et al.* 2018). Invertebrate pest invasions are associated with high social, economic and ecological costs (Kirk *et al.* 2013). Therefore, with the current pressures on agricultural productivity, pest outbreaks should be approached with urgency. Molecular genetic analyses have aided in the management of many lepidopteran pests, which has been useful in combatting resistance development to commercial pesticides and genetically modified crops (Korman *et al.* 1993; Silava-Brandào *et al.* 2015; Juric *et al.* 2017).

However, molecular genetic analyses can be costly, and the popular invertebrate sequencing gene, cytochrome oxidase I (COI), can be problematic when looking for slight differences amongst populations (Hey & Pinho 2012; Sun *et al.* 2012). The COI gene is useful in species identifications but is not as useful when trying to identify subtle molecular differences amongst populations within a species (Sun *et al.* 2012). This is because the COI gene is a highly conserved region that is very A-T rich, as well as exhibiting many indels and tandem repeats (Vila & Bjőrklund 2004). Thus, sequencing of the COI region is often not the best method for population studies. The use of genetic markers and advancement in DNA and PCR technology has led to the development of molecular techniques such as amplified fragment length polymorphism (AFLP) (Figure 2.1), randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (SSR) (Vos *et al.* 1995; Mueller & Wolfenbarger 1999).

These techniques can uncover cryptic genetic changes of closely related species and is particularly useful when identifying different populations of the same species (Vos *et al.* 1995).

The AFLP technique is arguably the best determination of genetic variation due to its greater resolution and reproducibility as well as the relatively inexpensive cost for degree of molecular knowledge output in comparison with SSRs and RAPD (Vos *et al.* 1995; Mueller & Wolfenbarger 1999; Mendelson & Shaw 2005).

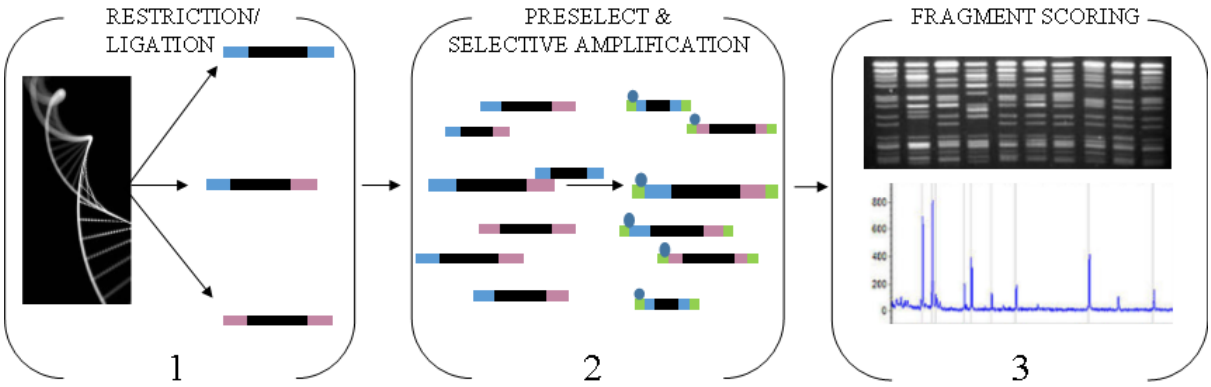


Figure 2.1. Schematic representation of AFLP analysis, showing the three-step process 1. Restriction digestion with rare cutter *Eco*RI and frequent cutter *Mse*I restriction enzymes (resulting in three types of fragments) and ligation of sticky ends; 2. Amplification of fragments, first through a pre-selection phase with no selective nucleotides, followed by a selective phase with three added nucleotides; 3. Analysis of fragments as electropherograms.

Microsatellite analyses such as AFLP are popular in lepidopteran population studies, as the results can facilitate control and resistance management, as well as gain insight to habitat expansion (Reineke *et al.* 1999; Han & Caprio 2002; Lindroth *et al.* 2017). This study used AFLP molecular markers to compare the genetic diversity and structures of FCM populations from laboratory colonies. The populations are named after the five geographical areas from which they originated; Marble Hall, Nelspruit, Addo and Citrusdal as well as a culture of mixed origin, named 'old'. However, the degree of relatedness and population structure between the laboratory populations, representatives of South African populations is unknown. Therefore, the aims of this experiment were to 1) determine if the laboratory colonies are still genetically distinct and 2) determine the population structure amongst the laboratory colonies and compare these to wild populations to verify that the laboratory-reared populations are still isolated for further regional attractiveness and mating compatibility studies.

2.2. Materials and Methods

2.2.1. Insect Colonies

Five laboratory colonies are maintained at a Rhodes University facility, kept at a constant temperature of 26°C ± 2°C, 50-60% relative humidity and a 12-hour photoperiod (L12:D12). Four of the colonies (Addo, Marble Hall, Citrusdal, Nelspruit) were established by Opoku-Debrah *et al.* (2013) from field collected larvae in Addo (33°34'S, 25°41'E, Eastern Cape), Marble Hall (24°58'S, 24°18'E, Limpopo), Citrusdal (32°36'S,19°01'E, Western Cape) and Nelspruit (25°28'S, 30°58'E, Mpumalanga). These colonies have been kept for more than 200 generations. The fifth 'mixed' culture was established by Moore (2002), and kept for more than 300 generations, consisting of field collected larvae from three different areas, Citrusdal (32°36'S, 19°01'E, Western Cape), Zebediela (24°16'S, 29°17'E, Limpopo) and the Eastern Cape. The colonies are maintained on artificial diet developed by Moore *et al.* (2014) and kept in separate rearing cages to prevent interbreeding.

2.2.2. DNA extraction

Fifth instar FCM from the five colonies previously mentioned were used for genetic analysis. Wild samples from Citrusdal, Addo and Clanwilliam, as well as laboratory-reared larvae from Xsit, a colony of mixed origin used for sterile insect technique, were used for genetic analysis. The DNA was extracted from similar amounts of tissue to ensure a comparable concentration of DNA across all samples. Genomic DNA was isolated from entire crushed larvae and preserved in 95% ethanol, according to standard procedures involving proteinase K digestion followed by a salt-extraction method (Hunt. 1997). The DNA concentration was determined using a NanoDrop™ 2000 Spectophotometer (Thermo scientific™). High quality DNA (260/280 = < 2.5) with a concentration of approximately 500 ng/µl was used, although samples containing as little as 110 ng/µl were used successfully (Appendix Table A1).

2.2.3. Amplified Fragment Length Polymorphism (AFLPs)

The AFLP protocol followed Paun & Schönswetter (2012) with modifications as described below.

2.2.3.1. Restriction/ Ligation (RL)

The adapter pairs were prepared as 50 µM *Mse*I and 5 µM *Eco*RI before being heated to 95°C for five minutes in separate vials and gradually cooled to room temperature over 10 minutes. Once cooled, the adapter pairs were centrifuged for 10 seconds and added to the master mix (Appendix Table A2). The master mix was comprised of 0.68 µl ddH_2O, 0.6 µl 400 U/µl T_4 DNA ligase (New England Biolabs), 1.1 µl 2× T4 ligase buffer (New England Biolabs), 1.1 µl 0.5M NaCl, 0.55 µl 1mg/ml BSA (New England Biolab), both the adaptor pairs as well as 0.02 µl 1U *Mse*I (New England Biolab) and 0.05 µl 5U *Eco*RI restriction enzymes (New England Biolab). Approximately 5.5 µl of the master mix was aliquoted into individual tubes with 5.5 µl of DNA (concentrations depending on sample). The final reaction volume of 11 µl was vortexed and centrifuged briefly before incubating at 37°C for three hours and then 17°C overnight. The efficiency of the restriction reaction was evaluated using a 1.5% agarose gel stained with ethidium bromide, containing 5 µl of the RL product, run for 20 minutes at 90 V (Figure 2.2). If the restriction/ ligation was successful, it was diluted 20-fold with ddH_2O to stop the reaction and stored at -20°C until further use.

2.2.3.2. Pre-selective PCR

A master mix was prepared using 2.42 µl of ddH_2O, 5 µl 1× Taq master mix (QIAGEN) and the pre-selective primers (Integrated DNA technologies Inc) (Appendix Table A3 and A4), which were pre-diluted to a concentration of 5 µM each. Approximately 8 µl of the master mix was aliquoted into individual tubes, with 2 µl of the diluted RL product. The final reaction volume of 10 µl was vortexed and centrifuged before running the following programme on the thermocycler: 1 cycle at 72°C for 2 minutes; 20 cycles at 94°C for one second, 56°C for 30 seconds, 72°C for two minutes; 1 cycle at 60°C for 30 minutes; held at 4°C. The efficiency of the pre-selective amplification was tested by running 5 µl of product on a 1.5% agarose gel (Figure 2.2). The final product was diluted 1:20 with ddH_2O.

2.2.3.3. Selective PCR

A master mix was prepared using 1.92 µl ddH_2O, 5 µl 1× Taq master mix (QIAGEN), as well as each of *Mse*I and *Eco*RI selective primers (Integrated DNA technologies Inc.) (Appendix Table A5). In accordance with Timm (2006) five primer pairs were used (Appendix Table A3) at a concentration of 1 µM. As multiple primers sets were used, a master mix for each primer

combination was prepared. Approximately 2 µl of the diluted pre-selective product was added to 8 µl of the selected PCR mix, resulting in a final volume of 10 µl. This final product was vortexed and centrifuged briefly before running the following programme on the thermocycler: 1 cycle at 94°C for 2 minutes; 9 cycles at 94°C for 1 second, 65°C (-1°C for every cycle until 57°C), 72°C for 2 minutes; 24 cycles at 94°C for 1 second, 56°C for 30 seconds, 72°C for 2 minutes; 1 cycle at 60°C for 30 minutes; held at 4°C. The final PCR product was frozen at -20°C and sent to Central Analytical Facilities (Stellenbosch, South Africa) for gel electrophoresis.

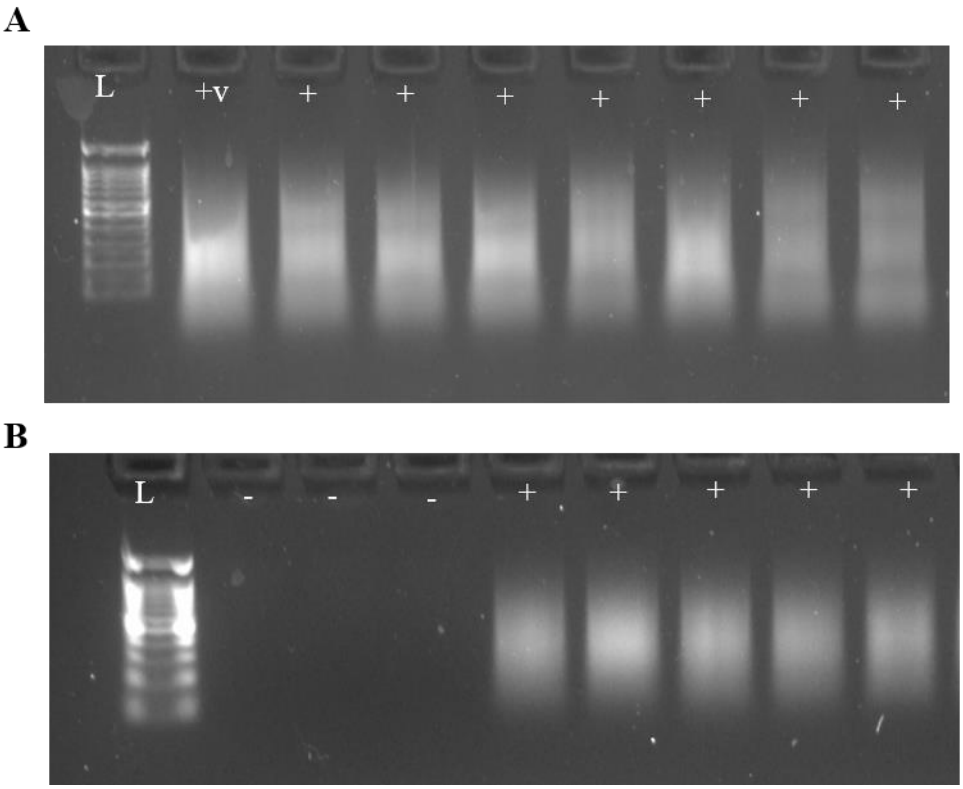


Figure 2.2. Images of 1.7% agarose gel showing A) successful RL reactions and B) successful and unsuccessful preselect reactions; if the reaction was unsuccessful, the analysis was redone until acceptable products were indicated (+v positive control (lamda DNA); + successful; - unsuccessful; L ladder).

2.2.4. AFLP analysis

2.2.4.1. Scoring of bands

The .ABI files received from the sequencer were analysed as electropherograms, using GeneMarker® version 2.7.0 (SoftGenetics LLC.). The filtering parameters were set as **Peak Detection Threshold**: Intensity>100, Percentage>1 Max, Local Region % > 1 Local Max; **Stutter Peak Filter**: (%): Left: 5, Right: 5; **Plus-A Filter**: selected; **Allele Evaluation Score**: Reject <1 Check 7< Pass; **Unconfidence at Rightside Score**: <30. These filtering parameters were chosen as they are considered conservative and minimise genotyping errors (Whitlock *et al*. 2008). From GeneMarker a Peak Table consisting of columns with data of the allele, marker, dye, size, height, ht-ratio, area, ar_ratio, quality, score, allele comments, start, end and difference was exported into RawGeno ver. 2 (Arrigo *et al.* 2009), run as a CRAN mirror of the R ver. 3.5.2 (2019) statistical platform (The R Foundation for Statistical Computing). The filtering parameters for RawGeno were set as follows: **minimum bin width**: 1.0 base pairs (bp); **maximum bin width**: 1.5 bp; **scoring ranges:** from 50 – 800 bp; **low frequency threshold**: 200 fluorescence units; **reproducibility**: 80%. As there were only five samples per population, all samples were replicated post-DNA extraction, to determine standard error rates and no blank samples were included.

2.2.4.2. Genetic diversity

The resulting binary matrix was imported and consolidated in Microsoft® Excel. The binary matrix was then converted to a pairwise similarity matrix to investigate the relationships among populations. The pairwise similarity matrix was generated via one-way Analysis of Similarities (ANOSIM); where R=1 was the most distant, and R=0 the most related. Genetic distance values were used to construct a dendogram, using the unweighted pair group method with arithmetic mean (UPGMA) metric in PAST®: paleontological statistics package ver. 3.0 (Hammer *et al.* 2001). At population level, the number of polymorphic bands (N_p) and the percentage of polymorphic bands (*PPB*) were calculated using GenAlEx v6.5 Excel software package (Peakall & Smouse 2005). Shannon Information Index (H_o), the observed number of alleles per locus (N_a), the effective number of alleles per locus (N_e) and Nei's genetic diversity (H_j) were calculated using POPGENE v3.0 (Yeh & Yang 1998; Fu *et al.* 2016).

2.2.5. Genetic structure

Relationships among individuals and populations were viewed as Principal Coordinates Analysis (PCoA) plots using Jaccard's permutation in the statistical software PAST, applying the Jaccard's permutation for binary genetic data as it excludes shared absence as a genetic character (Sokal & Michener 1953). A Bayesian model-based cluster analysis was conducted using STRUCTURE v2.3.4 (Falush *et al.* 2007). The software infers the genetic structure and defines the number of clusters in the data set. The correlated allele frequencies and admixed model were applied with 10 000 burnin and 250 000 Monte Carlo Markov Chain (MCMC) with 10 repetitions of the analysis. The admixed model was used, as it is the most efficient model to study intraspecific variation (Falush *et al.* 2007). Using an online programme, Structure Harvester v0.6.94, the results from STRUCTURE v2.3.4 were uploaded to determine the best supported K value, i.e. to determine the true number of natural populations. Structure Harvester uses two methods of determining K. The first technique uses ΔK to estimate true K when the peak of the score is at its highest (Evanno *et al.* 2005). The second technique uses L(K) to determine the best supported K, given the plateau of L(K) with high variance (Rosenburg *et al.* 2001).

2.2.5.1. Genetic differentiation

The relationships between populations were determined based on pairwise measures of genetic distance (*D*) and genetic identity (*I*), calculated using Nei's (1978) unbiased genetic distance algorithm with the software POPGENE v3.0 (Yeh & Yang 1998). Analysis of molecular variance (AMOVA) among and within populations was performed using GenAlEx v6 excel software package (Peakall & Smouse 2005) with 999 permutations. The population estimator PhiPT, which is an analogous statistic of F_{st} was calculated from the among-population variability observed during the AMOVA analysis, which is a measure of population differentiation for binary data (Timm *et al.* 2010).

2.3. Results

2.3.1. AFLP analysis and genetic diversity

An average of 314 loci per primer, with around 127 amplified fragments per individual were generated from five paired primer sets on 30 laboratory-reared individuals and 13 wild individuals through the optimised AFLP technique on FCM populations in South Africa (Table 2.1). The fragments generated ranged between 47 and 495 base pairs. The total percentage polymorphic loci for laboratory-reared populations ranged from 33.4% in Marble Hall to 52.9% in the Xsit laboratory-reared population. The percentage polymorphic fragments were significantly higher for wild populations at 75.3% for the Eastern Cape and 72.4% for the Western Cape. For genetic diversity amongst the laboratory-reared populations, Xsit had the highest genetic diversity (N_e =1.31; H_j =0.185; H_o =0.1681) closely followed by the Old population (N_e =1.31; H_j =0.184; H_o =0.1575), the lowest genetic diversity was found in the Marble Hall population (N_e =1.23; H_j =0.135; H_o =0.1282) (Table 2.2). The total genetic diversity for the laboratory-reared populations, as determined by the one-way ANOSIM, was high (R=0.77; P=0.001). Overall, the wild populations showed higher genetic diversity than the laboratory-reared populations, with Eastern Cape the highest (N_e =1.45; H_j =0.26; H_o =0.1963) followed by Western Cape (N_e =1.38; H_j =0.23; H_o =0.1867).

Table 2.1. Summary of primer performance with the total loci, mean number of fragments per individual and the max and minimum fragments generated by each primer combination. (E-EcoRI; M-MseI).

Primer Combo	E-	M-	Total loci	Mean fragment /individual	Max; Min
1	**CTG**	**CAC**	308	29 ±8,9	53 ; 17
2	**ATC**	**CTA**	324	40 ±13,33	74 ; 20
3	**AGT**	**CTA**	389	44 ±17,9	82 ; 2
4	**ATC**	**CAT**	327	40 ±15	74 ; 8
5	**GAC**	**GCA**	324	27 ±11	54 ; 12

Table 2.2. Genetic diversity of FCM distributed among populations in different geographical regions. Codes: N_p, Number of polymorphic loci; PPL, percentage of polymorphic loci; N_a, observed number of alleles per locus; N_e, effective number of alleles per locus; H_j, Nei's gene diversity index; H_o, Shannon information index.

Population	N_p	PPB (%)	N_a	N_e	H_j	H_o
Addo	765	45.78	1.4578	1.2961	0.1732	0.1571
Citrusdal	700	41.88	1.4188	1.2719	0.1595	0.1365
Nelspruit	696	41.56	1.4156	1.2751	0.1581	0.1336
Marble Hall	559	33.44	1.344	1.2318	0.1352	0.1282
Old	825	49.35	1.4935	1.3163	0.1849	0.1575
Xsit	885	52.92	1.529	1.3168	0.1852	0.1681
EC	1259	75.32	1.7532	1.4566	0.2649	0.1963
WC	1211	72.4	1.724	1.3837	0.23	0.1867

2.3.2. Population structure

The principal coordinate analysis (PCoA) for 25 laboratory-reared individuals of FCM revealed three groups (Figure 2.3): Individuals from Addo, Citrusdal and the Old populations; individuals from Nelspruit; and individuals from Marble Hall. The first principal vector explained 14.38% of genetic variance while the second principal vector explained 11.72%. Another PCoA was constructed to visualise the relationship between individuals from the wild populations (Figure 2.4). The two populations, Eastern Cape and Western Cape, grouped separately with the first principal vector explaining 10.61% and principal vector two explaining 8.94% of the variation. The laboratory-reared Xsit population was compared with the two wild populations on a separate PCoA (Figure 2.5), where it grouped apart from the Eastern Cape and Western Cape.

The STRUCTURE clustering revealed that the LnP(D) was greatest and ΔK reached its maximum when K=3, demonstrating that all populations fall into three clusters (Figure 2.6. and 2.7). The blue cluster included most individuals from all populations. The green cluster included individuals from Addo, Citrusdal and Old only, while the red cluster predominantly occurred in the Nelspruit population, with small representations in Addo, Marble Hall and Old. The UPGMA also suggested that populations or individuals gathered in three clusters that closely matched their geographical distribution (Figure 2.8.)

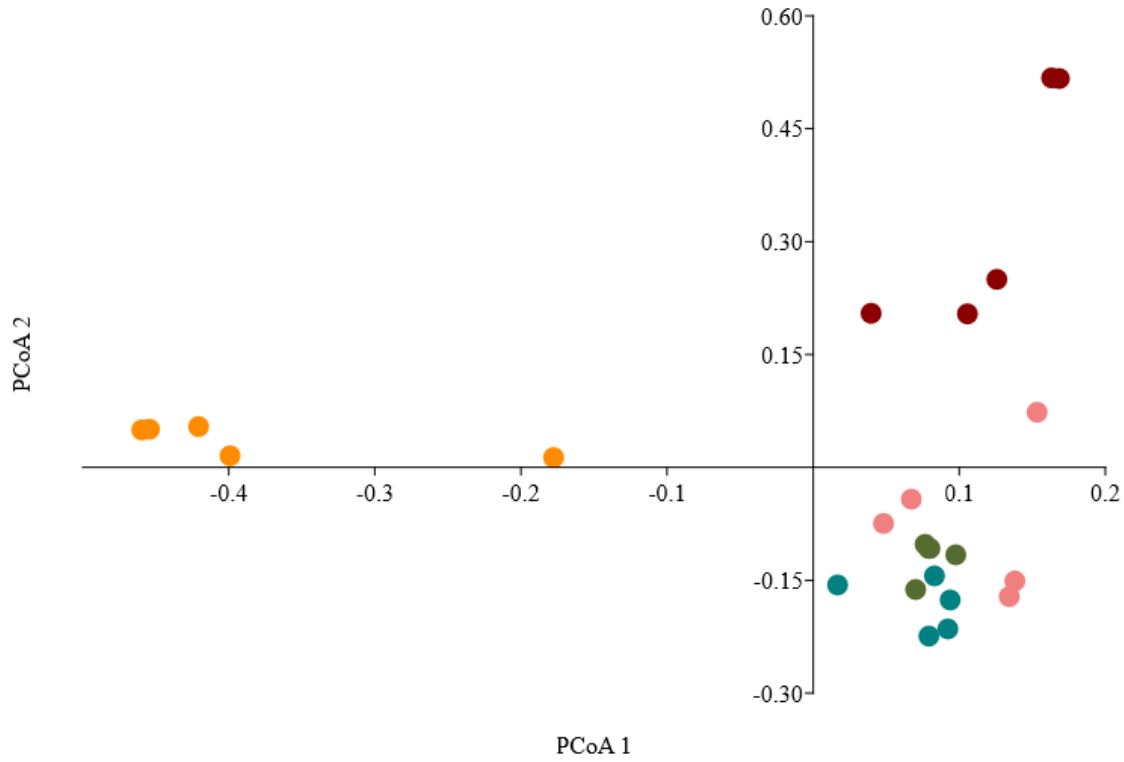


Figure 2.3. PCoA displaying the relationships of 25 individuals from five laboratory-reared populations of FCM (yellow = Nelspruit; red = Marble Hall; pink = Old; green = Citrusdal; blue = Addo).

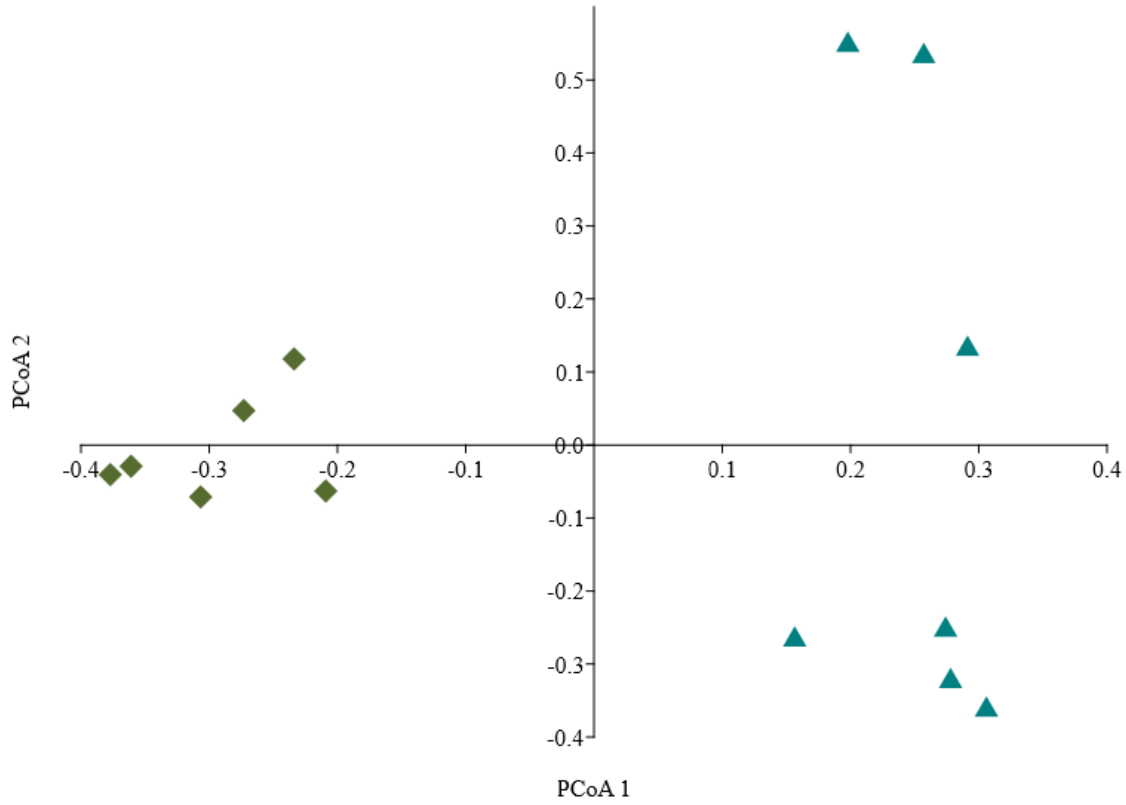


Figure 2.4. PCoA displaying the relationships of 13 field-collected individuals from populations originating from the Eastern Cape (blue triangle) and Western Cape (green diamond).

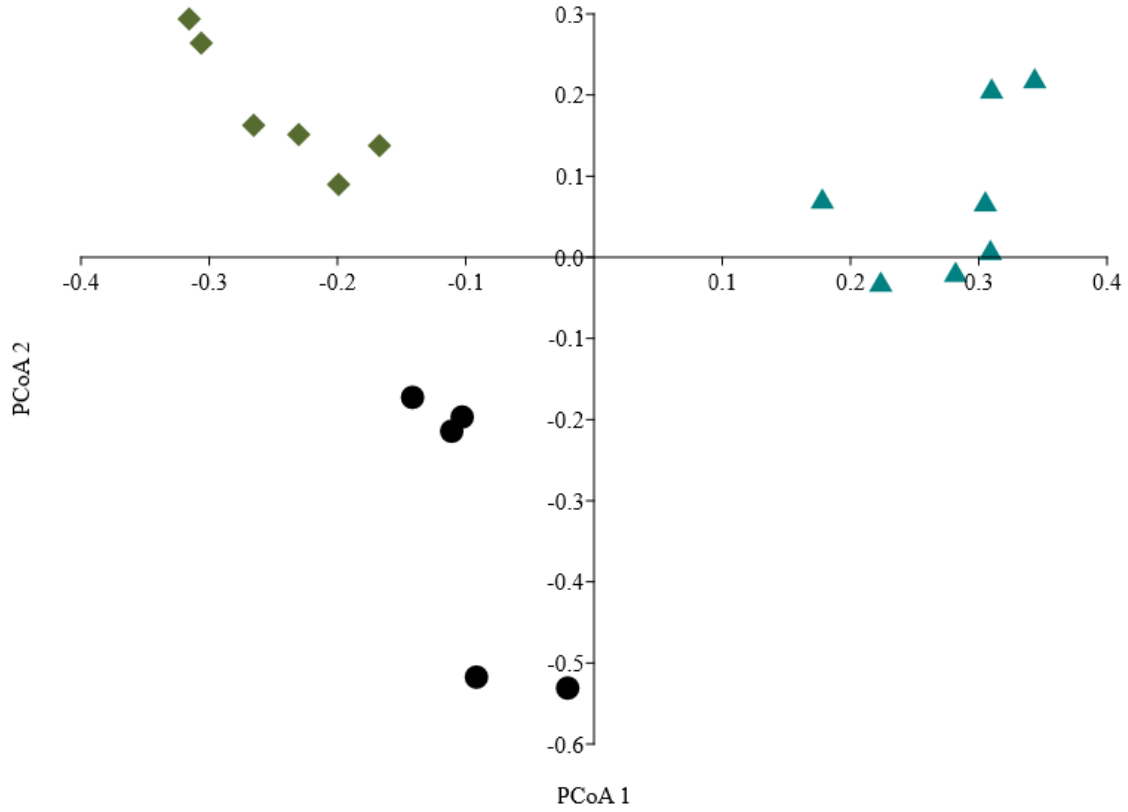


Figure 2.5. PCoA displaying the relationships of 13 field-collected individuals from populations originating from the Eastern Cape (blue triangle) and Western Cape (green diamond) as well as five individuals from a laboratory-reared culture called Xsit of mixed origin (black circle).

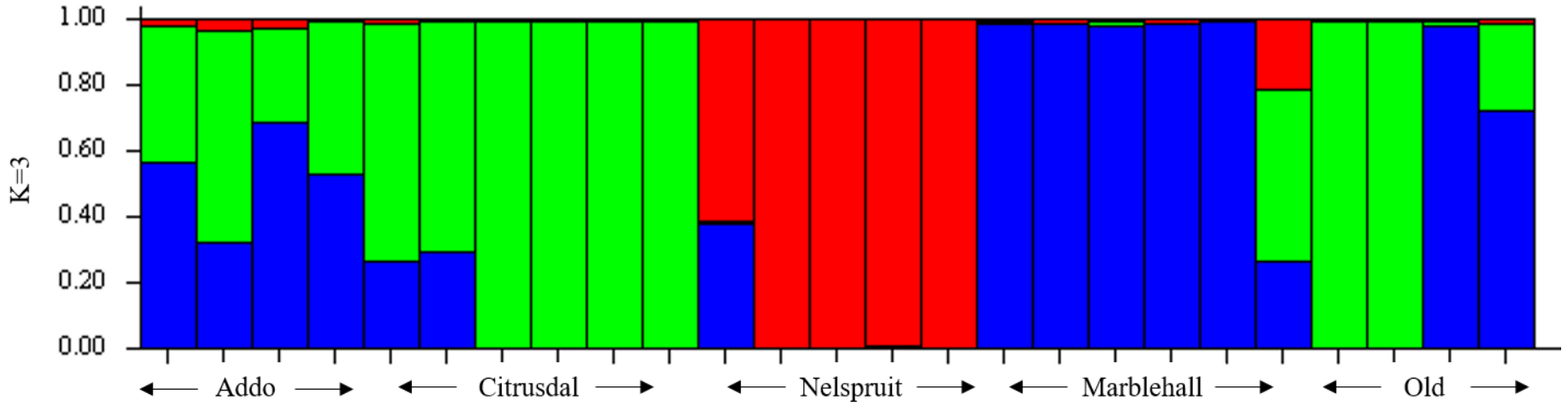


Figure 2.6. Bayesian assignment probabilities based on 25 FCM samples generated from AFLP data created by STRUCTURE v2.3.4. Each individual is represented by a single column divided into three *K* - genetic clusters.

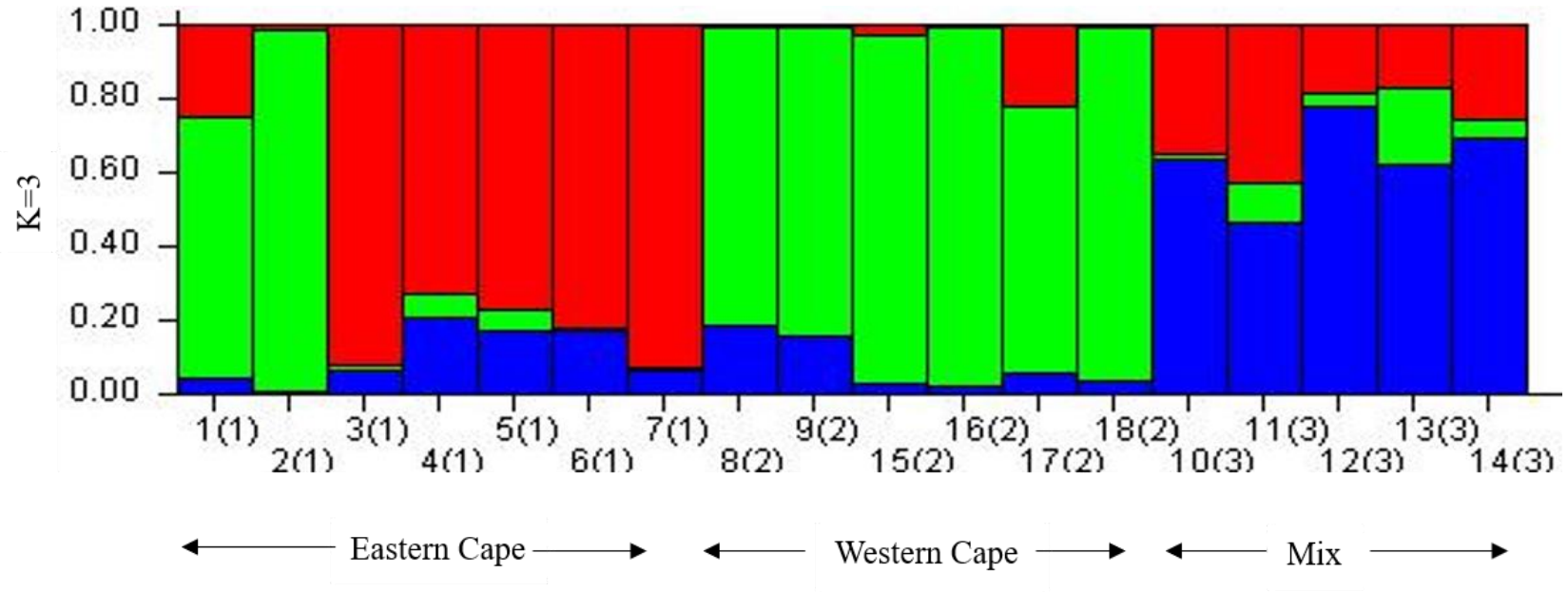


Figure 2.7. Bayesian assignment probabilities based on 18 FCM samples for two wild populations (Eastern Cape and Western Cape as well as a mixed origin population from Xsit) generated from AFLP data created by STRUCTURE v2.3.4. Each individual is represented by a single column divided into three *K* - genetic clusters.

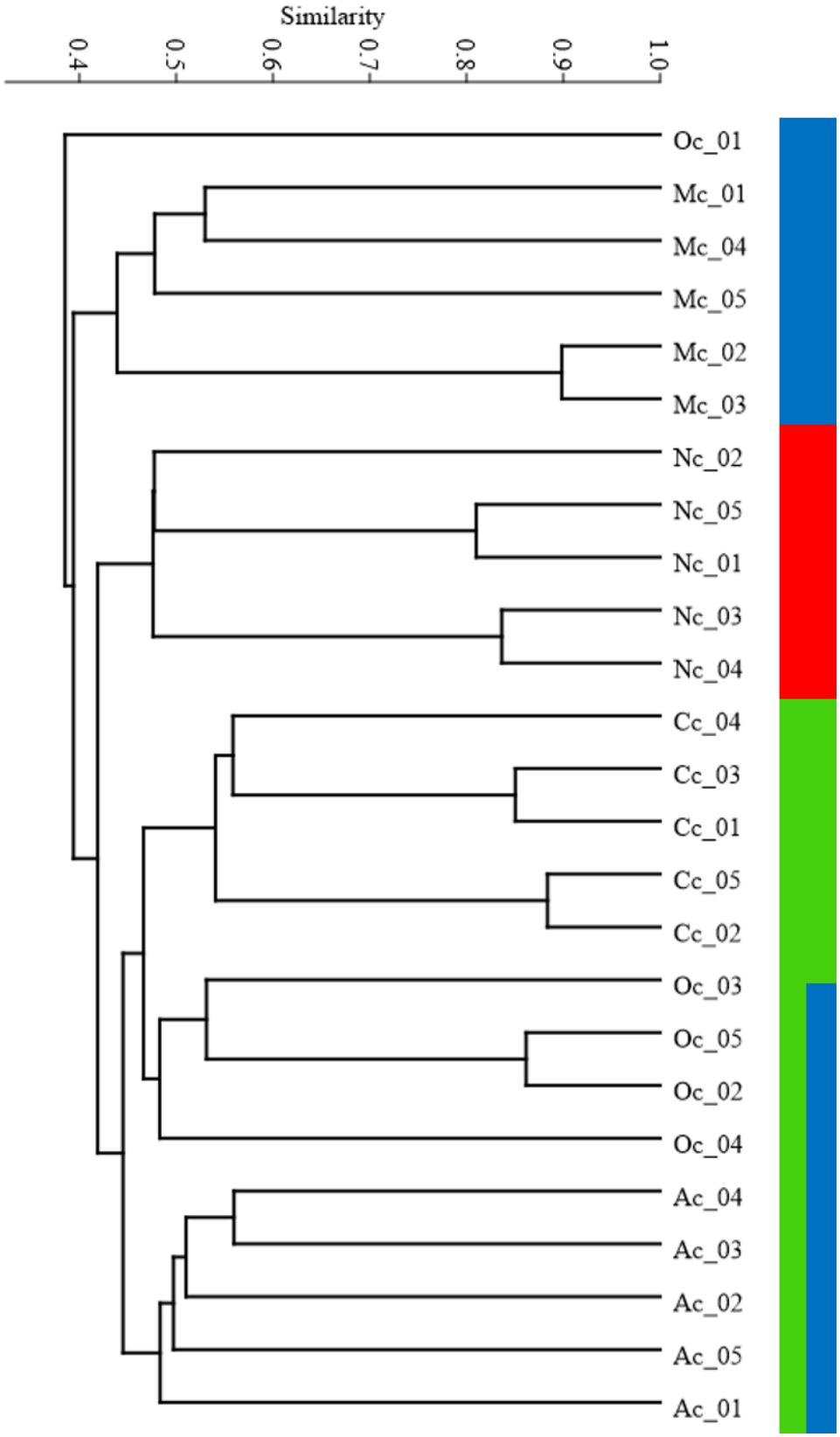


Figure 2.8. UPGMA tree of 25 individuals from five laboratory-reared populations in South Africa (Ac_ = Addo; Oc_ = Old; Cc_ = Citrusdal; Nc_ = Nelspruit; Mc_ = Marble Hall).

2.3.3. Genetic differentiation

Estimates of genetic identity ranged from 0.9545 (*D* = 0.0476) between Addo and Citrusdal to 0.9076 (*D* = 0.0764) between Addo and the Old population (Table 2.3). However, analysis of molecular variance (AMOVA) showed that 35.10% of the Nei's genetic differentiation occurred amongst populations, whereas the larger proportion of variation (75%) existed within populations (Table 2.4). It also suggested that more genetic variation occurred within populations (83.3%) than between the wild populations (Table 2.5).

Table 2.0.3. Genetic distance (below) and genetic identity (above) indices (Nei 1978), estimated using AFLP analysis for five South African FCM populations.

	Addo	Citrusdal	Nelspruit	Marble Hall	Old
Addo	----------	0.9545	0.9076	0.9213	0.9443
Citrusdal	0.0476	----------	0.9125	0.9236	0.9453
Nelspruit	0.0764	0.0871	----------	0.9355	0.9475
Marble Hall	0.0571	0.0592	0.0752	----------	0.9465
Old	0.0445	0.0461	0.0426	0.0452	----------

Table 2.0.4. Analysis of molecular variance for laboratory-reared FCM.

Source	df	Sum of Squares	Est.Var	%
Among pops (AR)	5	788.49	35.476	25
Within pops (AP)	20	3868.56	114.210	75
Total	25	4657.10	149.686	100
PhiRT (value/P<)	0.237 /0.001			

Table 2.0.5. Analysis of molecular variance for wild FCM.

Source	df	MS	Est.Var	%
Among pops (AR)	3	394.25	22.94	16.6
Within pops (AP)	15	257.10	257.10	83.3
Total	18		280.84	100
PhiRT (value/P<)	0.082 /0.003			

PhiPT = AP/(WP + AP) = AP/TOT
AP= Est. Var. Among Pops; WP= Est. Var. Within Pops.

2.4. Discussion

2.4.1. Optimisation of AFLP method

The AFLP technique is typically used for analysis in plants and microbes with comparatively little use in insect phylogeny. As AFLP had already been conducted successfully on FCM (Timm 2005), it was chosen over other microsatellite analyses (Vos *et al.* 1995; Mueller & Wolfenbarger 1999; Mendelson & Shaw 2005). However, developments in PCR technology as well as new primer labelling techniques and fragment analysis, allowed for optimisation of the AFLP method. The AFLP method described by Paun & Schonswetter (2012) adapted from Vos *et al.* (1995) was optimised for FCM in this study. High quality genomic DNA is very important in successful AFLP reactions with insect species (Reineke *et al*. 1998). Generally, CTAB is used and a phenol/chloroform extraction protocol (Makert *et al.* 2006). A high-salt DNA extraction procedure was used as previous studies have shown the importance of a step incorporating proteinase K digestion and the substitution of CTAB with SDS for insects (Paxton *et al.* 1996; Waldschmidt *et al.* 1997). Therefore, it was decided to use the high-salt DNA extraction protocol because it is well-suited for a high-throughput protocol due to its relative simplicity, low cost and the low toxicity of chemicals employed (Makert *et al.* 2006).

Instead of a two-step restriction/ligation, the whole genomic DNA was digested in one step using 2.5 U of MseI and 5 U of EcoRI, instead of 5 U for both restriction enzymes (RE). Increasing the amount of rare-cutter restriction enzyme is common practice for organisms with larger genomes such as insect-based AFLP methods (Reineke *et al.* 1998; Kazachkova *et al.* 2003; Makert *et al.* 2006). The concentration of primers was a critical step in generating more fragments. The number of fragments increased when preselect primers decreased from a concentration of 10 µM to 5 µM and selective primers increased from 1 µM each to 5 µM for MseI and 2.5 µM for EcoRI, respectively. Primers were labelled with photosensitive FAM instead of [γ-33P]dATP, as fragments were scored using an ABI sequencer instead of autoradiography gel electrophoresis as this latter process exposes the user to more radiation as well as leads to more human error when scoring bands (Makert *et al.* 2006; Holland *et al.* 2008).

2.4.2. Regional Genetic Diversity

Population genetic diversity creates an opportunity for adaptation to varied and changing environments. Analysis of the genetic structure at an intraspecific level can be an important indicator of colonisation success to new habitats and the capacity for future adaptive change or evolution (Zhang *et al.* 2008). For a widely distributed species such as FCM, there are more evolutionary opportunities, because of its high heterozygosity and low inbreeding depression in the wild. Therefore, FCM could more easily colonise and adapt under changing ecological habitats, as has potentially occurred in Israel (Te Beest *et al.* 2012; Levi-Zada *et al.* 2019). Five primer pairs were used for AFLP amplification on 38 individual genomic DNA samples from FCM, to detect the genetic diversity and to speculate on the relationships among them. Genetic diversity estimates and percentage polymorphisms showed that a high level of genetic diversity was present amongst South African populations of FCM. The highest estimates of genetic diversity based on various measures were consistently found in the wild Eastern Cape and wild Western Cape populations.

FCM is not considered indigenous in the Western Cape as it was twice accidentally introduced to the region in 1947 and 1974 (Newton 1998). Previous genetic analysis by Timm (2005) also confirmed high genetic diversity in the region (H = 1.599), confirming that the population did not suffer any bottlenecking effects that one might expect from an introduced population. In contrast, laboratory-reared population genetic diversity was consistently lower than the wild populations, with Marble Hall and Nelspruit exhibiting the lowest diversity. The laboratory-reared populations with a mixed origin, i.e. the Rhodes University Old colony and the Xsit colony showed higher levels of genetic diversity, however still considerably lower than the wild populations. This is not unexpected, as wild populations, even though they are found to be geographically isolated, still have the chance of mating with individuals from separate populations resulting in increased genetic diversity.

Even within regions, Timm (2005) found significant genetic diversity between populations within provinces. Therefore, laboratory colonies need to be reflective of entire regions and not necessarily one population from a region as is represented at Rhodes University. However, the Rhodes University laboratory-reared populations are still genetically differentiated from one another. As such, these colonies may still be used as representatives of regions for preliminary studies. Laboratory colonies are very important resources to conduct behavioural and physiological studies, especially on crop pests. The colonies can be maintained, and individuals

produced in regular intervals, preventing the need to wait for the appropriate seasons in order to collect insects from the field. However, the selection of individuals for colonies may lead to adaptations that are not present in the wild and therefore results may not directly transfer to wild populations.

2.4.3. Regional Population Structure

Typically, a high degree of gene flow is very common in well dispersing, polyphagous species, leading to low genetic variation among individuals and populations (Gloss *et al.* 2016). In this study, genetic variance mostly occurred within populations, and individuals from the same region were generally found to be more closely related to each other than individuals from different geographical regions. This is likely a reflection of the fragmented agricultural environment, separating FCM host ranges (Moore 2002). The fragmented environment will adversely impact dispersal and therefore reduce the opportunity of allelic exchange. Many lepidopteran pests in South Africa have formed locally adapted populations, due to fragmented host ranges and in part due to limited hosts, such as *Grapholita molesta* (Busck), *Cydia pomonella* (L.), *Thaumatotibia batrachopa* (Meyrick) and *Cryptophlebia peltastica* (Meyrick) (Meyrick 1930; Quilici 1988, Blomefield 1989; Blomefield & Geertsma 1990; Newton 1998). However, FCM has a far greater host range than these species. Therefore, it is more likely that dispersal may be affecting the population structure of FCM.

De Jager *et al.* (2013) reported the dispersal potential for FCM is over several hundred metres, but mark-recapture studies and field observations offer evidence that FCM is a poorly dispersing species (Stotter & Terblanche 2009). Another factor impacting the population structure of FCM is the cyclical outbreaks of the pest, as outbreaks usually occur from a single focal point and spread outwards. Therefore, the outbreak population has the potential to dominate and homogenise that region (Chapuis *et al.* 2008; Erlandson *et al.* 2019). For the Western Cape region, the source population is unknown, but due to the high genetic diversity it is possible that multiple introductions from different origins occurred in the region.

The AFLP technique has shown to be a good method for indicating differences between populations. However, due to the nature of gene flow amongst wild FCM, described as limited even between closely situated orchards (Timm 2005), AFLP may be too sensitive to use as a technique for comparing wild versus laboratory populations from the same region, unless one is careful to compare populations from the exact same location, down to the orchard. For

example, the laboratory-reared populations were originally collected from only one site, to compare these populations. Two hundred generations later, wild individuals also collected from only one site, different from the original collection site, would most likely result in two genetically distinct populations, if analysed through AFLP, despite both populations originating from the same region. The laboratory-reared colonies could benefit from introductions of wild individuals to maintain genetic homogeneity true to that region.

As Timm (2005) found no significant relationship between geographic distance and genetic distance, largely attributed to accidental introductions of the pest through human-aided dispersal (Giliomee & Riedl 1998, Newton 1998), a mantel test was not conducted in this study. However, the laboratory-reared Addo, Citrusdal and Old populations were comprised of similar genetic clusters in their population structure. Marble Hall was comprised of its own genetic cluster with representatives in all populations. The third cluster predominantly occurring in Nelspruit had little representation in other South African populations. This structuring shows little to no relationship between geographical distance and genetic structuring.

2.5. Conclusion

In conclusion, these results have shown genetically distinct populations of laboratory-reared FCM originating from geographically separate locations in South Africa. These laboratory-reared populations may thus be used as a model to test differences in regional sexual attraction and mating compatibility. The Old population will be presented as a fifth population and not a mixed group as originally intended, as the genetics revealed it is not genetically similar to all South African FCM populations. If differences in attraction between regions are found in the genetically distinct laboratory populations, this will likely be occurring in genetically distinct wild populations as well and the implications for IPM strategies involving semiochemical control will need to be assessed.

Chapter 3

Regional attractiveness between FCM males and females from different geographical regions in South Africa

3.1. Introduction

Previous research into the sex pheromones of female FCM has yielded conflicting results. Researchers found the ratios of the sex pheromone released by females differed at different geographical locations, and males were significantly more attracted to the pheromone blend from their own population (Read *et al.* 1968; Persoons *et al.* 1977; Angelini *et al.* 1981; Zagatti *et al.* 1983; Hall *et al.* 1984). Joubert (2017) found males originating from South Africa were more attracted to a synthetic pheromone lure comprised of the South African ratio of the pheromone than the ratio variants originating from Malawi and Ivory Coast. Further testing of regional attractiveness on a localised scale within the citrus producing regions of South Africa would be beneficial for semiochemical technologies relying on synthetic pheromone lures in providing a more localised form of control. Therefore, the aim of this chapter was to analyse the regional attractiveness between males and females from different geographic regions within South Africa, as well as to analyse the regional attractiveness of the sterile FCM colony from Xsit (Pty) Ltd.

3.2. Materials and Methods

3.2.1. Insect Colonies

All laboratory colonies were maintained in controlled environment rooms at Rhodes University (as described in Chapter 2). The laboratory colonies of Addo, Citrusdal, Marble Hall and Nelspruit originate from field collections and have been kept for over 200 generations (Opoku-Debrah *et al.* 2008). The fifth colony called the Old colony, is of mixed origin and has been kept for over 300 generations (Moore 2002). A population of mixed origin from Xsit (Pty) Ltd was used, with individuals irradiated with 150 Gy (Hofmeyr *et al.* 2015).

3.2.2. Experimental environment

The trials were conducted in a temperature controlled polytunnel at the Waainek facility at Rhodes University as per Joubert (2018), with minor modifications. The tunnel weas maintained above 20°C and monitored to ensure that trials were not conducted if night temperatures fell below 20°C, which may have affected the flight ability of the males (Stotter & Terblanche 2009); temperature data are not reported. Four replicates were conducted for each population, allocating a week between trials to allow for the males to perish before new males were released.

3.2.3. Attraction of adult males to virgin females from different populations

Yellow Delta Traps (18 cm × 18 cm) containing White Sticky Liners (Insect Science, Tzaneen) were used to house two one-day old virgin female moths in two plastic vials. The ends of the plastic vials were cut off and covered in meshing to ensure the female could not escape but the pheromone could be released into the environment. The vials containing the females were positioned with the open ends facing outwards on each side of the trap (Figure 3.1A. & 3.1B). Two one-day old virgin females per plastic vial were used as they would release an equivalent dose to a pheromone trap with 1:1 E/Z ratio at 1 mg dosage (Newton *et al.* 1993). One delta trap per colony containing the virgin females and one control trap were randomly positioned 5 m apart and 1.5 m from the ground (Figure 3.1C). The trial began at 5 pm (sunset) and consisted of releasing 400 males from the same laboratory colony (either Addo, Citrusdal, Marble Hall, Nelspruit or the Old colony) and were given the choice of the virgin females from the different populations. A total of four replicates per population was conducted, with the positioning of the traps randomised every replicate. The traps were checked every day for a week to monitor the experimental environment and to count the number of males captured. After five consecutive days of no new male captures, the environment was set up for a new replicate.

The same trial was conducted for the sterile males. A total of 600 sterile males were released, to account for reduced fitness, again, over five consecutive nights. The sterile moths were transported to Kirkwood from the Xsit Citrusdal mass rearing facility via cooling trucks and stored at 4°C upon arrival. The sterile moths were collected in the morning from the facility at Kirkwood and further transported to Rhodes University in a cooled container to keep the moths inactive to avoid damage to body parts leading to reduced fitness. Upon arrival at Rhodes University, 600 males were placed in Petri dishes and allowed to warm up for the duration of the afternoon ± 5 hours at room temperature until the trial commenced in the evening.

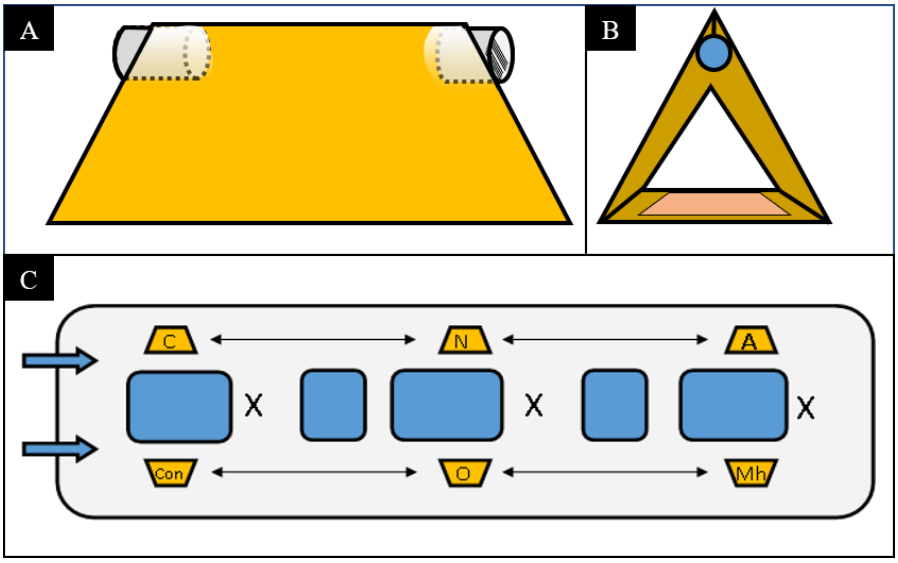


Figure 3.1. Experimental design showing **A)** the side view of the delta trap set up with plastic vials housing virgin females extending out slightly from each side of the trap, **B)** the front view of the delta trap with a sticky floor at the bottom, **C)** set-up of traps in the tunnel 5 m apart and approximately 4 m across (A-Addo; C- Citrusdal; Mh-Marble Hall; N-Nelspruit; O-Old; Con-Control) with × indicating release points and blue squares indicating water pools; release of the males was repeated four times for each population.

3.2.4. Statistical analysis

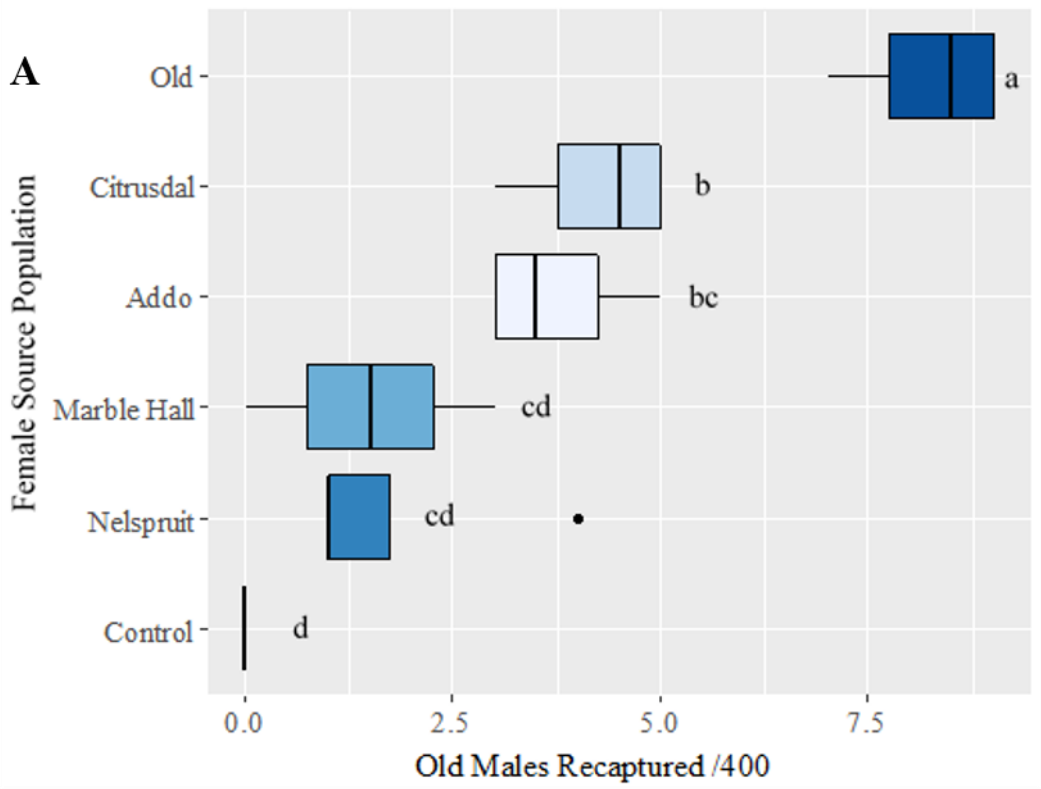
Data were checked for normality using Shapiro-Wilks normality test in R ver. 3.5.2 statistical platform (The R Foundation for Statistical Computing). The data were found to be non-normal, therefore the non-parametric Kruskal-Wallis H test and a single factor ANOVA was conducted. If significance occurred (P = <0.05), Tukey Post Hoc analysis was run.

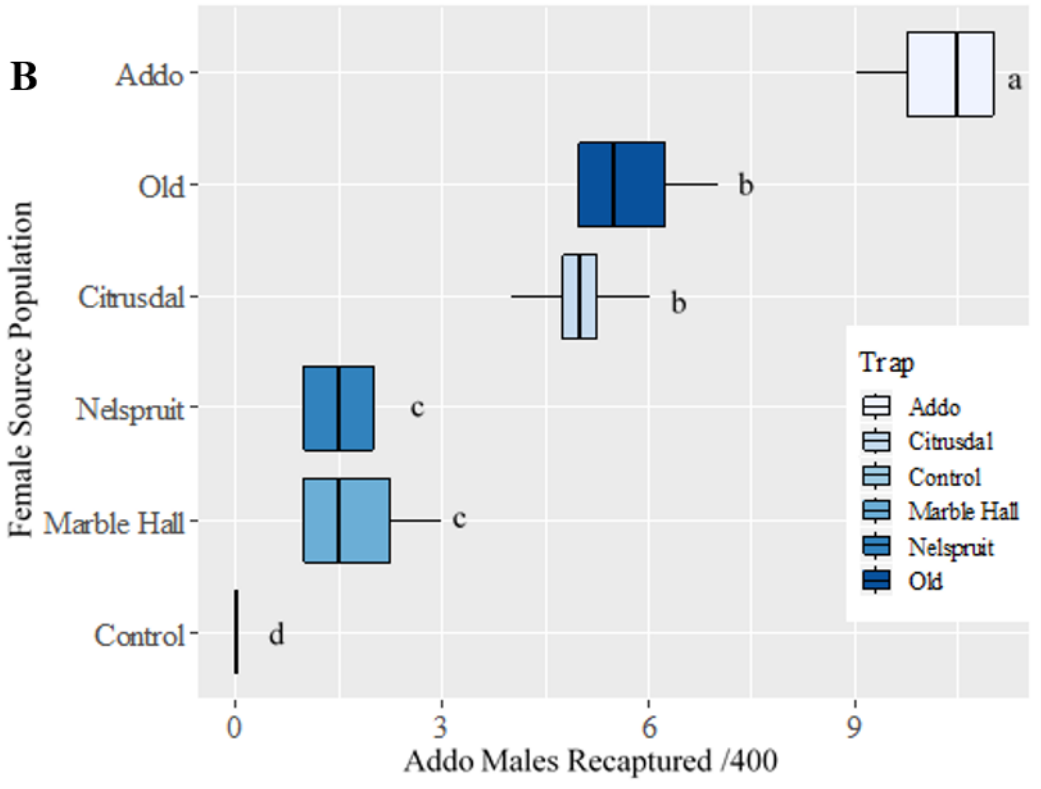
3.3. Results

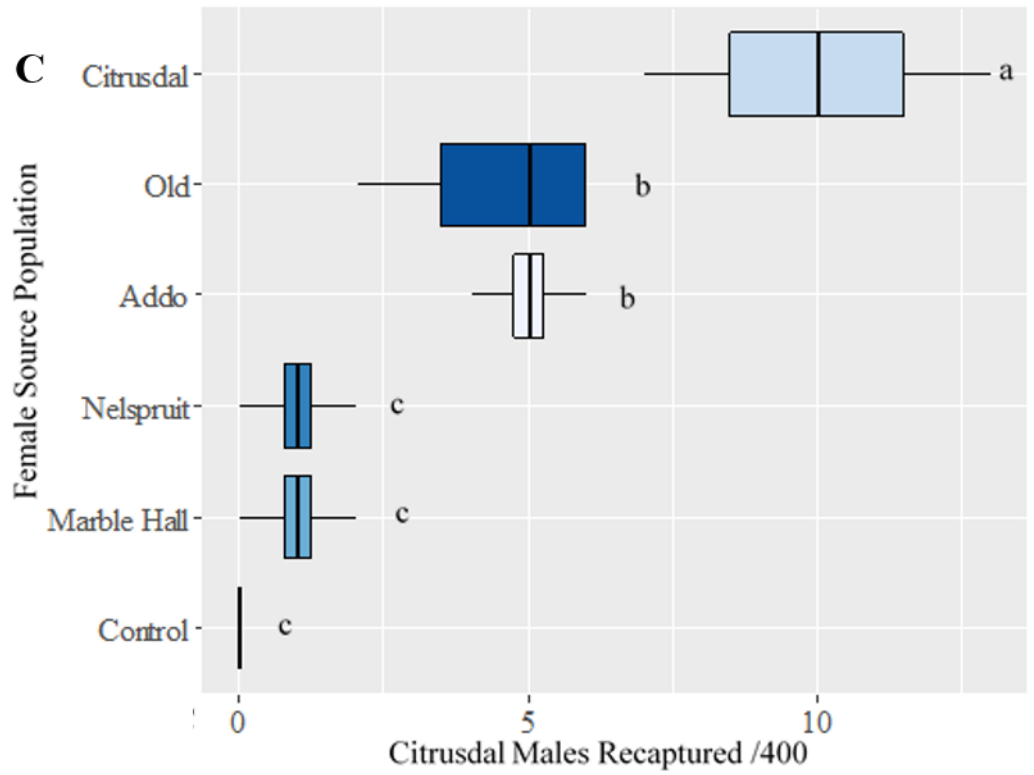
3.3.1. Mating Attraction Trials

Males were significantly attracted to females from the same population (P = <0.05) when given the choice of females from different citrus producing regions in South Africa (Figure 3.2). Old, Addo and Citrusdal were the top three most attractive populations to males of each of these populations (Figure 3.2A, B. and C, respectively). For the Marble Hall and Nelspruit males, no

clear second or third favourite was identifiable, however, the second most attractive population for both Marble Hall and Nelspruit males were females from the Old population (Figure 3.2D and E, respectively). There were no significant differences in attraction by the sterile Xsit males to females from any population. However, the capture rate for the sterile moths was considerably lower than the non-sterile laboratory-reared males, which may be the reason why a preference could not be measured.


A
Old
Citrusdal
Addo
Marble Hall
Nelspruit
Control
a
b
bc
cd
cd
d
Female Source Population
0.0
2.5
5.0
7.5
Old Males Recaptured /400


B
Addo
Old
Citrusdal
Nelspruit
Marble Hall
Control
a
b
b
c
c
d
Female Source Population
0
3
6
9
Addo Males Recaptured /400
Trap
Addo
Citrusdal
Control
Marble Hall
Nelspruit
Old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Citrusdal
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c
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Female Source Population
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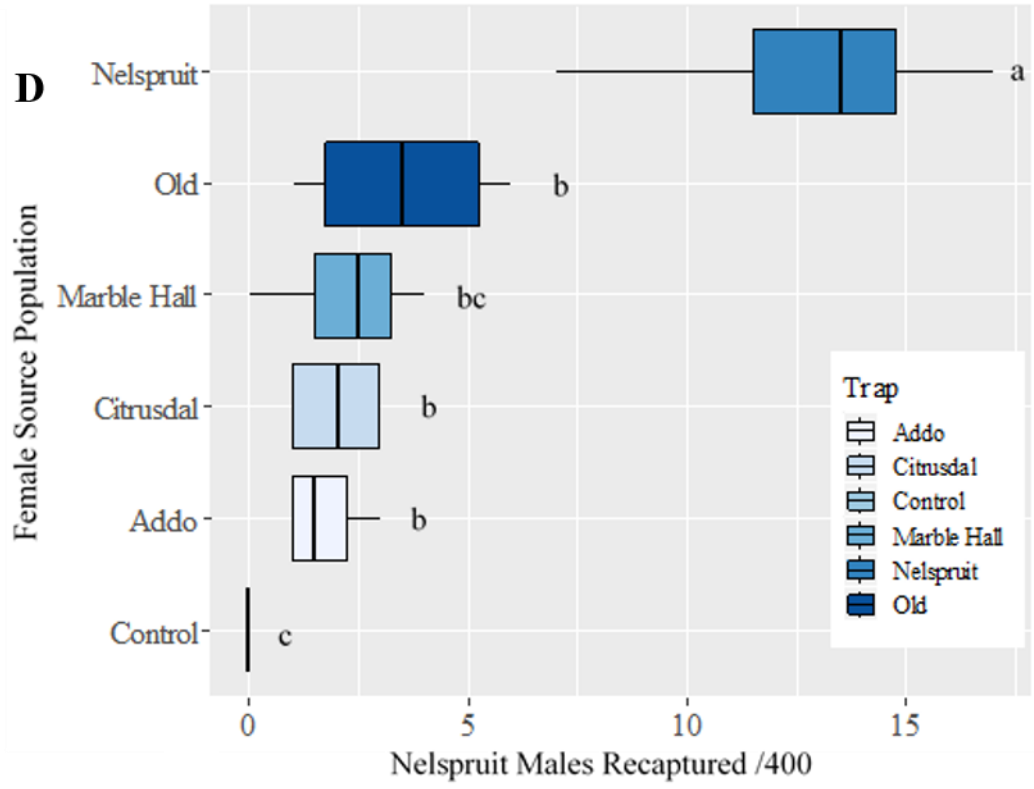
10
Citrusdal Males Recaptured /400


D
Nelspruit
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Marble Hall
Citrusdal
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Control
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c
Female Source Population
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Nelspruit Males Recaptured /400
Trap
Addo
Citrusdal
Control
Marble Hall
Nelspruit
Old

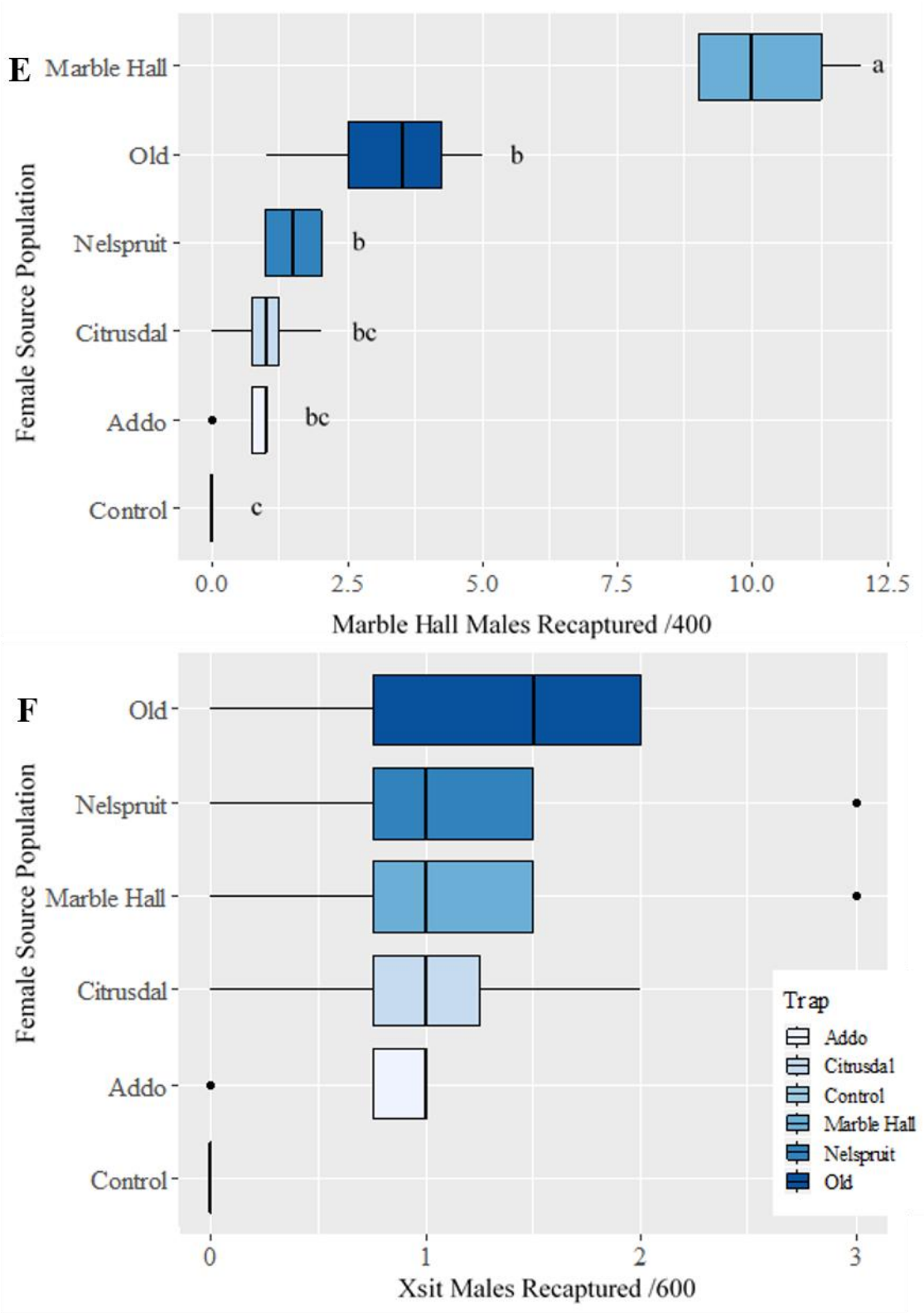

Figure 3.2. Box and Whisker plots sowing the means as well as highest and lowest number of male moths recaptured in the delta traps for males from the **A)** Old, **B)** Addo, **C)** Citrusdal. **D)** Nelspruit, **E)** Marble Hall, and **F)** Xsit populations using virgin females as lures. Significant difference (*P* = <0.05*)* indicated with a, b, c, and d.

3.4. Discussion

This chapter investigated the attractiveness of females from different citrus producing regions in South Africa, using laboratory-reared populations of different geographical origins. It also investigated the attractiveness of the females from the laboratory-reared populations to the sterile males from Xsit. A strongly significant attraction for females from their own population was found in males from different citrus producing regions of South Africa. No significant preference was found by the sterile moths for any of the females of different origin tested. However, recapture rates were very low even though higher numbers of males were released, relative to those released from the other laboratory colonies. Joubert (2018) was able to identify a clear preference of Citrusdal females to the Xsit males, however, it is unclear if at that time, the mixed Xsit population or the old population, which was largely comprised of individuals originating from Citrusdal, was used. Additionally, the Citrusdal males used by Joubert (2018) were not irradiated.

The attraction trial conducted by Joubert (2018) also recovered very few Xsit males in the traps, making it difficult to determine whether there were any significant differences in attractiveness. Joubert (2018) further investigated the attraction of sterile male moths to synthesised pheromones based on the ratio of isomers identified for populations from Ivory Coast, Malawi and a South African population. The number of sterile male recaptures significantly increased in the pheromone trap representative of South African females, leading to two conclusions. Firstly, the synthetic pheromones were better at luring the sterile males than the live females from the different FCM populations in South Africa, most likely due to the stronger release of the dispenser over the single female he used, and secondly, there was a regional attractiveness between the Xsit males and the South African female pheromone blend. Overall Joubert (2018) was successfully able to show a regional attractiveness of South African male to South African female FCM, whereas the results from this study clearly show a localised attractiveness between FCM populations within South Africa.

These findings suggest geographic variation of the sex pheromone is highly likely in FCM populations across South Africa. In previous studies where behavioural bioassays have found a significant attraction of males and females from the same population, chemical analysis revealed differences in the sex pheromone ratio, and likewise where behavioural bioassays have shown no significant preference from males to females of the same population, no variations in the sex pheromones were found (Tòth *et al.* 1992; Watanabe & Shimizu 2017). In South Africa, the pheromone dispenser was developed by Hofmeyr & Burger (1995) which

can be used continually for several months. The active ingredients are 50% (E)7 – 12:Ac (not found in the pheromone but is attractive alone, however this has recently come into question) and 50% (E)8 – 12:Ac + (Z)8-12:Ac in a ratio of 5:1 (Hofmeyr & Burger 1995; Levi-Zada *et al.* 2019). From Hofmeyr & Burger's (1995) study commercial pheromone lures were developed using a three component mixture of (E)7 – 12:Ac, (E)8 – 12:Ac and (Z)8-12:Ac in the ratio of 6:5:1 (Moore *et al.* 2014). Recent findings from Israeli researchers found no significant difference in FCM capture rate in traps adding (E)7-12:Ac in a citrus orchard in Israel, even though this was previously recommended to be added to baits in South Africa (Levi-Zada *et al.* 2019). In an Israeli citrus orchard, traps with added (E)7-12:Ac were found to have reduced numbers of males (Levi-Zada *et al.* 2019) even though this compound increased the capture rate in South African males. FCM was introduced to Israel through an accidental outbreak in the 1980s (Wysoki 1986). The population numbers remained low, however recently up to six interceptions of FCM was recorded in the EU leading threats from the EU to halt all citrus imports from Israel (EIA 2018). The seemingly increase in FCM populations in Israel, coupled with the knowledge that FCM populations are geographically structured (Timm *et al.* 2010) may be a result of the Israeli FCM population developing localised attraction different to that of the original South African population from which they originated (Wysoki 1986).

Overall the experiment may have been improved by increasing the number of sterile males released in order to account for the decreased physical fitness of these individuals. It would also be beneficial to repeat the experiment without the presence of the most attractive population to identify how successful males are at finding these females and to gain a clearer picture of regional attractiveness.

3.5. Conclusion

In conclusion, differences in population level attraction has been observed. Further chemical analysis into the variation of the sex pheromone will be beneficial in understanding the relationships between these populations. The results can be implemented into semiochemical-based technologies, such as pheromone trapping, to optimise capture rates in an area as well as mating disruption and for SIT, in its future expansion across South Africa's citrus producing regions.

Chapter 4

Mating compatibility between FCM males and females from different geographical regions in South Africa

4.1. Introduction

Optimising control methods is an important tool in managing pest populations below economically damaging levels. The highly successful sterile insect technique (SIT) used against FCM currently provides control in the Western Cape, Eastern Cape and Northern Cape citrus producing regions (Hofmeyr *et al.* 2019). The competitiveness of sterile moths is critical to their effectiveness in reducing wild populations of the pest. Sterile insect technique involves large scale production, irradiation and release of large numbers of sterile moths in the target area, who must then mate with wild partners (Hendrichs *et al.* 2002). For SIT to be successful, the sterile moths must compete successfully with wild males for wild females. Due to the success of SIT, extending the programme across South Africa to all citrus producing regions will be beneficial for protecting the industry against FCM.

Population level genetic assessments using the AFLP technique found genetically distinct populations of FCM amongst the Rhodes University laboratory colonies collected from Citrusdal, Addo, Marble Hall, Nelspruit and the Old colony of mixed origin (Chapter 2). Although these populations have been maintained under laboratory conditions for more than 200 generations, and most likely have high genetic differentiation from wild FCM populations, their genetic distinctness allows mating compatibility amongst genetically distinct populations to be evaluated. Regional attractiveness for the same population was also found in the laboratory colonies (Chapter 3), therefore evaluating mating compatibility between these populations will provide a greater understanding of how regional genetics and attractiveness influences overall mating success. This information can be used to improve current control strategies.

Thus, the aim of this chapter was to test the mating compatibility and success between the genetically distinct laboratory-reared populations, as a representative of wild moths at a prezygotic (pre-fertilization) level, as well as the mating compatibility and success of the sterile moths to each of the laboratory populations. The outcome of these experiments will be useful

in determining the level of localised management strategies needed for this species in South Africa.

4.2. Materials and Methods

4.2.1. Insect Cultures

The same laboratory colonies originating from Addo, Citrusdal, Marble Hall, Nelspruit and the Old populations of mixed origin that were used for experiments in Chapters 2 and 3, were used in this study. A population of mixed origin from Xsit (Pty) Ltd was used, with individuals irradiated with 150 Gy (Hofmeyr *et al.* 2015).

4.2.2. Choice Mating Compatibility Experiments

In a 3 m by 3 m cage, two day old males (n=30) and females (n=30) from two different populations were released and given the choice to either mate with individuals from the same population or individuals from a different population. Males and females were released at opposite corners of the cage with females upwind from the males. The moths were left to voluntarily depart from the container. Two treatments were evaluated, interpopulation (L-Lab) mating compatibility between the genetically distinct populations of FCM (L males × L females), and the effect of sterility (S-sterile) on the different populations (S males × L females). To distinguish between laboratory-reared populations, Calco Oil Red N-1700® was added to the diet using instructions as described by River Bioscience, where the dye is taken up by fat bodies of the moth, allowing populations to be distinguished either through being dyed or not. The dye does not affect the fitness of the moth (Davis 1973). All sterile moths were already treated with Calco Oil Red at Xsit. The trial was run during the night, beginning at 5 pm (sunset in winter). Checking for mating couples occured every half an hour and the trial ended when there was a whole hour of no coupling. Mating couples were captured and frozen overnight in preparation to determine the source population of each individual through dissecting the moth to identufy if the internal organs were dyed (one population) or not (other population). The experiment was repeated 3 times for each population combination (L_A -Addo; L_C -Citrusdal; L_O -Mixed; L_{MH} -Marble Hall; L_N -Nelspruit; S -Sterile).

The formulae used to calculate the various mating competitiveness indices were calculated as described in the FAO/IAEA/USDA (2003) Quality Control Manual. The suitability of the adults and the cage environmental conditions for mating were determined by calculating the participation in mating (PM), representing overall mating activity. According to FAO/IAEA/USDA, a PM of 0.2 is considered the minimum proportion of mating inclusion, i.e. at least 20% of the total amount of possible couples need to have mated. Further indices used to quantify sexual compatibility, performance and isolation between the adult populations were calculated (Cayol *et al*. 1999; Cayol *et al.* 2002; Taret *et al.* 2010). The Index of Sexual Isolation (ISI) accounts for the number of pairs obtained for each possible mating combination, and ranges from -1, where most couples consisted of the second population listed, to 1 where most couples consist of the first population listed. A value of 0 represents random mating (equal proportion of the four possibilities of mating, i.e. homotypic (AA and BB) or heterotypic (AB and BA), where A and B represent two different populations. To clarify the ISI value, two other indices that account for variations in mating propensity were calculated. The male relative performance index (MRPI) and the female relative performance index (FRPI) are measures of male and female mating propensity, regardless of their mating partners (Cayol *et al*. 1999). The values of these two indices also range from 1, where all matings are done by males (MRPI) or females (FRPI) of one type (the first population to be listed), through an equilibrium at 0 where there is an equal participation in mating by males (MRPI) or females (FRPI) of both types, to -1 where all matings are achieved by males (MRPI) or females (FRPI) of the other type (second population listed). The MRPI and FRPI explain the role of the males and females from two differing populations compared in each experiment, and thus complement the ISI very well (Taret *et al.* 2010). To calculate the mating performance of sterile males, the Relative Sterility Index (RSI), which is the proportion of wild females that mate with sterile males when sterile and wild males are present in a 1:1 ratio, was used (McInnis *et al.* 1996). Where SL is the number of matings between sterile males and laboratory females and LL is the number of matings between laboratory males and laboratory females. The RSI equals the number of sterile matings divided by the number of all matings, and its values can vary between 0 and 1, with 0.5 indicating equal performance of wild and sterile males. The formulae for the respective indices are:

$$PM = \frac{Number\ of\ pairs\ collected}{Number\ of\ females\ released}$$

$$ISI = \frac{(AA + BB) - (AB + BA)}{Total\ number\ of\ matings}$$

$$MRPI = \frac{(AA + AB) - (BB + BA)}{Total\ number\ of\ matings}$$

$$FRPI = \frac{(AA + BA) - (BB + AB)}{Total\ number\ of\ matings}$$

$$RSI = \frac{(SL)}{(SL + LL)}$$

4.2.3. No-choice Mating Compatibility Experiments

Five one-day old virgin females and one male were placed into a 30 cm × 30 cm container covered in material meshing for two consecutive nights. After 48 h the females were freeze killed at -20°C overnight in preparation for dissection. The females were then dissected and examined for the presence of spermatophores in their bursa copulatrix (Figure 4.1), using a simple dissecting microscope. This was repeated five times per mating combination. Males and females from the same population were regarded as the control treatment to compare the male's fitness with females from other populations. The controls were also used to compare spermatophore transfer between sterile and non-sterile males.

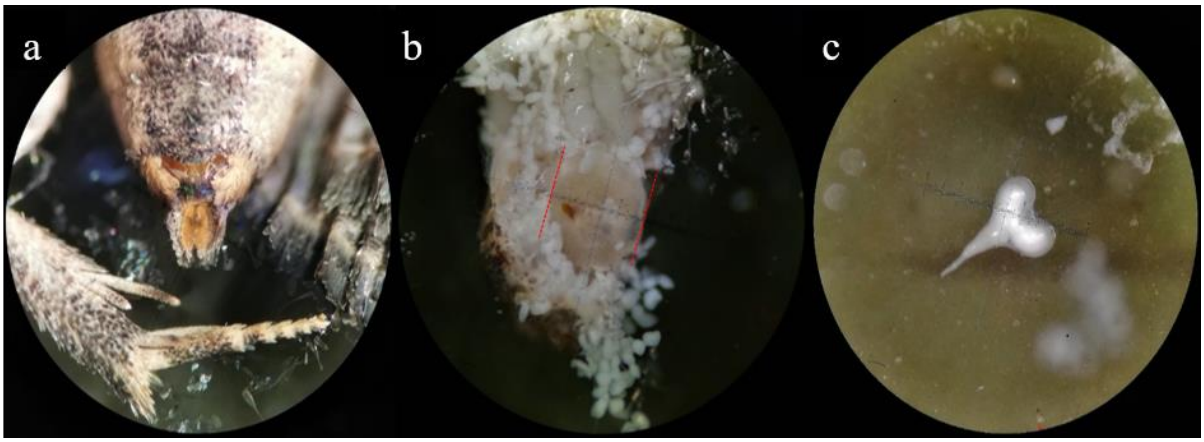

Figure 4.1. Microscopic pictures of a) the female external reproductive organ at 10X magnification, b) the bursa copulatrix at 10X magnification and c) the spermatophore at 40X magnification.

4.2.4. Data analysis

The indexes of competitiveness and compatibility for all laboratory-reared populations and sterile populations were assessed via a one-sample Student's *t* test to determine whether the Index (RSI, ISI, MRPI and FRPI) differed significantly from the theoretical value expected for equal competitiveness or random mating (Tejeda *et al.* 2017). In addition, an unpaired two-sample t-test was used to determine differences between the group means.

To evaluate the number of spermatophores per male per 48 h, a Shapiro-Wilks test for normality was used, followed by a general linear model using the Poisson family and log-link function to analyse the count data (McElduff *et al.* 2010) run on R ver. 3.5.2 statistical platform (The R Foundation for Statistical Computing). To determine significance between populations, a post hoc Pairwise Mann-Whitney U test was conducted.

4.3. Results

4.3.1. Choice compatibility experiments

4.3.1.1. Geographically isolated populations

Sexual competition between laboratory-reared populations found an average participation of mating (PM) of 0.52 or 52% of possible mating pairs in the cages, indicating conditions under which the tests were run were satisfactory (Table 4.1). In general, ISI values were close to 0. This suggests that the laboratory-reared populations are equally sexually compatible. However, four combinations showed significant divergence from the theoretical value for random mating (=0), and were therefore more sexually isolated: Addo vs. Nelspruit (ISI = 0.13; t_2 = 6.23; p = 0.02), Addo × Marble Hall (ISI = 0.11; t_2 = 4.72; p = 0.04), Citrusdal × Nelspruit (ISI = 0.11; t_2 = 4.95; p = 0.04), and Citrusdal × Marble Hall (ISI = 0.12; t_2 = 4.31; p = 0.04) (Table 4.2; Figure 4.2). In all significant combinations, there were more homogenous couples of Addo and Citrusdal than Nelspruit and Marble Hall. Focussing on the four significantly isolated treatments, MRPI and FRPI were analysed to further characterise sexual performance. Significant MRPI values were only found for Addo × Nelspruit (MRPI = 0,19; t_2 = 6.23; p = 0.02), Addo × Marble Hall (MPRI = 0.17; t_2 = 6.65; p = 0.02) and Citrusdal × Nelspruit (MRPI = 0.13; t_2 = 4.39; p = 0.04), indicating males originating from Addo and Citrusdal participated in more matings than Nelspruit and Marble Hall males. The MRPI and FRPI values for the

fourth significant cross, Citrusdal × Marble Hall, were inconclusive, as no significance for either MRPI or FRPI was found. No significance for FRPI was found in any combination tested, suggesting females of all populations presented a similar mating propensity in the cages.

4.3.1.2. Sterile vs. Non-Sterile Populations

As per the laboratory only treatment, more homogenous sterile couples were identified (16.2 ± 0.8) than heterogenous couples (12.7 ± 0.3) (Table 4.1, Figure 4.3). The average RSI for all combinations was 0.41 and not significantly different to the theoretical value for equal competitiveness. This indicated that sterile males were able to compete successfully with the non-sterile males for mates, even though the RSI was consistently less than 0.5, indicating the sterile males could never achieve at least 50% of all the matings. Mating compatibility was found to be significantly different from equal mating for Sterile × Marble Hall (ISI = 0.12; t_2 = 4.98; p = 0.01) and Sterile × Citrusdal (ISI = 0.13; t_2 = 3.96; p = 0.01). The MRPI for both combinations, Sterile × Marble Hall (MRPI = -0.11; t_2 = 4.14 p = 0.03) and Sterile × Citrusdal (MRPI = -0.13; t_2 = 6.41 p = 0.01) was significant, indicating that the non-sterile laboratory males outcompeted the sterile males in these two combinations. The FRPI for Sterile × Marble Hall was also found to be significant (FRPI = -0.08; t_2= 8.35; p = 0.01), indicating Marble Hall females also outcompeted sterile females. Negative values of MRPI and FRPI were found for all combinations tested, indicating non-sterile laboratory individuals engaged in more mating. However, as mentioned, this was only significant for Citrusdal and Marble Hall populations.

Table 4.1. The mean and standard deviation of homotypic couples (from same population) and heterotypic couples (consisting of individuals from different populations) and mating propensity (PM) per combination (n = 3).

Populations	Homotypic (AA/BB)	Heterotypic (AB/ BA)	Total couples	PM
		Trial 1. Intraspecific mating compatibility		
$L_A \times L_N$	17.3 ± 12.9	13.3 ± 0.6	30.7 ± 2.3	0,51 (±0.03)
$L_A \times L_C$	15.7 ± 0.6	15.3 ± 3.5	31 ± 3	0,52 (±0.01)
$L_A \times L_{MH}$	14.7 ± 2.5	11.7 ± 1.2	26.3 ± 3.5	0,44 (±0.06)
$L_A \times L_O$	17.33 ± 2.1	16.7 ± 1.2	34 ± 3	0,57 (±0.03)
$L_N \times L_C$	15 ± 2.6	12 ± 1	27 ± 3	0,45 (±0.04)
$L_N \times L_{MH}$	17.3 ± 1.2	13.7 ± 1.2	31 ± 1.1	0,52 (±0.02)
$L_N \times L_O$	17.3 ± 1.2	13.7 ± 1.2	33.3 ± 2.1	0,56 (±0.01)
$L_C \times L_{MH}$	18.3 ± 1.5	14 .3± 0.6	32.7 ± 1.5	0,54 (±0.07)
$L_C \times L_O$	17.7 ± 1.5	17 ± 1	34.7 ± 1.5	0,58 (±0.04)
$L_{MH} \times L_O$	16 ± 1.7	14.3 ± 1.5	30.3 ± 0.6	0,51 (±0.03)
		Trial 2. Sterile vs. non-sterile mating compatibility		
$S \times L_A$	15.3 ± 1.2	12.3 ± 1.2	27.7 ± 2	0.46 (±0.02)
$S \times L_N$	15.7 ± 2.1	12.5 ± 2.9	28.2 ± 3.8	0.48(±0.05)
$S \times L_O$	16 ± 1.5	13.3 ± 1.5	29.3 ± 3.1	0.49 (±0.07)
$S \times L_{MH}$	16.6 ± 1.5	13 ± 1	29.7 ± 1.5	0.42 (±0.04)
$S \times L_C$	17 ± 1.7	12.3 ± 1.2	29.3 ± 3.2	0.48 (±0.01)

S- Sterile; L-Lab of A- Addo; N- Nelspruit; C- Citrusdal; MH- Marble Hall; O- Old

Table 4.2. The mean, 95% confidence interval (CI) and *p*-value for the RSI, ISI, MRPI and FRPI indices for intraspecific mating compatibility (L × L) and sterile vs non-sterile mating compatibility (S × L) (n = 3).

	RSI			ISI			MRPI			FRPI		
Pop.	mean	**(95% CI)**	*p*	mean	**(95% CI)**	*p*	mean	**(95% CI)**	*p*	mean	**(95% CI)**	*p*
$L_A \times L_N$		-		0,13	**(0.05-0.27)**	0.02*	0,19	**(0.06 - 0.32)**	0.02*	-0,02	**(-0.11-0.07)**	0.42
$L_A \times L_C$		-		0,02	**(-0.31 - 0.34)**	0.82	-0,03	**(-0.22 - 0.17)**	0.59	0,00	**(-0.12- 0.12)**	0.93
$L_A \times L_{MH}$		-		0,11	**(0.01-0.23)**	0.04*	0,17	**(0.06 - 0.28)**	0.02*	0,03	**(-0.13 - 0.19)**	0.48
$L_A \times L_O$		-		0,02	**(-0.1 - 0.13)**	0.57	0,06	**(-0.15 - 0.27)**	0.34	0,02	**(-0.25 - 0.28)**	0.82
$L_N \times L_C$		-		0,11	**(0.02 - 0.25)**	0.04*	0,13	**(0.01 - 0.26)**	0.04*	0,04	**(-0.12 - 0.19)**	0.42
$L_N \times L_{MH}$		-		0,12	**(0.03 - 0.25)**	0.05	0,10	**(-0.33 - 0.52)**	0.43	0,08	**(-0.02 - 0.17)**	0.07
$L_N \times L_O$		-		0,04	**(-0.17 - 0.26)**	0.49	-0,02	**(-0.34 - 0.31)**	0.82	0,06	**(-0.13 - 0.15)**	0.58
$L_C \times L_{MH}$		-		0,12	**(0.01 - 0.24)**	0.04*	-0,02	**(-0.05 - 0.02)**	0.18	0,04	**(-0.15 - 0.22)**	0.46
$L_C \times L_O$		-		0,02	**(-0.13 - 0.16)**	0.65	-0,04	**(-0.21 - 0.14)**	0.48	-0,06	**(-0.17 -0.06**	0.17
$L_{MH} \times L_O$		-		0,05	**(-0.21 - 0.31)**	0.46	-0,01	**(-0.06 - 0.03**	0.42	-0,03	**(-0.32 - 0.25)**	0.68
$S \times L_A$	0.43	**(0.4-0.46)**	0.07	0.11	**(-0.08 – 0.3)**	0.12	-0.03	**(-0.19 - 0.12)**	0.46	-0.05	**(-0.37 - 0.26)**	0.54
$S \times L_N$	0.41	**(0.33-0.46)**	0.05	0,11	**(0.09 – 0.24)**	0.05	-0.04	**(-0.21 - -0.13)**	0.39	-0,07	**(-0.14 - 0.01)**	0.06
$S \times L_O$	0.44	**(0.31-0.55**	0.187	0.09	**(-0.02 – 0.18)**	0.05	-0.03	**(-0.22 - 0.17)**	0.61	-0.01	**(-0.26 - 0.24)**	0.82
$S \times L_{MH}$	0.4	**(0.35-0.44)**	0.01*	0.12	**(0.06 – 0.18)**	0.01*	-0.11	**(-0.18 - -0.02)**	0.03*	-0.08	**(-0.12 - -0.04)**	0.01*
$S \times L_C$	0.42	**(0.31-0.52)**	0.07	0,13	**(0.06 - 0.2)**	0.01*	-0.13	**(-0.19 - -0.08)**	0.01*	-0,05	**(-0.23 - 0.11)**	0.29

A- Addo; N- Nelspruit; C- Citrusdal; MH- Marble Hall; O- Old; *significantly different from theoretical value for random mating (0).

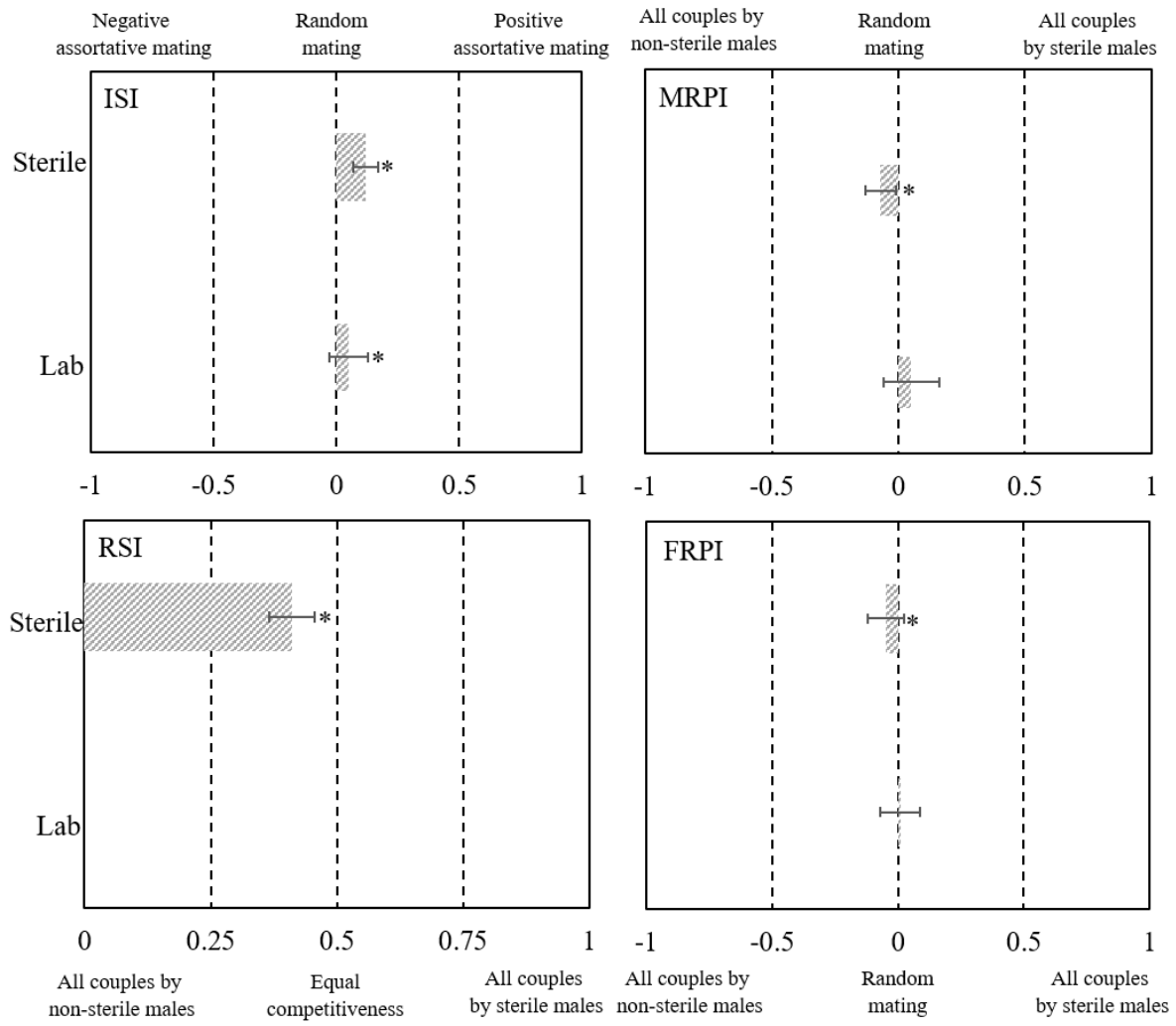

Figure 4.2. Comparisons of the index of sexual isolation (ISI), male relative performance index (MRPI), female relative performance index (FRPI) and relative sterility index (RSI), of sterile and laboratory (lab) reared populations. The asterisk indicates analysis by one sample Student's *t* test showed the mean differed significantly (<0.05) from the theoretical values expected for equal competitiveness (0.5) and random mating (0).

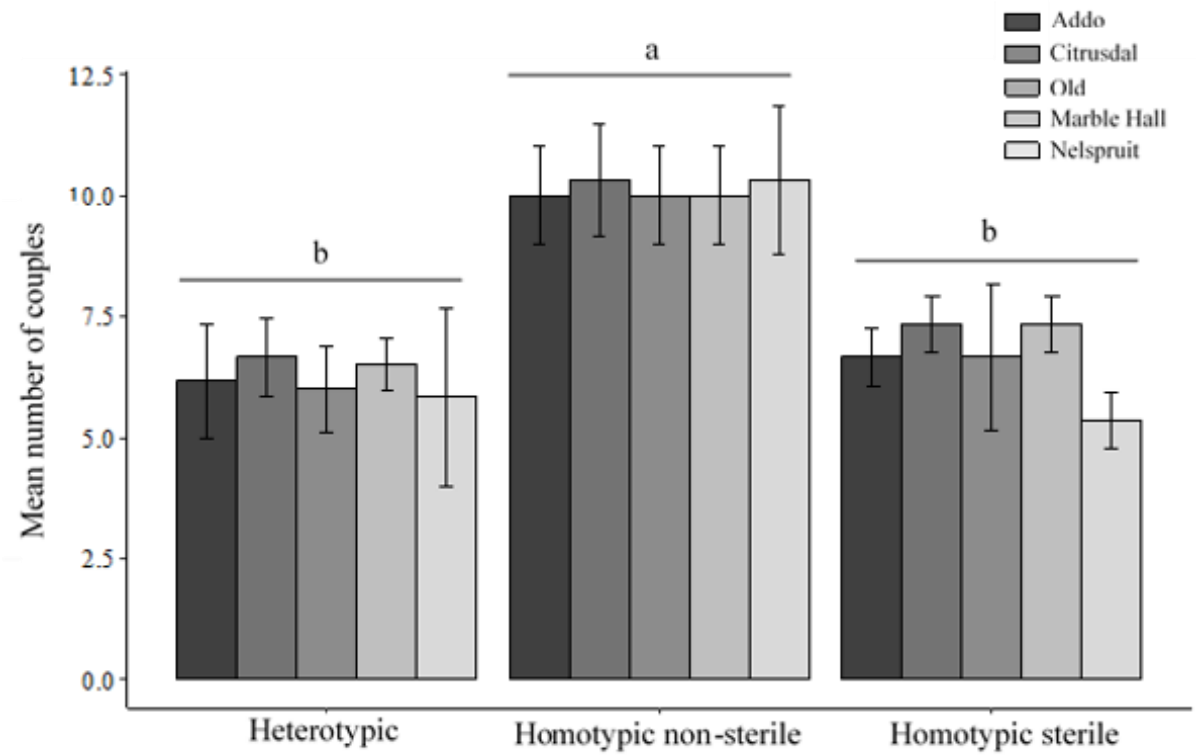

Figure 4.3. The mean number of couples (± SD), for heterotypic (S × L/L × S), homotypic non-sterile (laboratory) (L × L) and homotypic sterile (S × S) individuals. Different letters indicate significant difference between the groups (P = <0.05).

4.3.2. No-choice Spermatophore Trials

There was little variation in the number of spermatophores transferred by laboratory-reared males to females from all populations (Figure 4.4). Sterile males consistently transferred fewer spermatophores (n = 25; average = 0.28) than the non-sterile males (n = 150; average = 0.36) however, overall this was not significant apart from non-sterile Citrusdal males and the Xsit sterile males when paired with Old females (GLM: Z = 4.37; *p* = 0.01) (Figure 4.4).

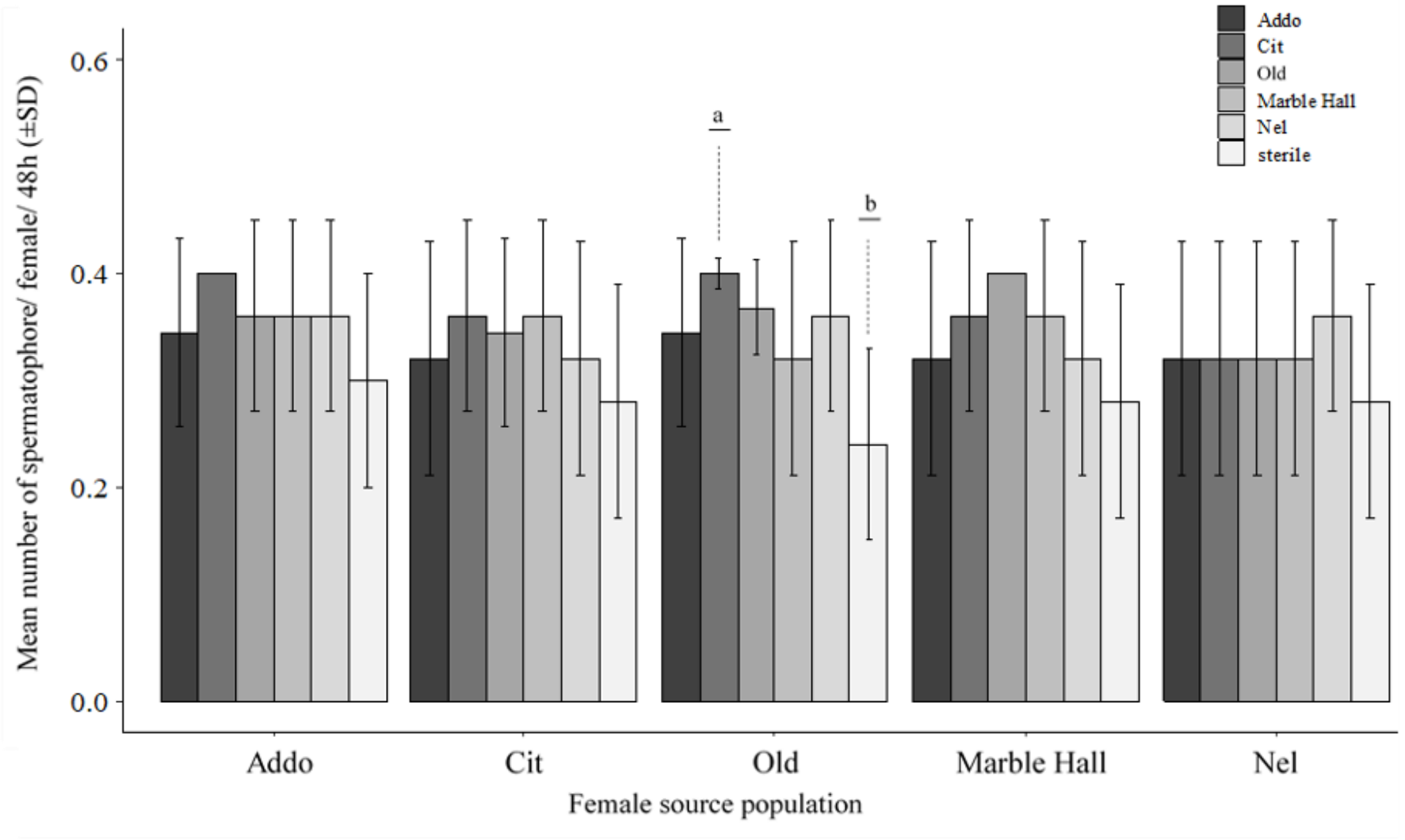

Figure 4.4. The mean number of spermatophore present per laboratory-reared female over 48-hours (±SD), comparing mating ability between non-sterile laboratory reared populations and sterile males from Xsit.

4.4. Discussion

Mass-rearing in an artificial environment can create atypical behaviour in these insects lessening their ability to compete with their natural counterparts in the field (Boller 1972). Therefore mass-rearing coupled with handling, irradiation procedures and transport all impact the fitness of these individuals which may lead to mating incompatibility between sterile and wild populations (Ahmad *et al*. 2018). It is important to assess mating compatibility between populations both treated and untreated for sterile insect technique as mating isolation is evident in many hybridising lepidopteran species (Leubke *et al*. 1988; Sperling 1990; Deering & Scriber 2002). Although many of these studies show sexual isolation at the interspecific level, it is arguably as important to evaluate sexual isolation at the intraspecific level, especially in species with a high pest status.

The following conclusions can be drawn from these results. Firstly, when given the choice between mating within their own population or with a different population, sexual isolation was observed between Addo × Nelspruit, Addo × Marble Hall, Citrusdal × Nelspruit and Citrusdal × Marble Hall. In these combinations, Nelspruit and Marble Hall males displayed reduced mating compatibility compared to Addo and Citrusdal. Secondly, sexual isolation also occurred between the sterile population when crossed with Marble Hall and Citrusdal, however, overall, the relative sterility index for these two populations remained close to the theoretical value for equal competitiveness between sterile and non-sterile males. Thirdly, sterile males had consistently lower recorded spermatophore transfer, and when comparing the overall spermatophores transferred of all sterile males and all non-sterile males, more spermatophores were present in females paired with non-sterile males, albeit not significant.

In cases where two populations were more isolated, as determined by the ISI, it was the MRPI which significantly differed from random mating indicating that males preferred females from the same population. This response from the males is likely a reflection of a difference in the sex pheromone (ratio of isomers) released from the females, as it is the male who actively seeks the female which calls for the male via the sex pheromone (Campion & Nesbitt 1989). Addo, Citrusdal, and the Old population were found to be the most genetically separate from Nelspruit and Marble Hall moths. In cases of higher than normal sexual isolation, it was these populations (Addo × Nelspruit, Addo × Marble Hall and Citrusdal × Nelspruit, Citrusdal × Marble Hall) that were found to be the least sexually compatible. However, when long range communication

is not a factor, males from all populations could successfully transfer similar amounts of spermatophore to females from all populations.

The results of the successful spermatophore transfer by sterile males are similar to previous studies measuring deleterious effects due to irradiation of males. Carpenter *et al.* (2009) found *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) males sterilised at 200 Gy showed no significant difference in spermatophore transferal (1.25 ± 1.39) compared to wild males (1.76 ± 1.52). White & Hutt (1975) also found *Cydia pomonella* L. (Lepidoptera: Tortricidae) males sterilised at 300 Gy had no significant difference in spermatophore transferal compared to wild males. Reported factors affecting the mating fitness of sterile males has been sperm transfer time, which was demonstrated in *C. pomonella* sterile males, long-distance transportation and chilling of the sterile moths (White & Hutt 1975; Carpenter *et al.* 2009). Differences in spermatophore transfer time between populations has not been found in FCM males, however long-distance transportation and chilling are two variables that affect the flight ability of FCM which may lead to reduced fitness (Nepgen *et al.* 2015). Further postzygotic testing involving fertility and fecundity of FCM originating from different regions in South Africa has previously been investigated and viable offspring were produced in all combinations tested (Mgocheki & Addison 2016; Van Steenderen *et al.* 2016; Joubert 2018). In only one study, a significant difference in egg hatch with combinations involving Marble Hall females was found (Joubert 2018). Therefore, sterile FCM males can successfully transfer sterile spermatophores at numbers similar to those of non-sterile males, reinforcing the efficacy of SIT as a control strategy for most, if not all citrus producing regions.

As FCM is a cryptic pest which, in the past, has been accidentally introduced into new regions (Western Cape and Israel), it would be beneficial to incorporate these FCM populations from Malawi, Ivory Coast and Israel into mating compatibility studies, especially since variations in the ratio of isomers of the sex pheromone has already been identified between these populations (Hall *et al.* 1984; Atygalle *et al.* 1986; Levi-Zada *et al*. 2019). An important note to reflect on is the inactivity of the laboratory-reared moths below 20°C. This study was originally conducted outside from March (average nightly temp = 15.1°C). However, after four failed trials, the cages were moved into a quarantine facility where temperatures were kept at 22°C. Multiple studies have cited the optimal copulating period for FCM as between 4-6 am or at least four hours after scotophase (Atygalle *et al.* 1986; Levi-Zada *et al.* 2019). This is viable in a laboratory where temperatures remain constant, however, in the wild, temperatures drop well

below 20°C by 4 am for most of the year where wild moths have a notably lower activity threshold (Daiber 1979). Therefore, it would be beneficial to test mating compatibility between laboratory-reared sterile and wild populations with a focus on temperatures reflective of the natural environment as well as measuring the peak calling times between laboratory and wild populations. Differences in peak calling times will have implications on pest management strategies such as SIT as the laboratory males will have reduced mating compatibility with wild females and therefore reduced competitiveness with wild males (Shi *et al*. 2018).

4.5. Conclusion

Overall the results demonstrate that mating compatibility via spermatophore transfer is successful for all FCM populations as well as the sterile population. The sterile males were outcompeted by males from Marble hall and Citrusdal resulting in a significant sexual isolation index. Further sexual isolation occurred between mating combinations of laboratory populations, Addo × Nelspruit, Addo × Marble Hall, Citrusdal × Nelspruit and Citrusdal × Marble Hall indicating that these populations have incipient pre-isolation mechanisms affected by local natural selection. These findings provide important information for semiochemical control technologies, especially the SIT program against FCM and support the potential to use and release anywhere in the country.

Chapter 5

Discussion

5.1. Thesis Overview

The genetic confirmation of five genetically isolated populations of FCM from laboratory-reared colonies maintained at Rhodes University using AFLP (Chapter 2) was an important step in order to be able to test sexual attraction and mating compatibility between FCM populations in South Africa. These five populations and the population used for the sterile insect technique (from Xsit), which was also found to be genetically isolated from the wild Eastern Cape and Western Cape populations (Chapter 2), were used for further trials. The first major objective of this study was to determine regional attractiveness between males and females from different geographic regions. A significantly higher attraction of males for females from their own population was found (Chapter 3). The second major objective of determining whether these translate into differences in mating compatibility between populations, found significant differences in sexual isolation between four population combinations, Addo × Nelspruit, Addo × Marble Hall, Citrusdal × Nelspruit and Citrusdal × Marble Hall. In all combinations, Addo and Marble Hall males outcompeted Nelspruit and Citrusdal males to participate in more mating events. When given no choice, there was no significant difference in spermatophore transfer by males to females from any population.

This chapter will discuss the implications of these key outcomes with regard to 1. the use of laboratory-reared colonies as representatives of field populations, 2. the possible pre-reproductive mechanisms relating to the prezygotic barriers of regional attractiveness, and lastly, 3. the implication these factors have on the implementation of control strategies, specifically semiochemical control, where pre-reproductive barriers may decrease overall efficacy. This will be followed by future recommendations and conclusions.

5.2. Laboratory colonies as representatives of field populations

Insects kept in laboratory colonies are a critical resource for conducting behavioural and physiological studies on crop pests. A large number of individuals can be accessed at regular intervals, and there is no need to wait for appropriate seasons, which can lengthen the research

time. However, these colonies comprise a small sample of the wild population and the optimised rearing conditions may lead to adaptations not present in the wild. Likewise, the wild population will experience different environmental pressures not present in the laboratory colony, leading to different adaptations (Erlandson *et al.* 2019). For this reason, the population structure and genetic diversity of the laboratory-reared populations was compared to the wild populations from the Western Cape and Eastern Cape. As is to be expected, the wild populations expressed greater polymorphism i.e. greater genetic diversity between individuals in that population, and overall wild population structure was comprised of two or three different genetic clusters, whilst the laboratory-reared populations were comprised of one or two. Similar to other studies investigating genetic diversity and population structure of laboratory and wild populations, the laboratory colonies had substantially lower observed genetic diversity (H_j) than the wild populations, again suggesting reduced genetic diversity in laboratory colony populations and genetic divergence from wild populations (Fritz *et al.* 2016; Erlandson *et al.* 2019). Therefore, it is important that genetic diversity is determined to understand the degree to which the results from laboratory-reared individuals can apply to wild individuals to prevent recommendations to wild populations that may not be entirely accurate.

Phenotypic effects and genetic effects are often recorded as key challenges faced in the mass rearing of insects. Phenotypic effects, or a decrease in vigour, could arise as a result of overcrowding and poor diet (Reynolds 2012). Genetic effects and changes in behaviour may arise as a result of a 'domestication' effect when long-term laboratory rearing natural selection favours the facility conditions, which in turn affects the reproductive or behavioural traits of the insect (Figure 5.1) (Bartlett 1984; Hoffman & Ross 2018). Whilst there was no formal selection in either the Rhodes University or the SIT FCM (Xsit) mass rearing populations, unintentional genetic pressures, such as inbreeding depression and random genetic drift, may have led to the loss of genetic variability, which can lead to loss of fitness and reduced field performance of the laboratory individuals (Figure 5.1) (Mackauer 1976). Other characteristics of inbred populations can be skewed sex ratios, due to reduced allelic diversity, reduced offspring and increased susceptibility to pathogens and disease (Zaviezo *et al.* 2017).

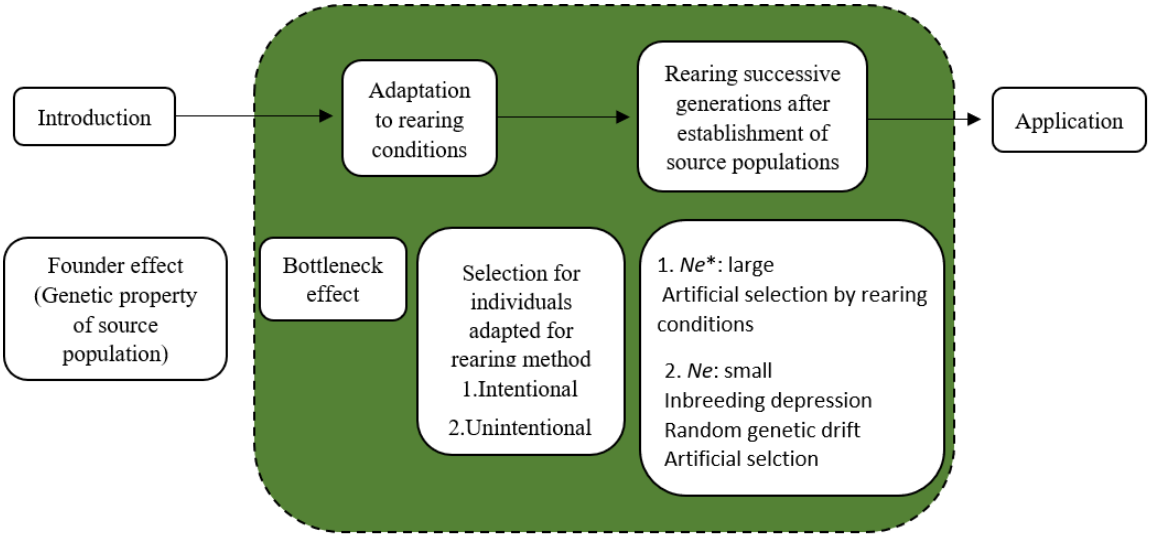

Figure 5.1. Steps during establishment of an insect rearing population and genetic aspects effecting reared populations. *: Effective population size. (Miyatake 1998).

5.3. Regional attractiveness as a mechanism of pre-reproductive speciation

The results from this trial, along with the variation in pheromone blend from geographically isolated populations of FCM in Malawi, Ivory Coast and South Africa, offer the first evidence of pre-reproductive isolation via the prezygotic barrier of sexual communication (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986). Differences in mating signals released by the female can lead to speciation via sexual isolation, due to communication breakdown (Panhuis *et al*. 2001). Prezygotic isolation through sexual selection is an important driver of speciation and relies on the premise that a divergence in sexual signals is connected with the speciation process (Panhuis *et al.* 2001; Wyatt 2003). It is considered to be part of ecological speciation, as this type of divergence is caused by interactions with the environment (i.e. geographically isolated populations) (Sobel *et al*. 2010; Rundle & Rowe *et al*. 2018).

The results from this research fall into the theoretical model that predicts that sexual conflict can drive reproductive isolation by decreasing the probability of mating events between individuals from allopatric populations (Wigby & Chapman 2006). Generally, the evolution of sexual communication is not fully understood in Lepidoptera. Long range sexual communication interference has the potential to be a powerful environmental variable that can exert strong directional selection on sex pheromone variants in female moths (Cardé *et al.* 1977; Löfstedt *et al.* 1991; Gries *et al.* 2001). Signal-to-noise ratios also play an important role

when there is a presence and abundance of species with similar chemical signals, resulting in a selection for females with the clearest, optimised pheromone blend. In evolutionary terms, divergence in sex pheromones equates to eventual discrimination in the choice of mates, and over time, total sexual incompatibility, leading to speciation (Lackey *et al.* 2018).

5.4. Impact of differences in regional attractiveness on semiochemical-based management

Monitoring, attract-and-kill, and mating disruption are all management options using the female synthetic sex pheromone as a trap or lure for FCM males. Their strategies are all different but the approach of using the synthetic female sex pheromone is the same. Therefore, the following problems associated with the use of pheromone lures and traps with regards to regional attractiveness are explored as well as the impacts of regional attractiveness and mating compatibility on SIT.

5.4.1. Monitoring with pheromone traps

The threshold value for trap catches in citrus orchards is determined at 10 males and the continuation of catching 10 males in a trap for a few weeks justifies the use of insecticidal application (Moore *et al.* 2009). This is an important value with regards to regional attraction assuming the lure has equal attractiveness to all populations, however as this study indicates, that is not likely. This could lead to greater population numbers than determined by monitoring and result in late or inadequate control implementation, possibly leading to damaged crops, loss of income and penalties against South African citrus farmers if the pest is reported in consignments sent for export.

5.4.2. Attract-and-kill

When FCM pressure is low, attract-and-kill technology may be used. Attract-and-kill is not as effective as mating disruption and is never used as a sole control method (Hofmeyr 2003; Kirkman & Moore 2007). Therefore, due to the pyrethroid which along with the sex pheromone aids in the attraction of the lure to males, this control strategy may not be as important in optimising to regional population than monitoring and mating disruption.

5.4.3. Mating disruption

Much like monitoring, mating disruption utilises synthetic sex pheromone however at a much more saturated level. The primary objective is to prevent potential mates from locating each other. However, this communication interference has also caused a strong selection in wild populations of codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), to change their sexual communication system, to the point where shifts in the wild pheromone occur (McNeil & Delisle 1993; Cardé & Haynes 1993). Resistance in codling moth to mating disruption was found in Japan where up to 1000 synthetic pheromone dispensers per ha were used consistently for 10 years, resulting in the wild females evolving to change their sex pheromone away from the synthetic pheromone and the males evolved to broaden their pheromone-receptor response (Duménil *et al.* 2014). A system called 'asymmetric tracking' has been widely accepted as a means for sexual communication to evolve. An example of asymmetric tracking occurred when a mutant line of cabbage looper moth, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), evolved in laboratory individuals and resulted in males from the unmutated (normal) line broadening the response towards the females from the normal line as well as the mutant blend after three generations of selection, demonstrating how rapidly sexual communication variations can occur (Evenden *et al.* 2002). The efficacy of mating disruption can be greatly reduced due to differing regional sex pheromones due to the narrow chemical communication system between males and the sex pheromone (Roelofs & Bjostad 1984). Therefore, if the unique isomeric ratio comprising the sex pheromone is not matched by the disruptor then the whole strategy may be at risk with similar consequences as discussed with monitoring.

5.4.4. Sterile Insect Technique

Sterile insect technique relies on sterile males to compete and mate with wild females (Hendrichs & Robinson 2009). Even though the technique is optimised to maintain the sterile moth's overall fitness (keeping radiation to the minimum efficient level and keeping the moths cool (inactive) during transport), the overall fitness of these moths due to the irradiation and transport process will never match the level of fitness of wild individuals. Many pests controlled by SIT have had reduced fitness, especially over different geographical ranges (Briceño *et al.* 2009; Ahmad *et al.* 2018), as the mass reared individuals are not subject to genetic drift and divergence under sexual selection as occurs in the wild. Overcoming these problems often involves choosing character traits best suited to match wild individuals and a genetically diverse origin population. This study also indicates that geographically isolated

populations have localised attraction, further reducing sterile individual fitness if the sterile population originates outside of the target area

5.5. Recommendations

5.5.1. Pheromone lures/ traps

Recommendations to improve monitoring, attract-and-kill as well as mating disruption are similar due to their use of a pheromone lure or trap. Further research into the exact ratios comprising each major component of the sex pheromone will need to be conducted and potentially developed into a localised pheromone lure to increase the efficacy of monitoring and mating disruption as management strategies. Due to the importance of monitoring and how IPM is developed based on the magnitude of the pest threat found in traps, monitoring traps should be of top priority to be optimised for local populations (Hofmeyr 2003). With regards to mating disruption, the current recommendations of the commercial product PheroLure (Insect Science) which was designed for mating disruption (current recommendations: place one pheromone lure source for every 4-6 ha, for 10 months at the start of production), already has recommendations in place that would minimise the pressure on females to alter their sex pheromone. This is important to prevent further intrapopulation sexual isolation (Duménil *et al.* 2014). The traps could also be optimised to the localised female FCM sex pheromone.

5.5.2. Sterile Insect Technique

It would be beneficial to investigate whether facilities mass rearing FCM for control, such as Xsit (Pty) Ltd, should maintain multiple populations or a mixed population. The Xsit mass rearing facility is able to produce up to 21 million insects per week (Hofmeyr *et al.* 2015). Maintaining insects is costly (in addition to the expensive irradiation treatment); therefore, a single population may be preferred over maintaining multiple populations, to keep production costs lower. The results from this study indicate that if a mixed population is preferred, periodic colony refreshment to maintain genetic heterogeneity will need to be conducted. Unfortunately, as there was no true 'mixed' population tested in this study, further testing of mating compatibility and regional attractiveness will need to be performed for this population. With particular focus on the Nelspruit and Marble Hall populations, as they were consistently outcompeted by Addo and Citrusdal males.

Developing a synthetic pheromone to release with the sterile males was not shown to improve mating compatibility in a previous study (Pélozuela *et al.* 2007). However, this has not been widely investigated. There is little information on increased efficiency of interpopulation mating of sterile to wild individuals, if the sex pheromone is released with the sterile individuals. Therefore, it would be beneficial to identify the sex pheromone blends of South African FCM populations and test the efficacy of sterile males in the presence of a synthetic pheromone and without it. This would be less costly than maintaining multiple populations of FCM in a rearing facility.

5.6. Further recommendations

Overall, this study would have benefited from better recorded information on laboratory-reared population origins, in particular from the sterile colony, as the origins for this population are unclear. Aside from the origins, information on the period of time maintained in the laboratory, as well as population crashes and sex ratios would be beneficial to further research conducted on these colonies in any capacity, whether it be genetics, behavioural or physiological. Analysis of the female sex pheromones using sample enrichment probes (SEP) and analysed through gas chromatography coupled to mass spectrometry (GC-MS) was investigated but results could unfortunately not be generated. Due to the sensitivity of the SEP, the experimental design still needs to be optimised to reduce 'noise' in the mass spectrometry analysis. The method was developed for the extraction of the female *Margarodes prieskaensis* Jakubski (Coccoidea: Margarodidae) sex pheromone and was chosen as it will be able to indicate exactly what, how much and in what quantities the sex pheromone is released by the female FCM (Burger *et al.* 2016). Although the extraction of the entire gland has previously been conducted, modern methods using GC-MS will be able to identify the compounds that are released and therefore important in sexual attraction rather than identifying all compounds present in the gland (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986; Hofmeyr & Calitz 1991; Burger *et al.* 2016). The experiment still needs to be conducted to ensure that it is the variation in the sex pheromone that is causing regional attractiveness, and not other potential factors such as differences in peak pheromone release time and diet. However, since variation in the sex pheromone has already been found in geographically isolated populations (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986; Hofmeyr & Calitz 1991), it is reasonable to conclude that it is the primary reason for regional attractiveness.

Another aspect not discussed, but still important to emphasise, is the limited knowledge of intraspecific deviation in sex pheromone blends of female Lepidoptera and differences in pheromone blends within populations, which have occurred in other lepidopteran species, such as codling moth (Duménil *et al*. 2014). Therefore, it may be beneficial to investigate the within population variation in the sex pheromone of FCM, as well as the males broadening response, which would contribute to a decrease in the efficacy of all established semiochemical-based management technologies.

5.7. Conclusion

In conclusion, assortative mating by geographical regions of false codling moth was found, which could be due to different environmental conditions leading to genetic differentiation. This indicates an unstable sexual communication system for FCM, which is subject to variation between populations, as has already been shown in FCM populations from other countries (Angelini *et al.* 1981; Hall *et al.* 1984; Attygalle *et al.* 1986). However, the primary reasons for this have not yet been identified. Evidence of mating disruption and interference with other closely related sympatrically occurring species has driven sex pheromone variation in other Lepidoptera, such as codling moth (Duménil *et al.* 2014). However, available information on intraspecific variation in the sex pheromone is limited, with most studies considering interspecific pheromone variation, due to the general assumption that lepidopteran sex pheromones have very low variation because of their importance as species-recognition signals (Duménil *et al*. 2014; Groot 2014). Overall, the fragmented agricultural environment that leads to sedentary populations of FCM, increases the chances of developing different sex pheromone signals and developing resistance against mating disruption and other semiochemical-based control techniques. Therefore, it is important to monitor these changes between populations and alter pest management strategies for optimised control.

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Appendix

Table A1. DNA concentrations used per sample in ng/µl from the various FCM populations.

Population	Sample ID	DNA concentration (ng/µl)	280/260
Addo (L)	A1	500	1.98
	A2	500	2.04
	A3	300	2.0
	A4	500	1.9
	A5	300	1.92
Nelspruit (L)	N1	500	2.16
	N2	200	2.2
	N3	200	2.07
	N4	200	1.99
	N5	400	1.89
Marble Hall (L)	M1	300	2.33
	M2	300	2.39
	M3	300	1.85
	M4	400	1.78
	M5	400	2.0
Citrusdal (L)	C1	300	2.22
	C2	300	1.95
	C3	300	2.22
	C4	300	1.98
	C5	200	1.91
Old (L)	O1	200	2.06
	O2	200	2.15
	O3	300	1.93
	O4	400	2.3
	O5	300	2.19
Addo (W)	EC1	220	2.04
	EC2	500	1.99
	EC3	300	2.36

	EC4	200	2.24
	EC5	200	2.19
	EC6	300	2.33
	EC7	300	2.28
Clanwilliam (W)	WC1	300	2.2
	WC2	300	1.99
	WC3	300	2.0
	WC4	300	2.21
	WC5	300	2.31
	WC6	300	2.22
Xsit (L)	X1	500	2.05
	X2	500	2.11
	X3	300	2.14
	X4	300	2.23
	X5	300	2.13

**(L) lab, (W) wild populations

Table A2. Reagents, concentrations and volume per sample for Restriction/ Ligation reaction.

Reagent	Stock Concen.	Working Concen.	Volume/sample
ddH2O	-	-	0.68
T4 ligase buffer	10X	10X	1.1 µl
NaCl	0.5M	0.5M	1.1 µl
BSA	10mg/ml	1mg/ml	0.55 µl
MseI adaptor	50µM	50µM	1 µl
EcoRI adaptor	5µM	5µM	1 µl
MseI RE	50U/µl	1U	0.02 µl
EcoRI RE	100U/µl	5U	0.05 µl
T4 DNA ligase	400U/µl	400U/µl	0.0015 µl

Table A3. Oligonucleotide adapters and primers used for AFLP analysis.

Primer	No. s. n.**	Code	Sequences (5'-3')
*Eco*RI adapter	-	*Eco*RI-A	(F)-CTCGTATGCGTACC
			(R)-CATCTGACGCATGGTTAA
*Mse*I adapter	-	*Mse*I-A	(F)-GACGATGAGTCCTGAG
			(R)-TACTCAGGACTCATC
*Eco*RI primer	+0	*Eco*RI-0	GACTGCGTACCAATTC
	+3 (6-FAM)	*Eco*RI-ATC	GACTGCGTACCAATTC +**ATC**
		*Eco*RI-AGT	GACTGCGTACCAATTC +**AGT**
		*Eco*RI-GAC	GACTGCGTACCAATTC +**GAC**
		*Eco*RI-CTG	GACTGCGTACCAATTC +**CTG**
*Mse*I primer	+0	*Mse*I-0	GATGAGTCCTGAGTAA
	+3	*Mse*I-CTA	GATGAGTCCTGAGTAA +**CTA**
		*Mse*I-CAT	GATGAGTCCTGAGTAA +**CAT**
		*Mse*I-CAC	GATGAGTCCTGAGTAA +**CAC**
		*Mse*I-GCA	GATGAGTCCTGAGTAA +**GCA**

** No. s. n. = Number of selective nucleotides

Table A4. Reagents, concentrations and volume per sample for pre-selection reaction.

Reagent	Stock Concentration	Working Concentration	Volume/sample
ddH20	-		1.92 µl
Taq MM	2x	1x	5 µl
MseI selective	100 µM	5 µM	0.54 µl
EcoRI selective	100 µM	1 µM	0.54 µl

Table A5. Reagents, concentrations and volume per sample for the selection reaction.

Reagent	Stock Concentration	Working Concentration	Volume/sample
ddH20	-	-	2.42 µl
Taq MM	2x	1x	5 µl
Primers (E + M)	100µM	5 µM	0.58 µl

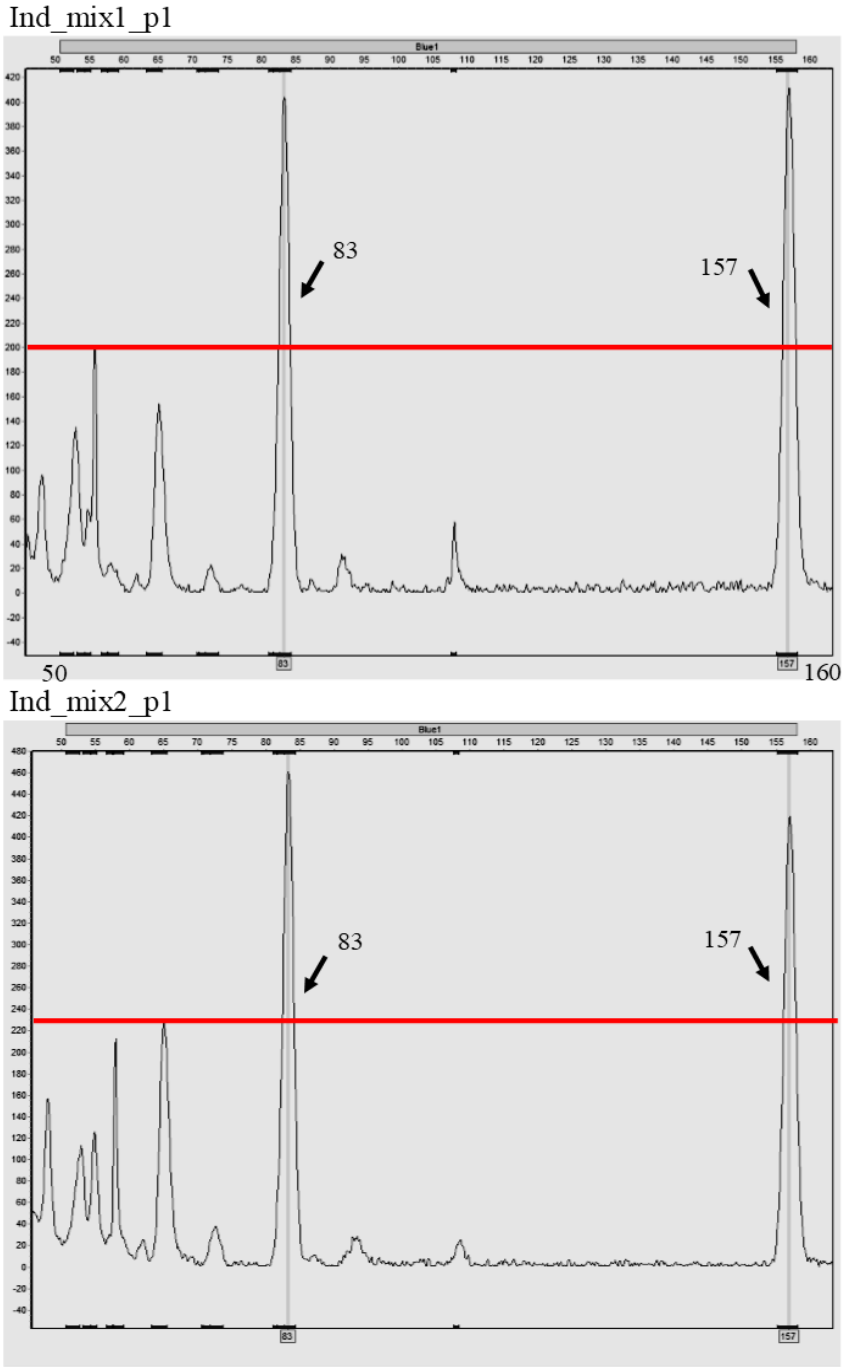

Figure A1. The differences in electropherograms generated by GeneMarker using the AFLP analysis on two individuals from the same population using the same primer, the red line illustrates the fluorescent threshold of 200 units, under which no fragments were used further.