Mechanisms of resistance and tolerance in African and European honeybees *Apis mellifera* L., against *Varroa destructor*

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Declaration

I, **Beatrice Tchuidjang Nganso** declare that the thesis/dissertation, which I hereby submit for the degree Doctor of Philosophy (Entomology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date: February 2019

Disclaimer

This thesis consists chapters that have been prepared as stand-alone papers either already published or as manuscripts yet to be submitted to scientific journals. Consequently, unavoidable overlaps and/or repetitions may occur. Additionally, I conducted all the experiments for the chapters organised in paper format with guidance from all the supervisors, Dr Ayuka T. Fombong at *icipe*, Dr Charles Stuhl and Dr Hans Alborn at the United States Department of Agriculture/Agricultural Research Service-Centre for Medical, Agricultural and Veterinary Entomology (USDA/ARS-CMAVE) in Gainesville, Florida in the United States of America.

Thesis summary

The honeybee, *Apis mellifera* L., is indispensable to global food security, poverty alleviation and natural biodiversity conservation. However, the ecto-parasitic mite *Varroa destructor* and its associated pathogens are one of the most serious threats to the health of honeybees, especially both wild and managed European honeybees found in Europe and North America. In contrast to European honeybees, their African counterparts appear to be minimally affected by these stressors. However, the underlying mechanisms that contribute to their survival against the mites remain mostly unknown. To test the hypothesis that resistant defence behavioural mechanisms are responsible for the survival of *A. m. scutellata* in Kenya, grooming and hygienic behaviours in this honeybee subspecies with those of *A. mellifera* hybrids of European origin found in the USA against the mite were compared in chapter two. The description of two newly damage patterns inflicted on mites by honeybees in both African and European honeybee colonies is highlighted. Additionally, the potential role of grooming behaviour as a tolerant defence mechanism that could reduce the detrimental effects of the mites in the savannah honeybee colonies was underscored, though the expression levels of hygienic behaviour were similar in both honeybee subspecies. However, both hygienic and grooming behaviours could not explain the lower mite-infestation levels recorded in *A. m. scutellata* colonies. To explain the low mite numbers recorded in *A. m. scutellata* colonies, chapter three explored the involvement of other potential resistant mechanisms including suppression of mite reproduction in worker brood cells of this subspecies. Low fertility, fecundity and numbers of mated female offspring were identified as adaptive resistance processes of

reduced *Varroa* mite reproductive success in *A. m. scutellata* colonies, which explained the slow mite population growth in colonies of this subspecies. Furthermore, mite offspring mortality in both sexes and absence of male offspring were identified as key factors to account for the low numbers of mated daughter mites produced in *A. m. scutellata* colonies. The relationship between *Varroa* mite-infestation levels on adult worker honeybees, grooming behaviour and titres of the insect juvenile hormone III (JH III) and that of its immediate biosynthetic precursor, methyl farnesoate (MF), MF + JH III, ratio of JH III to MF in the haemolymph of the African and European honeybees was explored in chapter four. Here, the results suggest that these hormones may not regulate these traits in the honeybee subspecies due to the absence of a significant correlation between them.

Overall, this study has revealed the behavioural mechanisms that partly confer survival strategies in this specific *A. m. scutellata* population against the mite without requiring any miticide treatment. The study has also revealed that JH III, MF, MF + JH III or ratio of JH III to MF may not be considered as potential biomarkers for some behavioural traits studied herein in honeybees. Nevertheless, additional studies are necessary to help shed more light in this interesting area.

Dedication

To my loving Nganso and Noumessi families and above all to God Almighty.

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Presentations and Publications from this thesis

Presentations

ICABH 2017: 19th International Conference on Apiculture and Bee Health (11 – 12th December 2017): Oral presentation. Title: **Assessment of tolerance mechanisms in African and European honeybees against *Varroa destructor***. Rome, Italy.

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International Bee Research Strategy Development Workshop (18-19th September 2017): Oral presentation. Title: **Assessment of tolerance mechanisms in African and European honeybees against *Varroa destructor***. International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya.

Publications

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CHAPTER ONE

General introduction and rationale of the study

General introduction

Pollinators, chiefly bees, but also butterflies, moths, flies, beetles, wasps, trips, birds, bats, mammals and lizards are indispensable to human welfare because they are important contributors to food production, food security, biodiversity conservation, farmer and beekeeper livelihoods globally (Potts et al. 2016). According to the United Nations Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) 2016 report, the economic value of these pollinators to global food production amounts to between USD$235 billion and USD$577 billion per annum. Among these pollinators, the honeybee *Apis mellifera* L. is the most important commercially managed bee that is used to boost the yield of agricultural food crops in the world (Klein et al. 2007, Kremen et al. 2007, Potts et al. 2010, 2016). The pollination services provided by insects, especially honeybees, to agricultural food crops are valued at €153 billion per annum worldwide (Gallai et al. 2009). In Africa, the economic value of insect pollination including honeybees to agricultural food crops is valued at €11.9 billion per annum (Gallai et al. 2009). In Western Kenya, for example, pollinators provide US$3.2 million in ecosystem services to eight crops including: beans, cowpeas, butternuts, sunflowers, monkey nut, tomatoes, capsicum and passion fruits (Kasina et al. 2009). Furthermore, the total economic value of pollination services rendered by managed honeybee colonies to the deciduous fruit industry of the Western Cape in South Africa was estimated to be US$312.1 million (Allsopp et al. 2008).

Honeybees are also essential for ecosystem stability and poverty alleviation because they pollinate wild flora and serve as a source of livelihood to many families through the sales of their products such as honey, royal jelly, propolis and wax (Klein et al. 2007, Allsopp et al. 2008, Potts et al. 2010, VanEngelsdorp and Meixner 2010, Raina et al. 2011). However, evidence exists of declines in honeybee populations, both wild and managed, particularly in North America and Europe over the past decade (VanEngelsdorp et al. 2008, Moritz et al. 2010, Potts et al. 2010, Pirk et al. 2014); while at the same time the demand for their pollination services on a global scale keeps on rising (Goulson et al. 2015).

The decline in honeybee populations has received eminent global attention because of the enormous risks it poses to global food security, economic development and ecosystem stability, particularly in countries where agriculture forms the backbone of the economy (reviewed in Steffan-Dewenter et al. 2005). This decline has spurred global research interests among government, private and public agencies as well as scientists to understand the factors responsible for large scale colony losses, and to provide solutions to them in order to improve and maintain the health of these keystone insect species (VanEngelsdorp et al. 2008, Moritz et al. 2010, Potts et al. 2010). Several interacting factors, which sometimes act synergistically, have been reported to be responsible for this honeybee decline. These include: parasites and pathogens, pesticides, lack of genetic diversity of honeybee colonies (especially those of European origin), climate change, poor nutrition and management, international trade in bee and non-bee products, political and economic disruptions brought by political instability (Potts et al. 2010, VanEngelsdorp and Meixner 2010, Kessler et al. 2015, Moritz and

Erler 2016,). Key among these factors is the ecto-parasitic mite, *Varroa destructor* Anderson and Trueman and its associated pathogens being the most destructive (Le Conte et al. 2010, Neumann and Carreck 2010, Rosenkranz et al. 2010, Francis et al. 2013, Zakar et al. 2014, Kielmanowicz et al. 2015).

The mite *Varroa destructor* is an invasive ecto-parasite of *Apis mellifera* worldwide (Fig 1.1) (reviewed in Nazzi and Le Conte 2016) which invaded *A. mellifera* colonies outside its native host range in Southeast Asia where it was originally restricted only to its natural host *Apis cerana* (Anderson and Trueman 2000). The infestations by the mites can have significant negative effects on susceptible *A. mellifera* populations, especially the ones of European origin, mainly because they lack or express poorly behavioural mechanisms displayed by the mite's original host to counter infestation (Ritter 1981, Fries et al. 1996). These behavioural mechanisms include: hygienic and grooming behaviours as well as entombing of drone broods. Hygienic behaviour is the ability of nurse honeybees to efficiently detect, uncap and remove dead or diseased/parasitised brood while grooming behaviour is the ability of individual honeybees to remove mites off their bodies or from those of their nest mates, thereby sometimes inflicting physical injuries to the mites during the removal process (Peng et al. 1987, Boecking and Spivak 1999, Rath 1999). Additionally, the mite reproduces only in the less abundant and seasonally occurring drone brood in colonies of *A. cerana*, whereas its reproduction takes place in both drone brood and the more abundant worker brood which occur throughout the year (or breeding seasons in temperate regions) in *A. mellifera* colonies (Rath 1999). As a result, beekeepers in the affected countries practice

periodic miticide treatment to prevent the collapse of honeybee colonies (Lee et al. 2010, Neumann and Carreck 2010, Rosenkranz et al. 2010).

Fig 1.1. A *Varroa* mite's family within a honeybee worker brood cell, approximately 11 days after the capping of the brood cell. Upper row from left to right: Protonymph, deutonymph, deutochrysalis. Lower row from left to right: freshly moulted young female, mother mite, adult male reported by Rosenkranz et al. (2010)

The life cycle of the mite *V. destructor* consists of two phases namely: a phoretic and a reproductive phase (Fig 1.2A) (Rosenkranz et al. 2010). During the phoretic phase, the mites are found on adult honeybees (mostly nurse honeybees) usually hidden under their sternites (Rosenkranz et al. 2010). They are either transported to brood cells for their reproduction or transferred horizontally to infest other individual honeybees or colonies (Rosenkranz et al. 2010, Huang 2012). This phase can last for five days to six

months depending on the availability of brood in the honeybee colony (Huang 2012). The reproductive phase of the mite takes place entirely in sealed brood cells and synchronises with the sealed brood development time of the host larvae (Martin 1994). During this phase, a foundress mite invades a worker brood cell with a 5th instar larva shortly before it is capped and lays her first unfertilised egg, ~ 60-70 h following cell capping (Fig 1.2B) (Ifantidis 1983, Martin 1994). This unfertilised egg develops into a male while the subsequent three to four fertilised eggs which are laid at approximately 30 h interval develops into females (Fig 1.2B) (Ifantidis 1983, Martin 1994). A female mite can lay up to five eggs in worker brood and up to six eggs in drone brood (Martin 1994). After hatching out of the egg, the mite offspring pass through two nymphal stages namely: the protonymph and deutonymph stages before the final moult into adult (Fig 1.2B and 1.1). Each nymphal stage is divided into a mobile and an immobile pharate phase, with the later stage called proto- and deutochrysalis (Fig 1.1). It takes about six to seven days for female and male mites to develop into adults (Martin 1994). In all developmental stages, the male mites are smaller than females and have longer legs in relation to the body size than those of female mites (Fig 1.1). They are light yellow in colour and their body shape is triangular. Female mites' body shape changes during development from oblong to transversely elliptical and their colour is reddish-brown to dark brown at adult stage (Fig 1.1) (Rosenkranz et al. 2010). Mating between the mite's offspring occurs within the sealed brood cells once they reach adulthood with the male mite dying shortly afterwards because his mouthpart or gnathosoma has been modified into a reproductive apparatus (Rosenkranz et al. 2010). The foundress mites together with one or two viable, mature and mated daughter mites attach themselves to

the young emerging honeybee leaving behind all the immature mites who die inside the cells. Therefore, a foundress mite is considered to reproduce successfully when one or two adults, mated and viable daughter mites emerge from the cell during each reproductive cycle (Fig 1.2B) (Ifantidis 1983, Martin 1994). Thus, the post capping brood developmental duration of worker brood and the mite offspring mortality in these cells are factors which can potentially influence the reproductive success of foundress mites (Martin 1994, Rosenkranz et al. 2010, Ardestani 2015). Alternatively, mites could be non-reproductive because they die in the cell without reproducing, produce no offspring, produce only male offspring or produce offspring that fail to reach maturity before the developing honeybee pupa hatches as an adult (Harbo and Harris 1999). While reproducing inside the brood cells, the mite and her offspring feed on the haemolymph of the developing pupae and the foundress together with the mature female offspring continue to feed on the adult honeybee after emergence from the cells (Rosenkranz et al. 2010). However, a recent study has reported that the mites feed primarily on the fat body of the individual honeybee (Ramsey and VanEngelsdorp 2017). In the course of feeding, the mites can transmit lethal pathogens to the individual honeybee (Rosenkranz et al. 2010), which affect the individual honeybee physically and physiologically (Aronstein et al. 2012, VanDooremalen et al. 2012, Annoscia et al. 2015).

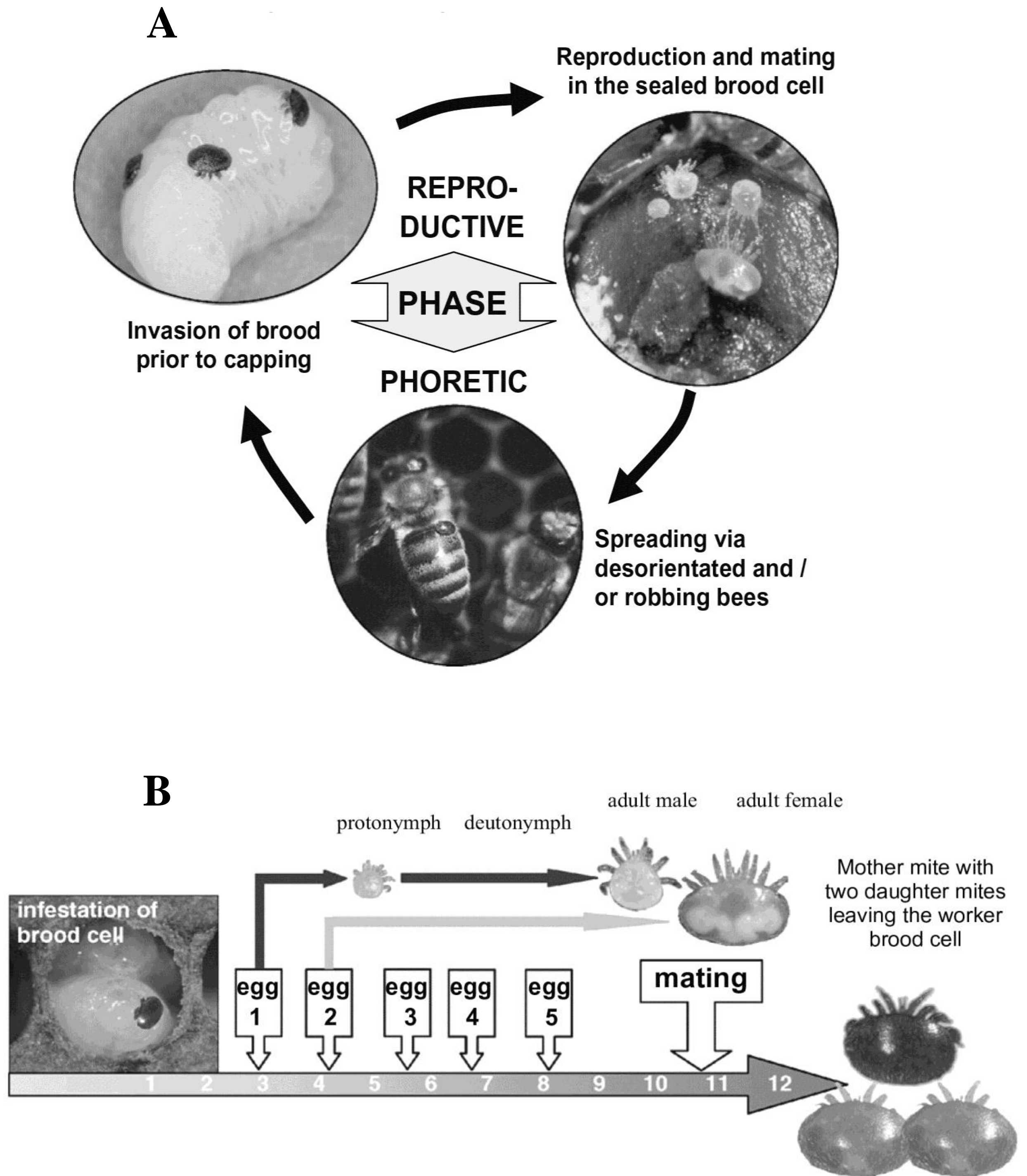

Fig 1.2. Life cycle (A) and reproductive phase (B) of the parasitic mite *Varroa destructor* adopted from Rosenkranz et al. (2010).

Previous studies carried out in Asia, Europe, South and North America have shown that *A. cerana* and some populations of *A. mellifera* displayed specific adaptive behaviours that enable them to co-exist with *Varroa* mite infestations for longer periods without requiring any in-hive miticide control treatment (Locke 2016, Brettell and Martin 2017, Oddie et al. 2017). These adaptive behaviours include hygienic and grooming behaviours, entombing of mites' infested drone brood cells, restriction of mite's reproduction in drone broods, suppression of the mite's reproductive success, shorter post capping time and less attractive brood for mites. Also, African honeybee populations have been reported to survive mite infestation without requiring any managerial inputs by beekeepers. For example, field studies by various researchers demonstrated that survival of the South African Cape honeybee *A. m. capensis* against *Varroa* mite was suggested to be linked to short post-capping stage, hygienic and grooming behaviours of this honeybee subspecies (Moritz 1985, Moritz and Mautz 1990, Allsopp 2006). Likewise, survival of the savannah honeybee subspecies *A. m. scutellata* against the mite was found to be associated with reduced population growth, low viral prevalence, short post-capping stage, low fertility, fecundity and reproductive success of *Varroa* mite foundresses (Moritz 1985, Strauss et al. 2013, Strauss et al. 2015, Strauss et al. 2016). Interestingly, the East African honeybee population of *A. m. scutellata* has also been reported to survive *Varroa* mite parasitism, requiring no chemical treatment even when coexisting with other pathogens responsible for the losses of colonies in Europe and North America (Frazier et al. 2010, Muli et al. 2014). However, it is unknown whether survival of this specific African savannah honeybee population is associated with tolerance (the ability to limit the detrimental effects of the

mite) or resistance (the ability to reduce the reproductive fitness of the mite) as part of its behavioural defense mechanisms or both (Schmid-Hempel 2011).

Rationale of the study

Worldwide, there is widespread consensus of the need to increase our understanding of the impact of *Varroa destructor* and its associated pathogens on the health of honeybee populations to develop solutions, which will help to improve and maintain their health and ultimately colony survival. On the African continent, for example, the presence and spread of the mite *Varroa destructor* and its associated pathogens is of significant concern as they might negatively affect the health of African honeybees in the long term as shown in their European counterparts. Therefore, there is an urgent need to understand the potential resistant and tolerant behavioural defense mechanisms that contribute to the survival of African honeybee populations as they interact with the mites (Dietemann et al. 2009, Pirk et al. 2016). This basic knowledge is crucial for early identification of traits that sustainably mitigate colony loses caused by *Varroa* mites. Additionally, this knowledge might pave the way for more collaborative research, which if rigorously pursued to the end, could restore the global health of honeybees, improve food security, diversify livelihood opportunities thereby reducing poverty and conserve biodiversity. Thus, the overall goal of this research study is to investigate the possible resistant and tolerant behavioural defense mechanisms that contribute to the survival of the African savannah honeybee, *A. m. scutellata* found in Kenya against *V. destructor*. Additionally, this study sought to search for possible hormonal biomarkers

specific to any resistance or tolerance behaviour that could be used for *Varroa* mite-infestation diagnosis.

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CHAPTER TWO

Hygienic and grooming behaviours in African and European honeybees-New damage categories in *Varroa Destructor*

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Abstract

Varroa destructor is an ectoparasitic pest of honeybees, and a threat to the survival of the apiculture industry. Several studies have shown that unlike European honeybees, African honeybee populations appear to be minimally affected when attacked by this mite. However, little is known about the underlying drivers contributing to survival of African honeybee populations against the mite. We hypothesised that resistant behavioural defenses are responsible for the survival of African honeybees against the ecto-parasite. We tested this hypothesis by comparing grooming and hygienic behaviours in the African savannah honeybee *Apis mellifera scutellata* in Kenya and *A. mellifera* hybrids of European origin in Florida, USA against the mite. Grooming behaviour was assessed by determining adult mite infestation levels, daily mite fall per colony and percentage mite damage (as an indicator of adult grooming rate), while hygienic behaviour was assessed by determining the brood removal rate after freeze killing a section of the brood. Our results identified two additional undescribed damaged mite categories along with the six previously known damage categories associated with the grooming behaviour of both honeybee subspecies. Adult mite infestation level was approximately three-fold higher in *A. mellifera* hybrids of European origin than in *A. m. scutellata*. However, brood removal rate, adult grooming rate and daily natural mite fall were similar in both honeybee subspecies. Unlike *A. mellifera* hybrids of European origin, adult grooming rate and brood removal rate did not correlate with mite infestation levels on adult worker honeybee of *A. m. scutellata.* However, they were more aggressive towards the mites than their European counterparts. Our results provide valuable insights into the tolerance mechanisms that contribute to the survival of *A. m. scutellata* against the mite.

Introduction

Varroa destructor Anderson and Trueman (Acari: Varoidae) is an ecto-parasitic pest of the Western honeybee, *Apis mellifera* L. (Hymenoptera: Apidae). It feeds on the fat body of both immature and adult honeybees while transmitting lethal pathogens (Ramsey and VanEngelsdorp 2017, Rosenkranz et al. 2010) causing severe physical and physiological injuries to individual honeybees (Locke 2012). In the absence of appropriate control measures, honeybee colonies heavily infested with the mites succumb within 1-2 years (Rosenkranz et al. 2010). Interestingly, the mite is a relatively harmless pest on its native host, the Eastern honeybee *Apis cerana*, found mainly in Asia (Hepburn and Radloff 2011). *A. cerana* has efficient defensive mechanisms including hygienic and grooming behaviours to limit the mite's reproduction in drone brood cells only which are generally less abundant than worker brood cells in a colony and do not occur throughout the year (Peng et al.1987, Rath 1999, Boecking, and Spivak 1999). The mite is an invasive pest of the Western honeybee, *A. mellifera* which occurs elsewhere in the world (reviewed in Nazzi and Le Conte 2016). Unlike *A. cerana*, the mite reproduces successfully in both worker and drone broods of *A. mellifera* (Rosenkranz et al. 2010). Additionally, the absence of certain adaptive behavioural and physiological mechanisms that are present in its original host, has made the Western honeybee highly susceptible to the mite (Ritter 1981). Pathogens associated with the mite are considered responsible for the decline of managed honeybee colonies especially in Europe and North America (Boecking and Genersch 2008, Le Conte et al. 2010, Neumann and Carreck 2010, Francis et al. 2013). As a result, beekeepers in most of the affected countries substantially depend

on in-hive chemical treatments to keep mite populations below economic thresholds so as to prolong survival of the colonies (Boecking and Genersch 2008, Lee et al. 2010, Rosenkranz et al. 2010).

Previous studies carried out in Asia, Europe, South and North America have shown that *Apis cerana* and some populations of *Apis mellifera* have developed specific adaptive behaviours that enable them to co-exist with *Varroa* mite infestations (Locke 2016). These adaptive behaviours include hygienic and grooming behaviours, entombing of mites' infested drone brood, restriction of mite's reproduction in drone broods, suppression of the mite's reproductive success, shorter post capping time and less attractive brood for mites.

In grooming behaviour studies, an estimate of the percentage of damage inflicted on mites by honeybees is used as a measure of the bee's grooming behaviour (Bienefeld et al.1999). This estimation can also be inferred from a damage classification scheme developed by Corrêa-Marques et al. (2000) comprising six different categories: a) damaged legs, b) hollow in the dorsal shield, c) carcass-empty dorsal shield, d) damage shield + damaged legs, e) hollow in the dorsal shield + damaged legs, and f) damaged shield. On the other hand, hygienic behaviour is measured as the rate at which nurse bees remove dead or diseased brood (Spivak and Reuter 1998).

Studies have shown that African honeybee populations survive mite infestation without requiring any managerial inputs by beekeepers. For example, field studies by various researchers demonstrated that survival of the South African Cape honeybee *A. m. capensis* against *Varroa* mite was linked to short post-capping stage, hygienic and grooming behaviours of this honeybee subspecies (Moritz 1985, Moritz

and Mautz 1990, Allsopp 2006). Likewise, survival of the savannah honeybee subspecies *A. m. scutellata* against the mite was found to be associated with reduced population growth, low viral prevalence, short post-capping stage, low fertility, fecundity and reproductive success of *Varroa* mite foundresses (Moritz 1985, Strauss et al. 2013, Strauss et al. 2015, Strauss et al. 2016). Interestingly, the East African population of *A. m. scutellata* has also been reported to survive *Varroa* mite parasitism, requiring no chemical treatment even when coexisting with other pathogens responsible for the losses of colonies in Europe and North America (Frazier et al. 2010, Muli et al. 2014). However, it is unknown whether survival of this specific African savannah honeybee population is associated with tolerance (the ability to limit the detrimental effects of the mite) or resistance (the ability to reduce the reproductive fitness of the mite) as part of its behavioural defense mechanisms or both (Schmid-Hempel 2011). To test the hypothesis that resistant defense mechanisms confer coping and survival strategies in this specific population of *Apis mellifera scutellata*, we compared the grooming and hygienic behaviours in this honeybee subspecies with those of *A. mellifera* hybrids of European origin found in the USA against the mite.

Materials and methods

Study sites

The study was conducted in Nairobi, Kenya from August - September 2015 (the cooler- dry season) and in Gainesville, Florida, United States of America in April

2016 (spring). These periods are characterised by reduced brood rearing in both savannah and European honeybees (Hood 2000, Raina and Kimbu 2005). All the colonies were housed in standard Langstroth hives containing 3 to 4 brood combs and were not treated with acaricides to reduce mite infestations.

In Kenya, seventeen (17) queen right colonies were selected at two sites namely Kithimani (1°8' S, 37°25 E) (N=10) and Kilimanbogo (1°8' S, 37°21' E) (N=7) both located within the county of Machakos. These two apiaries were 7.4 Km apart and, contained colonies that originated from locally captured swarms. The colonies in this neighbourhood host *A. m. scutellata* (Hepburn and Radloff 1988, Raina and Kimbu 2005, Muli et al. 2014). In Gainesville, Florida, USA, twenty colonies (20) were selected, with ten (10) each at the University of Florida apiary (29.62°38'N, 82.35°21'W) and USDA- ARS-CMAVE apiary (29.63°38'N, 82.36°21'W). These apiaries were ~ 1.6 Km apart and were bred from honeybee stocks purchased from local commercial queen breeders. The honeybee colonies in the USA were hybrids of different European subspecies (Ellis, personal communication).

Molecular identification of *Varroa* mite strains

To confirm the strain of *Varroa* mites present in the savannah and hybrids of European honeybee colonies, two honeybee colonies were randomly selected among the colonies used at the individual apiaries in Kenya and the USA. Five living mites per colony (N = 5) were collected from adult worker honeybees on the brood area of the comb using the standard sugar-roll method (Dietemann et al. 2013) and preserved in 95 % ethanol for DNA analysis at the USDA-ARS-CMAVE in Gainesville, Florida, USA. In total, eight mites were analysed, that is, two mites

per single colony in each apiary using the methods detailed below. Genomic DNA was extracted from individual mites using the DNeasy Tissue Kit (Qiagen, USA) per the manufacturer's protocols for the spin-column protocol for Cultured Animal Cells with the following slight modifications: (i) all volumes were reduced to half; (ii) incubation was at 70 °C for 1 hour; (iii) final elution was in 50 µL of Buffer AE. Nucleic acid concentrations were measured in each sample and three fragments from the cytochrome oxidase I (cox1), cytochrome oxidase III (cox3) and ATP synthase 6 (atp6) mitochondrial genes were amplified by polymerase chain reaction (PCR). The primers of these selected mitochondrial genes were purchased from Integrated DNA Technologies, Coralville, Iowa, USA (Navajas et al. 2008). The amplified gene fragment, primer name, primer sequences, product size base pairs (bp) and the annealing temperature for each fragment are presented in S1 Table. Reactions were carried out in 50 µl containing 1X buffer, 0.05 U Taq polymerase (Invitrogen), 0.2 mM dNTPs, 0.4 µM of each oligonucleotide primer, 1.5 mM of $MgCl_2$ and 1 µl of sample DNA. The positive control was gDNA from *Varroa destructor* samples identified at the study sites in Gainesville, Florida, USA. Cycling conditions involved initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds and annealing for 30 seconds and extension at 72 °C for 1 minute. The amplicons were analysed by gel electrophoreses on a 1.5 % agarose gel run for 2 hours at 90 volts. PCR products were cleaned-up using the DNA Clean and Concentrator™-5 kit (Zymo Research) and bi-directionally sequenced by Macrogen (Maryland, USA). Sequences were edited with BioEdit Version 7.2.5.0 software (Hall 1999). Sequences obtained from

individual mites were compared with those at National Center for Biotechnology Information (NCBI) using the online tool BLASTn to identify the *Varroa* mite strain. Species-level identification was determined when sequences exhibited ≥ 99 % identity.

Assessment of grooming behaviour in honeybees of African and hybrids of European origin

Prior to the grooming behaviour experiments, the level of infestations with *Varroa* mites on approximately hundred (100) adult worker honeybees in each colony was determined using the standard sugar-roll method (Dietemann et al. 2013). The percentage of *Varroa* mite infestation rates in adult honeybees was determined by taking the number of *Varroa* mite collected divided by 100 adult worker honeybee and then multiplied by 100 (Allsopp 2006, Strauss et al. 2015).

Grooming behaviour was assessed in the selected colonies in Kenya and USA using the screen bottom board method. Prior to the beginning of the study, the original bottom board of each colony was replaced with a modified bottom equipped with a retractable floor and covered with a screen mesh fine enough to permit only the passage of mites through its openings, thereby restricting the honeybees to further inflict damages on fallen mites. Cardboard white paper coated with sticky non-toxic petroleum jelly (Vaseline®) was smeared on the retractable floor to intercept falling mites and to protect them from being further damaged by predators such as ants, the small hive beetle and wax moth larvae. Natural fallen mites were collected every 24 hours from the debris on the bottom board using a fine Camel hairbrush for a duration of 7 days and examined for injuries under a Leica S6E stereo microscope

(×40 Magnification). The damaged mites were further grouped into different damage categories using the classification of mite's damages (Corrêa-Marques et al. 2000). The percentage of damaged mite in each colony was determined by dividing the number of damaged mites by the total number of dropped mites collected at the end of the collection period. The average daily natural fallen mite/per colony was determined by dividing the total number of natural fallen mites by the number of days mites were collected (Strauss et al. 2015).

Assessment of the source of physical damage on fallen mites in *A. m. scutellata* colonies

We investigated whether the recorded mite damages on the screen bottom boards of colonies were due to honeybee's grooming behaviour or other agents such as ants, small hive beetle or wax moth larvae. *Varroa* mites were collected from the savannah honeybee colonies using the standard sugar-roll method (Dietemann et al. 2013) from a subset of colonies at the Kithimani's apiary in Kenya and freeze-killed at - 80 °C for 30 minutes. They were subsequently observed under a dissecting microscope to ensure that none was damaged before the beginning of the experiment. The dead, undamaged mites were marked on the dorsal shield with two permanent markers of different colours, blue and black. Three colonies (N=3) were used for this experiment and grease oil was spread on the wooden platforms to restrict ants present from accessing the hives. In each colony, ten (10) black, marked mites were introduced on a white, glossy cardboard coated with sticky non-toxic petroleum jelly (Vaseline®) (to protect fallen mites from being further damaged by predators such as ants and wax moth larvae); and twenty (20) blue, marked mites were introduced

in the brood area of one frame. Fallen mites were collected after 24 hours from the debris on the bottom board using a fine Camel hairbrush and examined for injuries under a Leica S6E stereo microscope (×40 Magnification). The experiment was repeated three times.

Assessment of hygienic behaviour in honeybees of African and hybrids of European origin

Hygienic behaviour was assessed in the selected colonies (N=17) at each apiary in Kenya and in nine colonies (N=9) at each apiary in the USA using the standard freeze-killed brood assay method using liquid nitrogen to freeze-kill young pupae (white- to purple-eyed stage with no cuticular tanning) as described by (Büchler et al. 2013). The number of fully removed freeze-killed brood cells from the test patch was recorded after a period of 24 and 48 hours and expressed as the percentage of the total brood containing cells at the start of the experiment.

Ethical considerations

For field study in Kenya, written informed consents were obtained from the apiary owners. In the United States of America, we used apiaries managed by the USDA/ARS-Centre for Medical, Agricultural and Veterinary Entomology, Gainesville and the University of Florida.

Statistical analyses

Statistical analyses were performed using R-Software version 3.2.5 (R Development Core Team 2015) and the alpha level was set at 0.05. In Kenya, six colonies absconded during the experimental period including five from Kithimani and one from

Kilimanbogo apiaries respectively. Consequently, these colonies could not be monitored for the entire duration of the experiment. In the USA, none of the colonies absconded during the entire monitoring period. Data from Kithimani and Kilimanbogo apiaries were pooled to obtain average total mite dropped, percentage of damaged mites, *Varroa* mite-infestation per 100 adult worker honeybees and the proportion of removed freeze-killed brood at 24 and 48 hours in the African savannah honeybee colonies. Likewise, data from the USDA-CMAVE and experimental farm of the University of Florida apiaries were pooled to obtain similar information in the colonies of honeybee hybrids of European origin. The count data were analysed using generalised linear model (GLM) with log link and binomial distribution error to compare the factors: total number of fallen mites, *Varroa* mite-infestation level and the daily mites fall between both honeybee subspecies. Meanwhile, the proportion data were analysed using generalised linear model (GLM) with logit link and binomial distribution error to compare the factors: percentages of damaged mites, different types of damages and freeze-killed brood removed at 24 and 48 hours between both honeybee subspecies. The effect of a factor for a GLM is reflected in the deviance (likelihood ratio test statistic) that has an appropriate chi-square distribution; hence the chi-square values are presented as test statistics. The Mann-Whitney-Wilcoxon test was used to compare the ratio of total natural fallen mite/*Varroa* mite-infestation level on adult worker honeybee between both honeybee subspecies. Spearman's rank order correlation analysis was conducted to establish the existence of a relationship between the percentage of mite damage (overall and categorical damage types), total

natural fallen mite, daily natural fallen mites/colony and brood removal (after 24 and 48 hours) to *Varroa* mite-infestation level on adult worker honeybee in each study site.

Results

Molecular identification of *Varroa* mite strains

Varroa destructor was the only *Varroa* mite species detected in the colonies of *A. m. scutellata* and *A. mellifera* hybrids of European origin and all the haplotypes belonged to the Korean strain (K1 haplotype).

Assessment of grooming behaviour in honeybees of African and hybrids of European origin

An infestation rate of 5 ± 1.4 mites/100 adult worker was recorded in the surviving African savannah honeybees which was significantly lower (~three-fold less) than the infestation rate in the susceptible hybrids of European origin honeybees at 14 ± 2.3 mites/ 100 adult workers (df = 32: F = 10.90; P = 0.001, Table 2.1).

A total of 126.6 ± 3.2 natural fallen mites/colony was collected from the bottom boards of the African savannah honeybee (Kithimani; N = 8 colonies; Kilimanbogo; N = 6 colonies) compared to 110.6 ± 4.2 natural fallen mites/colony collected from the bottom boards of the hybrids European origin honeybees (USDA and University of Florida apiaries (Gainesville): N = 10 colonies in each), which were not significantly different (df = 236: F = 1.97; P = 0.16). Similarly, the daily natural mite fall/colony (df = 32: F = 0.28; P = 0.60) and the percentages of damaged mites (df = 236: F = 0.04; P = 0.84) recorded in both honeybee subspecies colonies were not significantly different (Table

2.1). The ratio of total natural mite fall/mite infestation level was significantly higher in the African savannah honeybee colonies than those recorded in the hybrids of European origin honeybee colonies ($W = 52$, $P = 0.002$). There was no significant correlation between the daily natural mite fall/colony (Spearman's rank correlation: $r = -0.17$, $P = 0.55$; Fig 2.1), total natural mite fall (Spearman's rank correlation: $r = -0.17$, $P = 0.57$; Fig 2.1) and mite infestation level/colony in the African savannah honeybee colonies. In contrast, a significant and positive correlation was detected between the daily natural mite fall/colony (Spearman's rank correlation: $r = 0.48$, $P = 0.03$; Fig 2.1), total natural mite fall (Spearman's rank correlation: $r = 0.47$, $P = 0.04$; Fig 2.1) and mite infestation level/colony in the hybrids of European origin honeybee colonies. Also, there was no significant correlation between the percentage of damaged mites or the percentage of the different types of damages and the *Varroa*-infestation levels in the African savannah honeybees (Fig 2.1). A similar result was obtained in the hybrids of European origin honeybees with the exception that there was a significant negative correlation between damage to the mite's dorsal shield or idiosoma and the adult worker honeybee mite infestation rates (Spearman's rank correlation: $r = -0.46$, $P = 0.04$; Fig 2.1).

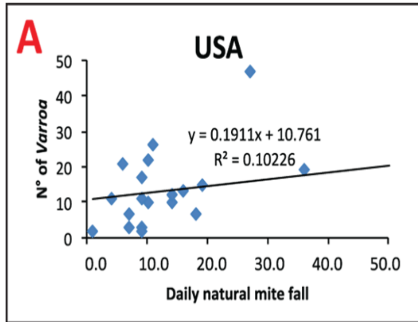
Different categories of damages to the mite were recorded in this study (Fig 2.2) including two additional previously undescribed damage categories to the mite namely; damaged empty dorsal shield and damaged legs + damaged gnathosoma + damaged shield (Fig 2.2B and 2.2C). These additional damage categories were present in colonies of both African savannah and hybrids of European origin honeybees (Table 2.2). Damaged leg (total or partial loss of one or more legs) was the predominant type of physical injury to the mite recorded in the hybrids of European origin honeybee colonies

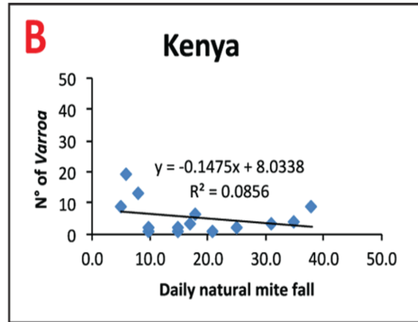
and this was significantly different from those found in the African savannah honeybee colonies (df = 236: F = 9.23; P = 0.003, Table 2.2). In the African savannah honeybee colonies, damaged legs + damaged gnathosoma was the predominant type of mite injury found and this was significantly different from those found in colonies of European hybrids (df = 236: F = 5.14; P = 0.02, Table 2.2).

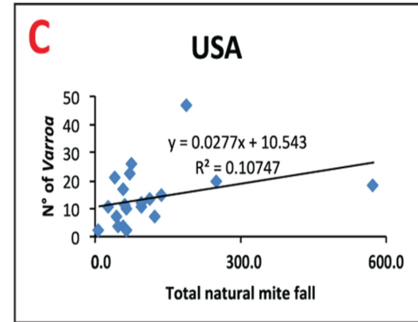
Table 2.1. Mean ± standard error of mite infestation rates, daily mite fall and percentage of damaged mites on adult honeybee workers in colonies of *A.m. scutellata* and *A. mellifera* hybrids of European origin.

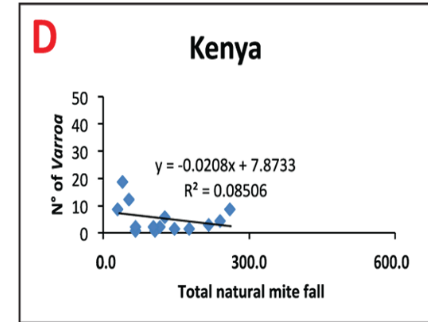
			Mean ± SE		
Sites	**Honeybee species**	**Number of colonies**	**Mite infestation rate/100 adult worker bees (3 replicates/colony)**	**Daily mite fall/colony**	**% damaged mites**
Kenya	*Apis mellifera*	14	5.0 ± 1.4	18 .1 ± 2.8	21.3 ± 1.7
USA	*Apis mellifera* hybrids of European origin	20	14 ± 2.3	15.8 ± 3.9	21.3 ± 2.4
P-Value[a]			0.001	0.60	0.84

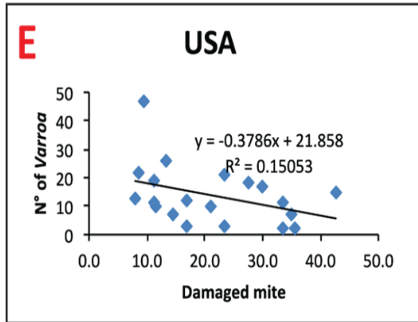
a p values were calculated using the generalised linear model (GLM) with log or logit links

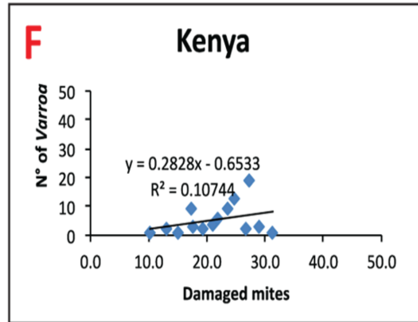

A
USA
y = 0.1911x + 10.761
R² = 0.10226
N° of Varroa
Daily natural mite fall

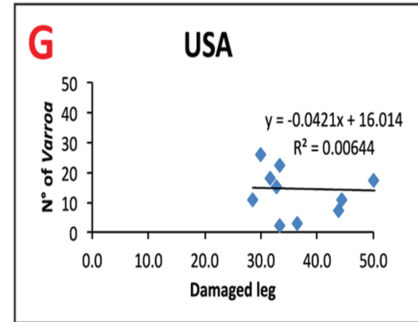

B
Kenya
y = -0.1475x + 8.0338
R² = 0.0856
N° of Varroa
Daily natural mite fall

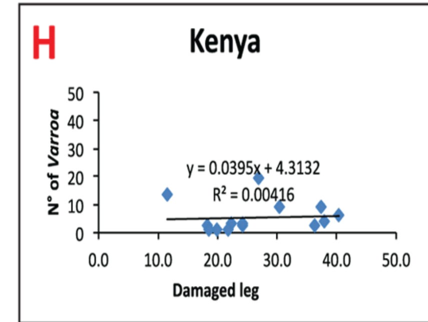

C
USA
y = 0.0277x + 10.543
R² = 0.10747
N° of Varroa
Total natural mite fall

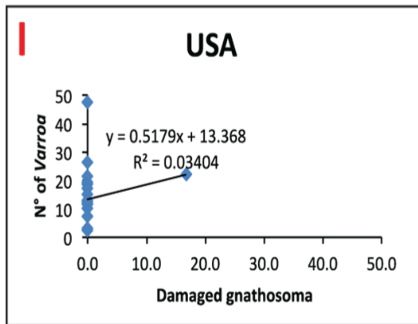

D
Kenya
y = -0.0208x + 7.8733
R² = 0.08506
N° of Varroa
Total natural mite fall

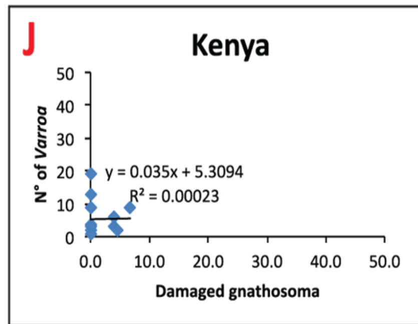

E
USA
y = -0.3786x + 21.858
R² = 0.15053
N° of Varroa
Damaged mite

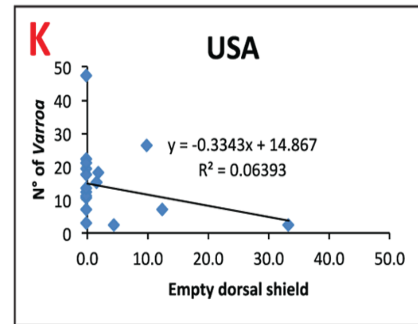

F
Kenya
y = 0.2828x - 0.6533
R² = 0.10744
N° of Varroa
Damaged mites

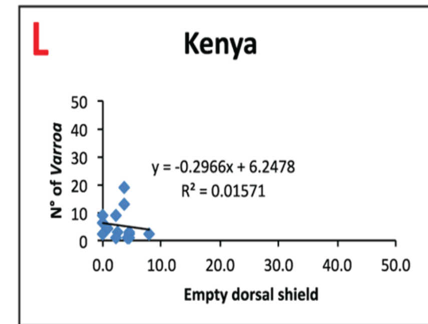

G
USA
y = -0.0421x + 16.014
R² = 0.00644
N° of Varroa
Damaged leg


H
Kenya
y = 0.0395x + 4.3132
R² = 0.00416
N° of Varroa
Damaged leg


I
USA
y = 0.5179x + 13.368
R² = 0.03404
N° of Varroa
Damaged gnathosoma


J
Kenya
y = 0.035x + 5.3094
R² = 0.00023
N° of Varroa
Damaged gnathosoma


K
USA
y = -0.3343x + 14.867
R² = 0.06393
N° of Varroa
Empty dorsal shield


L
Kenya
y = -0.2966x + 6.2478
R² = 0.01571
N° of Varroa
Empty dorsal shield

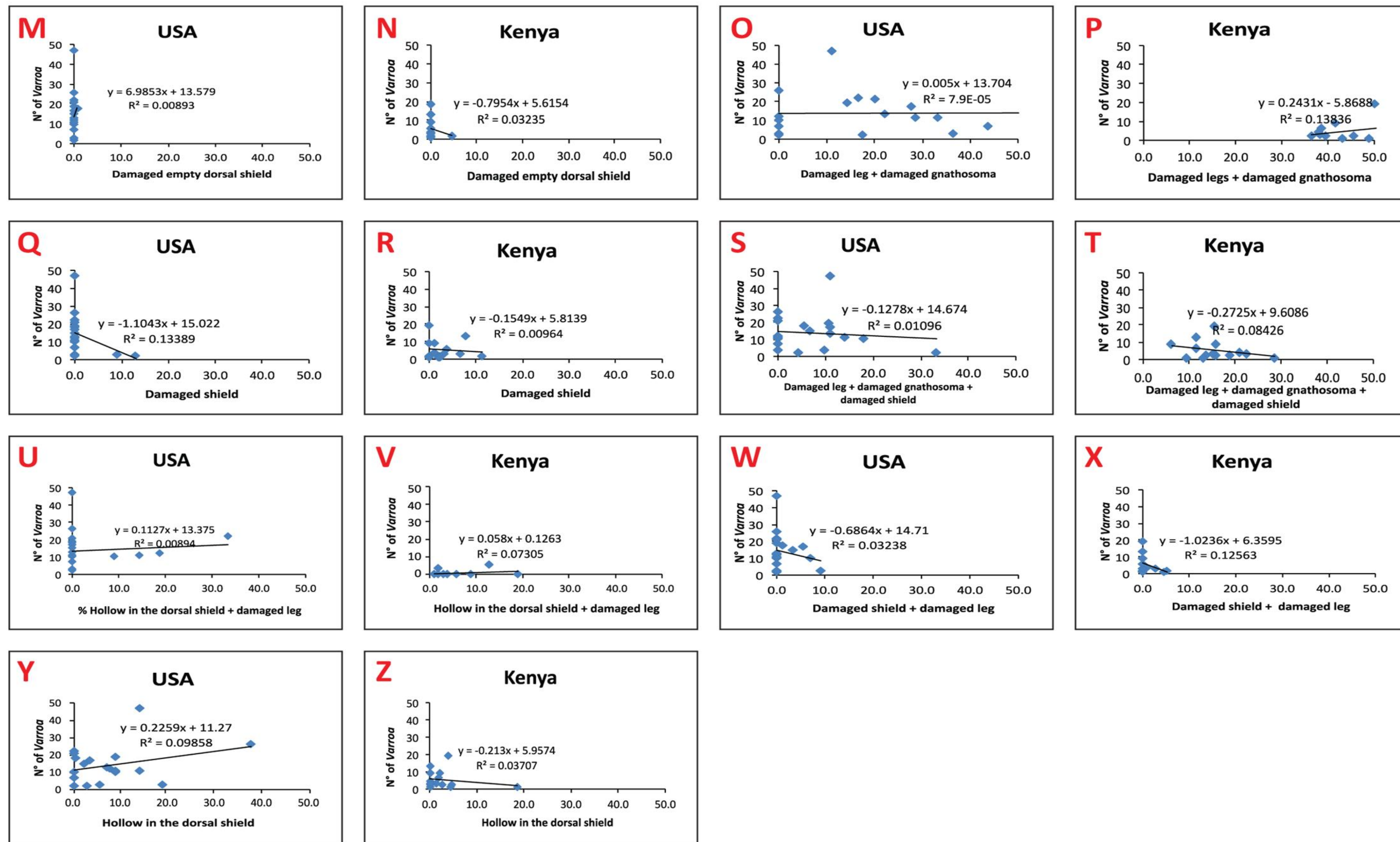


Fig 2.1. Correlation between daily natural mite fall, total natural mite fall, percentage damaged mites, different categories of damage to the mites and *Varroa*-mite infestation level per colony in honeybees of African and European origin in Kenya and USA respectively.

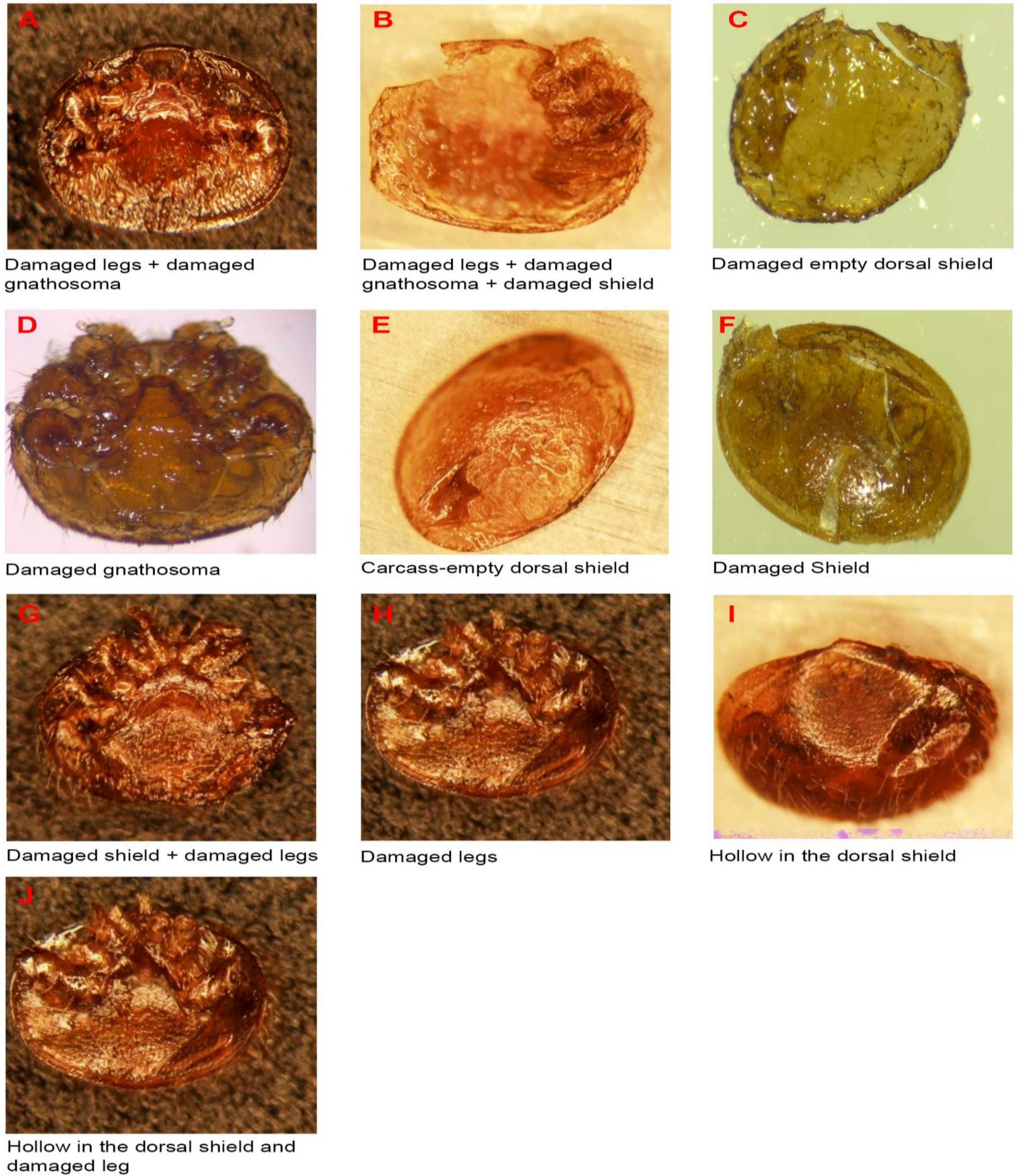


Fig 2.2. Photographs showing the different damage patterns in mature female *Varroa destructor* mite (×40 Magnification). (A and D) Damaged categories from literature (Ruttner and Hanel 1992, Lodesani et al.1996, Rosenkranz et al. 1997). (B and C) Additional damage categories reported in this study. (E-J) Previously known classification of damage to the mites reported by Corrêa-Marques et al. (2000).

Table 2.2 Percentages (mean ± SE) for the different categories of damages to *Varroa destructor* recorded in the colony debris of *A.m. scutellata* and *A. mellifera* hybrids of European origin in Kenya and USA respectively.

Category of damage	Kenya (%)	USA (%)	P-Value[a]
Damaged legs (DL) = total or partial loss of one or more legs	5.7 ± 0.6	10 ± 1.4	0.003
Hollow in the dorsal shield (HDS) = Depression in the dorsal shield	0.5 ± 0.2	2.3 ± 0.7	0.01
Empty dorsal shield (EDS)-carcass = mites that lacked all legs and all or almost all of the ventral shields, generally only the dorsal shield remained	0.6 ± 0.2	0.1 ± 0.7	0.01
Damaged shields (DS) = loss of dorsal shields, fissures in and loss pieces of the dorsal shield	0.6 ± 0.2	0.3 ± 0.2	0.001
Damaged shield + damaged legs (DS + DL)	0.2 ± 0.1	0.4 ± 0.2	0.46
Hollow in the dorsal shield + damaged legs (HDS + DL)	0.1 ± 0.1	0.4 ± 0.2	0.08
Damaged gnathosoma (DG) = loss of chelicerae and/or pedipalps	0.3 ± 0.1	0.1 ± 0.1	0.001
Damaged empty dorsal shield (DEDS) # = fissures in and loss of pieces of empty dorsal shield	0.1 ± 0.1	0.01 ± 0.01	0.44
Damaged legs + damaged gnathosoma (DL + DG) #	9.5 ± 0.7	6.1 ± 1.2	0.02
Damaged legs + damaged gnathosoma + damaged shield (DL + DG + DS) #	3.7 ± 0.4	1.6 ± 0.7	1.9e-10

#New damage categories observed in this study
a p values were calculated using the generalised linear model (GLM) with logit links

Assessment of the source of physical damage on fallen mites in *A. m. scutellata* colonies

Out of the 90 marked, undamaged, dead mites introduced, four blue (6.7%) and four black (13.3%) marked mites were damaged, representing only 8.9% damaged mites of the overall mites introduced. The damages inflicted on the blue marked mites were likely caused by worker honeybees, while damages inflicted on the black marked mites may have been caused by other agents (e.g. wax moth larvae, small hive beetle adults and ants) found on the white, glossy cardboard fitted on the bottom board of colonies. We recorded only two types of damages to the mites in this experiment namely: damaged legs and damaged legs + damaged gnathosoma.

Assessment of hygienic behaviour in honeybees of African and hybrids of European origin

Brood removal rates at 24 and 48 hours were not significantly different between colonies of the African savannah (24 hours = 66.5 ± 8.3% and 48 hours = 81.0 ± 6.2%, mean ± SE) and the hybrids of European origin honeybees (24 hours = 59.1 ± 4.9% and 48 hours = 77.0 ± 3.9%, mean ± SE) (24 hours: $F = 0.65$, $df = 27$, $P = 0.43$; 48 hours: $df = 27$, $F = 0.42$, $P = 0.52$). There was a significant positive correlation between the mite infestation level of adult bees and the brood removal rate at 48 hours (Spearman's rank correlation: $r = 0.48$, $P = 0.04$) though no correlation (Spearman's rank correlation: $r = 0.37$, $P = 0.13$) was detected at 24 hours in the hybrids of European origin. In colonies of the African savannah honeybee, there was no correlation between the mite infestation level of adult bees and the brood removal rate at 24 hours (Spearman's rank correlation: $r = -0.43$, $P = 0.19$) and 48 hours (Spearman's rank correlation: $r = -0.30$, $P = 0.38$).

Discussion

Grooming behaviour

Our study suggests that European and African honeybees express similar grooming behaviour since the percentage of damaged mites recorded on the bottom boards of both subspecies were similar. However, the phoretic mite numbers in both honeybee populations were different, approximately three-fold more in the European than in the African honeybee colonies. With more phoretic mites, one would expect to find more fallen and damaged mites on the bottom board; however, these values were not significantly different between both honeybee subspecies (Table 2.1). The absence of a significant correlation between the total natural mite fall, the percentage of damaged mites, the different categories of damage to the mite and *Varroa* mite-infestation levels suggests that grooming behaviour may not explain the variability in *Varroa* mite-infestation levels recorded in the African and European honeybees. Moreover, these measures of grooming behaviour (percentage of damaged mites or different categories of damage to mite) might not be sensitive enough to assess grooming behaviour at the colony level as previously thought (Lodesani et al. 1996, Corrêa-Marques et al. 2000). It is important to note that of the total mite population recorded on the bottom boards of honeybee colonies, not all mites which are groomed off by honeybees are damaged (Rosenkranz et al. 1997). It is likely that damaged mites may also result from hygienic removal of infested capped brood by honeybees, and interactions with other arthropods in the colony such as the small hive beetle, wax moth and/or ants (Rinderer 1986, Rosenkranz et al. 1997, Bienefeld et al. 1999). The ratio of total natural mite fall/mite infestation level, which represents a fraction of the total mite removed by

honeybees off their bodies relative to the total mite population present in their colonies (Branco et al. 2006), was significantly higher in the African savannah honeybee colonies than those recorded in the colonies of their European counterparts. It appears that, the African savannah honeybee which maintains lower mite colony infestations displays a more efficient grooming behaviour than its European counterpart. This finding corroborates results of previous studies which showed that colonies of *Varroa*-resistant *A. mellifera* subspecies also maintain lower mite loads and record a higher percentage of injured mites than their susceptible counterparts (Guzman-novoa et al. 2012, Invernizzi et al. 2015).

To further characterise the differences in grooming behaviour between subspecies, we analysed the levels and patterns of damage in fallen mites using the previously known classification of damage to mites (Corrêa-Marques et al. 2000). We found that the number of mites with only damaged legs was significantly higher in the European honeybee colonies than in colonies of the African counterpart. On the other hand, the numbers of mites with damaged legs and damaged gnathosoma were significantly higher in the African honeybee colonies than the European counterpart. This category of damage was first recorded in mites found in *A. m. carnica* colonies in Austria (Ruttner and Hanel 1992) but not included as a separate category of damage in the previous classification of damage to mites (Corrêa-Marques et al. 2000). In the present study, the category described as legs and gnathosoma damage, was the second most frequent category of damage recorded in mites found in the European honeybee colonies. Moreover, we found two additional undescribed damage categories in mites namely; damaged empty dorsal shield and damaged legs + damaged gnathosoma +

damaged shield in both honeybee subspecies and occurring more frequently in the African savannah honeybee than in the European honeybee. Overall, these results suggest that a higher aggressive behaviour is displayed by the African savannah honeybee than by their European counterparts towards the mite. Taken together, these results provide additional insights into the grooming behaviour of different subspecies of honeybees.

Based on recommendations for the control of *Varroa* mite in European honeybee colonies in the USA, interestingly, we observed that, the *Varroa* infestation levels recorded in the savannah honeybee colonies were high enough to warrant miticide treatment (Traynor et al. 2016). Surprisingly, none of the colonies of *A. m. scutellata* used in the present study showed any signs of collapse. Typically, beekeepers in this region encountering such populations of *Varroa* mite in honeybee colonies neither administer any mite control measures (Muli et al. 2014) nor is done by beekeepers elsewhere on the rest of the African continent (Pirk et al. 2016). The *Varroa* infestation levels recorded in *A. m. scutellata* colonies in Kenya was similar to those recorded in colonies of the same honeybee subspecies found in South Africa (Mortensen et al. 2016) and no deleterious effects caused by the mites were reported (Allsopp 2006, Strauss et al. 2015, Mortensen et al. 2016, Pirk et al. 2016). The suppression of the mite reproductive output and the lower viral prevalence within honeybees and mites have been demonstrated to explain the slow rate of mite growth in *A. m. scutellata* colonies and their healthy appearance in colonies in South Africa (Strauss et al. 2013, Strauss et al. 2016). Hence, other factors such as suppression of the mite's reproductive success and/or lower viral prevalence within honeybees and mites might better explain the variability in the mite infestation levels observed between both *A. mellifera*

subspecies (Mondragon et al. 2005, Locke and Fries 2011) and should be evaluated in future studies.

Hygienic behaviour

Our study suggests a similar expression of the hygienic behaviour trait in the European and African savannah honeybee since we recorded similar levels of brood removal rates in both honeybee populations. Our findings corroborate results of a previous study Locke and Fries (2011) which also found a similar expression of hygienic behaviour between the Gotland mite-surviving and the local mite-susceptible honeybee populations in Sweden. Hygienic behaviour appears not to explain the lower mite infestation rates observed in the savannah honeybee since we recorded no association between the brood removal rate and *Varroa* mite infestation levels. Our results differ from previously results reported by Muli et al. (2014) which found that colonies of *A. m. scutellata* which displayed higher levels of hygienic behaviour had lower levels of *Varroa* mite infestation. These dissimilarities could be due to different climatic zones in which both studies were conducted and this might underline genotypic differences (Meixner et al. 2015). Nonetheless, hygienic behaviour is known to be variable since it can be strongly influenced by environmental and in-hive factors (Rosenkranz et al. 2010). Thus, the association between this behaviour and *Varroa* mite loads in the savannah honeybee known to have a wide distribution range in Kenya (Raina and Kimbu 2005) and the continent need to be investigated further.

On the other hand, the significant positive correlation detected between mite infestation rate and brood removal at 48 hours in European honeybee colonies implies that more

parasitised/non-parasitised brood are removed under high *Varroa* parasitism. Our results suggest that hygienic behaviour or brood removal rate is a response to the degree of diseased or parasitised brood found inside the brood cells. We expect that during spring, a period characterised by the early stages of brood production and lowest mite numbers in colonies (Hood 2000), few mites will move inside the cells to reproduce, leading to a reduced removal of parasitised brood cells and vice-versa during mid or late summer (Hood 2000). However, previous studies reported that European honeybee colonies bred for hygienic behaviour were more efficient at removing *Varroa*-infested brood only under low mite parasitism and maintain lower mite loads on both adult honeybees and within worker brood cells than unselected colonies (Spivak and Reuter 1998, Spivak and Reuter 2001, Ibrahim and Spivak 2006). Under high parasitism (> 15 % of both worker brood and adult honeybees), these colonies are unable to remove parasitised brood cells efficiently, requiring periodic miticide treatments to reduce their collapse (Spivak and Reuter 2001). As has been reported in breeding programs with Russian honeybees, hygienic and *Varroa*-sensitive hygienic honeybees in the USA, none of these honeybees have provided full protection for susceptible European honeybee colonies against *Varroa* mite infestation (Locke 2012). As such, they periodically require application of in-hive miticide to control the mite (Locke 2012). It appears that under high mite parasitism, honeybees invest significant resources into feeding their broods in order to obtain the next generation sub-optional worker honeybees than into other tasks such as grooming or hygienic behaviour to remove infesting mites. Another explanation could be that, the build-up of large levels of odour cues released by parasitised broods which signal removal of diseased or

parasitised brood cells in the colony might cause habituation and a reduction in receptor sensitivity to further detect odours (Spivak and Gilliam 1998, Masterman et al. 2000, Spivak and Reuter 2001). Nevertheless, a long-term longitudinal study would help shed more light on the hygienic behaviour in both the African savannah honeybee and their European counterparts.

Conclusions

In host-parasite interactions, host tolerance is defined as the ability to limit the detrimental effects of the parasite, while host resistance is the ability to reduce the reproductive fitness of the parasite (Schmid-Hempel 2011). In the present study, we found two additional undescribed damage categories in mites which occur more frequently in the African savannah honeybee than their European counterpart. Grooming behaviour was better expressed in *A. m. scutellata* than in *A. mellifera* hybrids of European origin and hence, a potential tolerant mechanism displayed by the African savannah honeybee towards *V. destructor* attack. However, hygienic and grooming behaviours did not significantly differ between subspecies with respect to *Varroa* mite-infestation levels recorded. Suggesting that, other resistant mechanisms such as suppression of mite reproductive success and/or lower viral prevalence within honeybees and mites might play an important role in honeybee responses to mite infestation. The observed differences in *Varroa* mite-infestation levels and grooming behaviour between the African and European honeybees recorded herein could have environmental and genetic bases since these traits can be strongly influenced by environmental, colony and genetic factors (Currie and Tahmasbi 2008, Arechavaleta-

velasco et al. 2012, Rinderer et al. 2013, Hamiduzzaman et al. 2017). Thus, future studies are warranted to help shed more light on the influence of these factors on the expression level of these behaviours in African and European honeybees.

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Supporting information

S1 Table. Primers used for molecular analysis for identification of *Varroa* species and haplotype. Amplified gene fragment, product size base pairs (bp) and annealing temperatures (Ta) are indicated (Navajas et al. 2008).

Fragment	Primer name	Primer sequences (5'-3')	Size (bp)	Ta (◦C)
cox1	10KbCOIF1	CTT GTA ATC ATA AGG ATA TTG GAAC	929	52
	6,5KbCOIR	AAT ACC AGT GGG AAC CGC		
atp6-cox3	6KbATP6F	GAC ATA TAT CAG TAA CAA TGAG	818	52
	16KbCOIIIR	GAC TCC AAG TAA TAG TAA AACC		

CHAPTER THREE

Low fertility, fecundity and numbers of mated female offspring explain the lower reproductive success of the parasitic mite *Varroa destructor* in African honeybees

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Abstract

Although *Varroa destructor* is the most serious ecto-parasite to the honeybee, *Apis mellifera* L., some honeybee populations such as *Apis mellifera scutellata* in Kenya can survive mite infestations without treatment. Previously, we reported that grooming behaviour could be a potential tolerant mechanism expressed by this honeybee subspecies towards mite infestation. However, both hygienic and grooming behaviours could not explain the lower mite-infestation levels recorded in these colonies. Here, we investigated the involvement of other potential resistant mechanisms including suppression of mite reproductive output in worker brood cells of *A. m. scutellata* to explain the low mite numbers in their colonies. Higher infertility rates (26–27%) and percentages of unmated female offspring (39–58%) as well as lower fecundity (1.7–2.2, average offspring produced) than measured in susceptible honeybee populations were identified as key parameters that seem to interact with one another during different seasons to suppress mite reproduction in *A. m. scutellata* colonies. We also identified offspring mortality in both sexes and absence of male offspring as key factors accounting for the low numbers of mated daughter mites produced in *A. m. scutellata* colonies. These results suggest that reduced mite reproductive success could explain the slow mite population growth in *A. m. scutellata* colonies.

Key words: *Varroa destructor*, reproduction, resistance, African honeybees.

Introduction

Varroa destructor Anderson and Trueman is the most serious ecto-parasitic mite that has significantly contributed to the decline of the Western honeybees (*Apis mellifera* L.), both wild and managed, particularly in Europe and North America (Neumann and Carreck 2010, Francis et al. 2013, Smith et al. 2014, Kielmanowicz et al. 2015). The mite invaded *A. mellifera* colonies outside its native host range in Southeast Asia where it was originally restricted only to its natural host *Apis cerana* (reviewed in Nazzi and Le Conte 2016). The infestations by the mites can have significant negative effects on susceptible *A. mellifera* populations, especially the ones of European origin, mainly because they lack or poorly express the behavioural mechanisms displayed by the mite's original host to counter infestation (Ritter 1981, Fries et al. 1996). These behavioural mechanisms include: efficient hygienic and grooming behaviours as well as entombing of drone broods (Peng et al. 1987, Boecking and Spivak 1999, Rath 1999). Additionally, the mite reproduces only in the less abundant and seasonally occurring drone brood in colonies of *A. cerana*, whereas its reproduction takes place in both drone brood and the more abundant worker brood which occurs throughout the breeding season in *A. mellifera* colonies (Rath 1999). As a result, beekeepers in the affected countries practice periodic miticide treatment to prevent the collapse of honeybee colonies within 1 or 2 years (Lee et al. 2010, Neumann and Carreck 2010, Rosenkranz et al. 2010).

The reproductive cycle of *Varroa* mite takes place entirely in sealed brood cells and synchronises with the sealed brood development time of the host larvae (Martin 1994). A foundress mite invades a worker brood cell shortly before it is capped and lays her

first unfertilised egg, ~60–70 h following cell capping (Ifantidis 1983, Martin 1994). This unfertilised egg develops into a male while the subsequent three to four fertilised eggs which are laid at approximately 30 h interval each develop into females (Ifantidis 1983, Martin 1994). A mite can lay up to five eggs in worker brood and up to six eggs in drone brood (Martin 1994). It takes about 6 and 7 days for female and male mites, respectively, to develop into adults (Martin 1994). Mating between the mite's offspring occurs within the sealed brood cells once they reach adulthood with the male *Varroa* mite dying shortly afterwards. The foundress mites together with one or two viable, mature and mated daughter mites attach themselves to the honeybee that emerges from the cell leaving behind all immature mites which ultimately die inside the cells. Therefore, a foundress mite is considered to reproduce successfully when one or two viable, mature and mated daughter mites emerge from the cell during each reproductive cycle (Ifantidis 1983, Martin 1994). Thus, the duration of the post-capping stage of worker brood and the mite offspring mortality in these cells are factors which can potentially influence the reproductive success of foundress mites (Martin 1994, Rosenkranz et al. 2010, Ardestani 2015). Alternatively, mites could be considered non-reproductive because they die in the cell without reproducing, produce no offspring, produce only male offspring or produce offspring that fail to reach maturity before the developing honeybee pupa hatches as an adult (Harbo and Harris 1999). While reproducing inside the brood cells, the mite and her offspring feed on the fat body of the developing pupae and the foundress together with the mature female offspring continue to feed on the adult honeybee after emergence from the cells (Ramsey and VanEngelsdorp 2017). In the course of feeding, the mites can/often transmit lethal

pathogens to the individual honeybee (Rosenkranz et al. 2010), which affects it physically and physiologically (Aronstein et al. 2012, VanDooremalen et al. 2012, Annoscia et al. 2015).

However, some *A. mellifera* populations are reported to display behavioural mechanisms including hygienic and grooming behaviours and suppression of mite reproductive success. These allow them to coexist with the mites for longer periods without requiring any in-hive miticide treatment (Peng et al. 1987, Fries et al.1996, Calderón et al. 2010, Calderón et al. 2012, Locke et al. 2012, Strauss et al. 2013, Strauss et al. 2016). For example, previously we had shown that, the surviving African savannah honeybee, *Apis mellifera scutellata* (Lepeletier) in Kenya maintains a lower mite colony infestation (~3-fold lower) than their susceptible *A. mellifera* hybrids of European origin found in the USA (Nganso et al. 2017). Furthermore, they also express a higher grooming behaviour towards the mite than their European counterparts, although both honeybee subspecies express similar levels of hygienic behaviour. However, both hygienic and grooming behaviours could not explain the lower mite infestation levels recorded in *A. m. scutellata* colonies. Grooming behaviour was identified as a potential tolerant mechanism displayed by the African savannah honeybee towards infestation by the mite. Suggesting that other resistant mechanisms such as suppression of mite reproduction might explain the lower mite population growth observed in colonies of the savannah honeybee. The suppression of the reproductive success of *Varroa* mite in the worker brood cells by *A. mellifera* populations is considered a crucial adaptive resistant mechanism (Fries et al. 1994, Harris et al. 2003, Martin and Medina 2004, Mondragon et al. 2006). It explains the

slow rate of mite population growth within their colonies and slight variations in this trait could underline resistance development towards the mite. The suppression of the mite reproductive output which translates into lower mite fertility, fecundity and reproductive success in worker brood cells has been found to explain honeybee resistance towards the mites in various populations. These populations include *A. m. scutellata* in South Africa (Strauss et al. 2016), Africanized honeybees in Brazil (Calderón et al. 2012) and the oldest *Varroa* tolerant European honeybee populations, *A. m. ligustica* in the island of Fernando de Noronha in North-eastern Brazil (Brettell and Martin 2017). The Avignon and Gotland honeybee populations in France and Sweden, respectively (Locke and Fries 2011, Locke et al. 2012), the Russian honeybee population in the USA (de Guzman et al. 2008) and the Norwegian honeybee population (Oddie et al. 2017) have also been reported to reduce the mite reproductive fitness. In the present study, we aimed to investigate mite reproduction in worker brood cells of *A. m. scutellata* to explain the low mite numbers recorded in their colonies.

Materials and methods

Study sites

The study was conducted in Nairobi, Kenya in November 2015 (the short rainy season), January 2016 and February 2018 (the hot dry season). The hot dry season is characterised by a drastic reduction or cessation in brood rearing while the short rainy season is characterised by increased brood rearing in savannah honeybee colonies (Raina and Kimbu 2005). All the colonies were housed in standard Langstroth hives

containing 3–4 brood combs and were not treated with acaricides to reduce mite infestations.

Four and 14 (14 = 7 colonies used in each hot dry season) queen right colonies of *A. m. scutellata* were selected at an apiary in Kithimani (1°8′S, 37°25E) during the short rainy and hot dry seasons, respectively. While three colonies were selected at an apiary in Kilimanbogo (1°8′S, 37°21′E) during the short rainy season. Both apiaries are located within the county of Machakos and hosted *A. m. scutellata* colonies that originated from locally captured swarms (Hepburn and Radloff 1988, Raina and Kimbu 2005, Muli et al. 2014).

Assessment of *Varroa* mite reproduction in worker brood cells

To quantify *Varroa* mite reproductive output, we used the method described by Strauss et al. (2016) with slight modifications. Briefly, 200 worker brood cells containing pupae at the molting stage were inspected in each colony (Martin, 1994). All the colonies in each of the apiary were screened for brood at this stage and only positive colonies were used. These were four colonies in November 2015, seven colonies in January 2016, seven colonies in February 2018 at the apiary in Kithimani and three colonies in November 2015 at the apiary in Kilimanbogo. We used this stage because at the time of emergence of the young honeybees from the worker cells, the foundress mites have already completed their reproduction and it becomes easy to estimate their reproductive output. To determine *Varroa* mite reproduction, we initially generated count data on the number of foundresses, mature daughter mites, immature daughter mite and males in each infested cell. We used only singly infested cells to determine the reproductive success of the mites in worker brood cells of *A. m. scutellata* (Rosenkranz et al. 2010).

For each infested cell, we further collected data on infertility (alive and dead foundresses with no offspring), fertility (production of offspring), fecundity (number of offspring produced), number of viable, mated and mature daughters and presence (alive and dead) or absence of adult males. The mating status of the daughter mites was determined by the simultaneous presence of one live mature daughter and one live adult male in a worker brood cell during an inspection of infested cells (Rosenkranz et al. 2010, Locke et al. 2012, Strauss et al. 2016, Brettell and Martin 2017). We also determined the fecundity and number of mature mated female offspring produced in cells infested by two or more foundress mites.

Assessment of the post-capping duration of worker brood

The duration of the post-capping stage of worker brood was determined in three colonies at the apiary in Kithimani. Two frames containing approximately 300 mature worker larvae prior to capping were removed from the central region of each colony and marked. Snap shots were taken to record the position of all sealed and unsealed worker broods after which the marked frames were returned to their colonies. The frames were then inspected twice a day (morning and evening) to record worker cells that were capped and these worker cells were monitored until the honeybees emerged from them. A total of 657 worker brood cells were recorded. During each inspection period, photographs were taken. The number of brood that emerged from the worker cells and the number of days they took to emerge were recorded to determine the average duration of the sealed worker brood stage of *A. m. Scutellata* through a thorough analysis of the photographs.

Statistical analyses

Statistical analyses were performed using R-Software version 3.2.5 (R Development Core Team 2015) and the alpha level was set at 0.05 (Pirk et al. 2013). The generalised linear model (GLM) with logit link and binomial distribution error was used to examine the differences in the percentage of fertile and infertile foundress mites. This statistical analysis was also used to examine differences in percentage of foundress mites with viable mated daughter mites, unmated daughter mites and only male produced per cell and per foundress among the short rainy (November 2015) and hot dry seasons (January 2016 and February 2018) at the apiary in Kithimani. To compare the average number of offspring and mated daughter produced per cell and per foundress among the short rainy and hot dry seasons at the apiary in Kithimani, we used the GLM with log link and binomial distribution error. We also used the GLM with log link and binomial distribution error to compare the average number of offspring and mated daughter produced per cell and per foundress in worker cells infested by 1 or 2–4 foundresses in each season in the colonies of the African savannah honeybee.

Results

Assessment of *Varroa* mite reproduction in worker brood cells

Reproduction in singly infested cells

The patterns of *Varroa* mite reproduction during the different seasons of assessment in colonies of *A. m. scutellata* are presented in Tables 3.1 and 3.2.

The percentage of infertile mites was significantly lower during the hot dry season (January 2016) than the short rainy (November 2015) and hot dry (February 2018) seasons at the apiary in Kithimani (df = 16: $\chi^2 = 0.64$; P = 0.001, Table 3.1). However, there were no significant differences in the average number of offspring produced per cell (df = 16: $\chi^2 = 0.02$; P = 0.89, Table 3.1) and foundress (df = 16: $\chi^2 = 0.07$; P = 0.80, Table 1) among these seasons at the same apiary. There were also no significant differences in the average number of mated daughter mites produced per cell (df = 16: $\chi^2 = 1.63$; P = 0.20, Table 3.1) and foundress (df = 16: $\chi^2 = 2.45$; P = 0.12, Table 3.1) among these seasons at the same apiary. Likewise, there were no significant differences in the percentage of viable mated daughter mites produced per cell (df = 16: F = 0.002; P = 0.97, Table 3.1) and foundress (df = 16: F = 0.002; P = 0.97, Table 3.1) among these seasons at the apiary in Kithimani. The percentage of only male produced per cell (df = 4: $\chi^2 = 0.33$; P = 0.57, Table 3.1) and foundress (df = 4: $\chi^2 = 0.28$; P = 0.60, Table 3.1) were also not significantly different among these seasons at the apiary in Kithimani. Furthermore, the percentage of unmated daughter mites produced per cell (df = 13: $\chi^2 = 12.13$; P = 0.001, Table 3.1) and foundress (df = 13: $\chi^2 = 12.11$; P = 0.001, Table 3.1) was significantly lower during the hot dry season (February 2018) than the short rainy (November 2015) and hot dry (January 2016) seasons at the apiary in Kithimani.

Table 3.1. Comparison of the reproductive parameters of *Varroa* foundress mites produced per cell and per fertile foundress in singly infested worker brood cells in *A. m. scutellata* during the hot dry and short rainy seasons at the apiary in Kithimani, Kenya

Parameters	**Hot dry season (January 2016)**	**Hot dry season (February 2018)**	**Short rainy season (November 2015)**	***P*-value**[a]
Per single infested cell, Fertile and infertile (Total inspected cells)	n = 39 (1400)	n = 99 (1400)	n = 41 (800)	
Fertility	92%	74%	73%	
Infertility	8%	26%	27%	**0.001**
Viable and mated female offspring	62%	54%	29%	0.97
Unmated female offspring	39%	16%	49%	**0.001**
Non-viable female offspring due to adult daughter and male dead, adult male dead and missing	23%	13%	29%	**0.04**
Immature offspring	16%	3%	20%	**0.002**
Male only	8%	5%	7%	0.57
Average number of offspring produced (mean ± S.D)	2.2 ± 1.0	1.9 ± 0.6	1.7 ± 0.3	0.89
Average number of mated daughter produced (mean ± S.D)	0.5 ± 0.3	0.5 ± 0.2	0.3 ± 0.1	0.20
Per fertile foundress only	n = 36	n = 73	n = 30	
Viable and mated female offspring	67%	73%	40%	0.97
Unmated female offspring	42%	22%	66%	**0.001**
Non-viable female offspring due to adult daughter and male dead, adult male dead and missing	25%	18%	40%	**0.04**
Immature offspring	17%	4%	26%	**0.002**
Male only	9%	7%	10%	0.60
Average number of offspring produced (mean ± S.D)	2.7 ± 1.5	2.7 ± 0.5	2.4 ± 0.2	0.80
Average number of mated daughter produced (mean ± S.D)	0.5 ± 0.3	0.7 ± 0.2	0.4 ± 0.1	0.12

[a]*p* values were calculated by generalised linear model (GLM) with log and logit links.

Table 3.2. Reproductive parameters of *Varroa* foundress mites produced per cell and per fertile foundress in singly infested worker brood cells in *A. m. scutellata* during the short rainy season at the apiary in Kilimanbogo, Kenya.

Parameters	**Short rainy season (November 2015)**
Per single infested cell, Fertile and infertile (Total inspected cells)	n = 35 (600)
Fertility	91%
Infertility	9%
Viable and mated female offspring	49 %
Unmated female offspring	58%
Non-viable female offspring due to adult daughter and male dead, adult male dead and missing	52%
Immature offspring	6%
Male only	3%
Average number of offspring produced (mean ± S.D)	2.1 ± 0.3
Average number of mated daughter produced (mean ± S.D)	0.6 ± 0.6
Per fertile foundress only	n = 32
Viable and mated female offspring	53%
Unmated female offspring	62%
Non-viable female offspring due to adult daughter and male dead, adult male dead and missing	56%
Immature offspring	6%
Male only	3%
Average number of offspring produced (mean ± S.D)	2.4 ± 0.5
Average number of mated daughter produced (mean ± S.D)	0.7 ± 0.8

Reproduction in multiply infested cells

During the hot dry season (January 2016) at the apiary in Kithimani, the mites reproduced in all the 9 cells infested with 2 live foundresses and a total of 34 offspring were produced, with 3.8 ± 0.3 (mean ± S.D) offspring produced per cell (Fig 3.1A). There was no significant difference in the average number of offspring produced per cell (df = 10: $\chi^2 = 1.46$; P = 0.23) and per foundress (df = 10: $\chi^2 = 2.45$; P = 0.12)

as well as, the average number of mated daughters produced per foundress (df = 10: χ^2 = 0.70; P = 0.40) between multiple and singly infested worker cells (Fig 3.1A). However, the average number of mated daughters produced per cell was significantly higher in multiply infested worker cells than in singly ones (df = 10: $\chi^2 = 5.07$; P = 0.02) (Fig 3.1A).

During the hot dry season (February 2018) at the apiary in Kithimani, the mites reproduced in 62 of the 64 cells infested with 2-4 live foundresses and a total of 170 offspring were produced, with 2.7 ± 1.4 (mean ± S.D) offspring produced per cell (Fig 3.1B). There was no significant difference in the average number of offspring (df = 12: $\chi^2 = 0.36$; P = 0.55) and the average number of mated daughter (df = 12: $\chi^2 = 0.0$; P = 1) produced per cell between multiply and singly infested worker cells (Fig 3.1B). However, the average number of offspring (df = 12: $\chi^2 = 9.64$; P = 0.002) and the average number of mated daughter (df = 12: $\chi^2 = 9.70$; P = 0.002) produced per foundress were significantly lower in multiply than singly infested worker cells (Fig 3.1B).

During the short rainy season (November 2015) at the apiary in Kithimani, there was reproduction in 10 out of the 11 worker cells infested with 2-3 live foundresses and a total number of 26 offspring were produced, with 2.6 ± 1.0 (mean ± S.D) offspring produced per cell (Fig 3.1C). There was no significant difference in the average number of offspring produced per cell (df = 6: $\chi^2 = 1.33$; P = 0.25) and per foundress (df = 6: $\chi^2 = 1.97$; P = 0.16) as well as, the average number of mated daughter produced per cell (df = 6: $\chi^2 = 1.05$; P = 0.31) and per foundress (df = 6: $\chi^2 = 0.0$; P = 1) between multiply and singly infested worker cells (Fig 3.1C).

During the short rainy season (November 2015) at the apiary in Kilimanbogo, the mites reproduced in all the 8 worker cells infested with two live foundresses and a total of 27 offspring were produced, with 3.4 ± 0.5 (mean ± S.D) offspring produced per cell (Fig 3.1D). There was no significant difference in the average number of offspring produced per cell (df = 4: $\chi^2 = 0.53$; $P = 0.47$), per foundress (df = 4: $\chi^2 = 0.08$; $P = 0.78$) as well as, the average number of mated daughter produced per cell (df = 4: $\chi^2 = 0$; $P = 1$) and per foundress (df = 4: $\chi^2 = 0.2$; $P = 0.65$) between multiply and singly infested worker cells (Fig 3.1D).

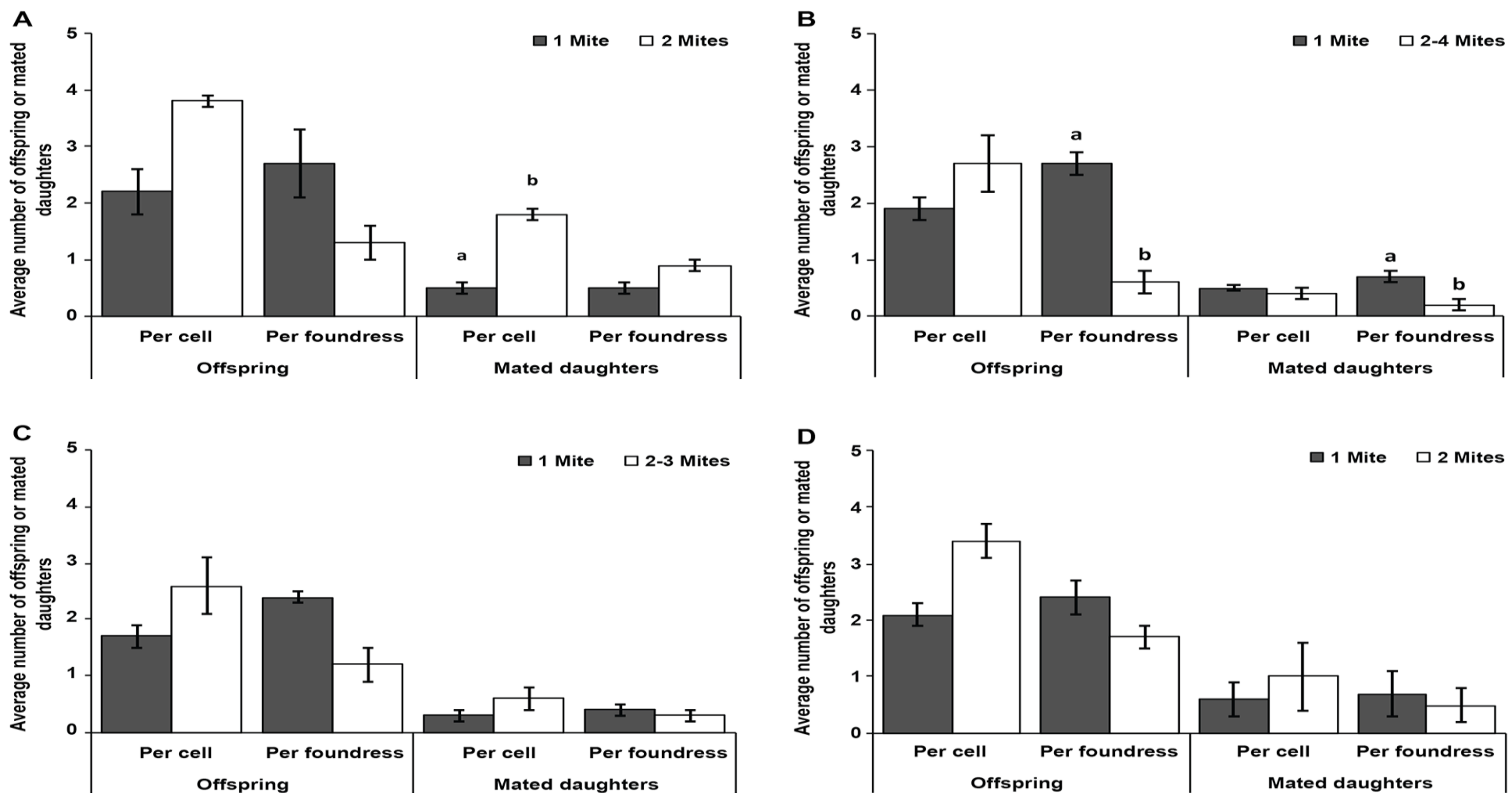

Fig 3.1. The average number of offspring and mated daughters (mean ± S.E) produced per cell and per foundress in singly and multiply infested worker brood cells in *A. m. scutellata* during the hot dry seasons (January 2016 and February 2018) at Kithimani (A) and (B) respectively, short rainy season (November 2015) at Kithimani (C) and short rainy season (November 2015) at Kilimanbogo (D). Only fertile foundresses were considered. Pair of bars with letters indicates significant effects for each category.

Assessment of the post-capping duration of worker brood

The average duration of the post-capping developmental time of *A. m. scutellata* worker brood was 265.2 ± 0.04 hours (11 days).

Discussion

Mite reproduction in singly infested worker cells

In colonies of the African savannah honeybee, we recorded a higher infertility rate for the mites during the short rainy (November 2015) and the hot dry (February 2018) seasons which are characterised by increased and reduced brood rearing, respectively, at the apiary in Kithimani (26–27%). In contrast, a lower infertility rate of the mites was recorded during the hot dry season (January 2016) at the same apiary (8%) which was similar to the infertility rate recorded during the short rainy season at the apiary in Kilimanbogo (9%). The amount of brood present in honeybee colonies is a host feature that is known to significantly influence the fertility and the population dynamics of the mites (Lodesani et al. 2002). It appears that when brood is available in the colonies, features of the mites such as the reproductive capacity during their lifetime and lifespan might also influence their reproductive rate and population dynamics in honeybee colonies (Rosenkranz et al. 2010). Despite the variability in the fertility rates of the mites observed in worker brood cells of *A. m. scutellata*, the reproductive success of foundress mites remained similar to those reported in other surviving honeybee populations (Medina and Martin 1999, Locke and Fries 2011, Calderón et al. 2012, Locke et al. 2012, Strauss et al. 2016, Brettell and Martin 2017, Oddie et al. 2017). Thus, these results suggest a strong suppression of mite reproduction in worker brood

cells of *A. m. scutellata* in Kenya and this could be a plausible explanation for the low mite numbers recorded previously in colonies of this honeybee subspecies (Nganso et al. 2017).

In this study, we found that the post-capping duration of worker brood of *A. m. scutellata* could not explain the lower reproductive success of the mites recorded in their colonies. Up to 3–5 eggs were laid and 1–2 viable, mature and mated daughter mites emerged in worker brood cells of this honeybee subspecies. This finding suggests that when oviposition is initiated, up to five eggs are laid and there is sufficient time for one and sometimes two daughter mites to emerge from the worker cells of *A. m. scutellata* according to *Varroa* developmental charts (Martin 1994). Interestingly, we identified high infertility rates (26–27%) and percentage of unmated female offspring (39–58%) as well as low fecundity (1.7–2.2, mean number of eggs laid) as exciting parameters that appears to explain the lower mite reproductive success in colonies of the savannah honeybee studied herein (Tables 3.1 and 3.2). These parameters seem to interact with one another during different seasons to reduce the number of viable female offspring produced in worker brood cells of the African savannah honeybee. The low mite fecundity recorded in this study was similar to those reported in worker brood cells of the surviving *A. m.* scutellata population in South Africa (1.7 ± 0.3, mean ± S.D) (Strauss et al. 2016); though it is much lower than those reported in other surviving or susceptible honeybee populations (3.1–4.9, mean number of eggs laid) (Medina and Martin 1999, Martin 2001, Alattal et al. 2006, Locke and Fries 2011, Calderón et al. 2012, Locke et al. 2012, Brettell and Martin 2017). Also, an increase in the percentage of infertile mites over time (from 13 to 30%) has been reported as a parameter that

suppresses the mite reproduction in worker brood cells of the surviving *A. m. scutellata* population in South Africa (Martin and Kryger 2002, Strauss et al. 2016). Furthermore, we identified offspring mortality for both sexes and absence (missing) of male offspring as key factors that appear to be responsible for the high number of unmated daughters produced in the African savannah honeybee colonies (23–52%). Mite offspring mortality has also been reported as a major factor that accounts for the lower mite reproductive output and population growth in the surviving Africanized honeybee colonies in Brazil (Mondragon et al. 2006, Calderón et al. 2010, Calderón et al. 2012). Though the fertility of the mites in these Africanized honeybee colonies is currently reported to be at the same level as in European honeybee colonies. Offspring mortality or absence (missing) within the worker brood cells has been reported to be due to failure to locate the single feeding site established by the foundress mite on the developing honeybee brood (Donzé and Guerin 1994, Donze et al. 1996). The disturbance or damage of the first egg which is usually male when the pre-pupae molts into pupae has also been reported to explain offspring mortality or absence in these worker cells (Donzé and Guerin 1994, Donze et al. 1996, Calderón et al. 2010, Calderón et al. 2012).

Mite reproduction in multiply infested cells

The reproduction of mites in multiply infested cells can also influence their reproductive success and population growth in honeybee colonies (Rosenkranz et al. 2010). In this study, we observed that the number of offspring produced per individual mite in multiply infested cells was generally lower than those produced in singly infested cells in *A. m. scutellata* colonies though the difference was only significant

during the hot dry season (February 2018) (Fig 3.1). Additionally, there was a general reduction in the number of female offspring produced per foundress in multiply than singly infested cells in colonies of this honeybee subspecies though the difference was only significant during the hot dry season (February 2018) (Fig 3.1). However, the number of female offspring produced per cell was generally higher in multiply than singly infested cells in the savannah honeybee colonies though the difference was only significant during the hot dry season (January 2016) (Fig 3.1). In multiply infested cells where competition for food resources is expected, the fecundity and reproductive success of individual mites is generally reduced compared with those of singly infested cells (Fuchs and Langenbach 1989, Martin 1995, Martin and Medina 2004, Mondragon et al. 2006). The higher reproductive success of the mites recorded in multiply infested cells in this study might be due to the lower incidence of offspring mortality and absence recorded in multiply infested cells than those of singly infested cells (Strauss et al. 2016). Moreover, daughter mites have a greater chance to mate successfully before emerging from multiply infested cells because more than one adult male can be produced (Martin 1995). In this study, however, only a single male offspring was produced in all multiply infested cells of *A. m. scutellata*. Therefore, the probability that all the daughter mites produced in these cells will receive sufficient sperms before emerging from the cell is questionable. Hence, though the reproductive success of mites remains high in these cells, there could be a chance that not all the daughter mites will receive sufficient sperm from the male before emerging from the cell (Donze et al. 1996, Wendling et al. 2014). Our findings corroborate results of a previous study which also reported a significant reduction in the number of offspring produced per individual

mite in multiply infested worker cells compared to singly infested ones. Though the number of mated daughters produced per cell was higher in multiply infested cells compared to singly infested cells in *A. m. scutellata* colonies in South Africa (Strauss et al. 2016).

In conclusion, the *A. m. scutellata* population studied herein showed evidence of resistance towards mite infestation. This translates into the strong suppression of the mite reproductive success recorded in worker brood cells. This lower reproductive output was mainly due to the high mite infertility rates and percentage of unmated daughter mites as well as low mite fecundity recorded in infested cells. The mortality of adult male and female offspring and the absence (missing) of male offspring in a considerable number of worker brood cells were identified as major factors responsible for the lower production of mated daughters in the savannah honeybee colonies. The consistency of results regarding mite reproduction in two geographically distinct *A. m. scutellata* populations (South Africa, Strauss et al. 2016 and Kenya, this study) suggests general adaptations towards *V. destructor* within African honeybees, most likely due to the higher number of wild colonies and lack of miticide use in their colonies (Pirk et al. 2017). Nonetheless, because the number of multiply infested cells recorded in this study was low, we recommend that the data should be treated with caution. Further verification of the reproductive values of the mites obtained herein and in other *A. m. scutellata* populations distributed in other climatic zones in Africa need to be undertaken, as it will shed more light on the evolution of tolerance and resistance mechanisms towards *Varroa* mites on the continent.

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Conflicts of interest

None

Ethical standards

Not applicable

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CHAPTER FOUR

Juvenile hormone III and Methyl farnesoate in the haemolymph of African and European honeybees: Possible biological role in their behaviour towards the ecto-parasitic mite *Varroa destructor*

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Abstract

Although the insect juvenile hormone III (JH III) has been shown to regulate several physiological processes in honeybees *Apis mellifera* L., little information is known about its regulatory role in honeybee's behaviour towards the most damaging ecto-parasitic mite *Varroa destructor*. Moreover, recent study identified the immediate biosynthetic precursor of JH III, methyl farnesoate (MF) in the haemolymph of insects including honeybees and suggested that both hormones could play a role in insect behaviour. Here we showed, using liquid chromatography-quadrupole time of flight-mass spectrometry (LC-QToF-MS) that the titres of JH III and MF fluctuate across seasons with resultant significant effects on the total concentrations of MF + JH III in the haemolymph of the African savannah honeybee *Apis mellifera scutellata* in Kenya. However, the levels of JH III, MF, MF + JH III and ratio of JH III to MF did not correlate with mite-infestation rates on adult worker honeybees in colonies of *A. m. scutellata* and *A. mellifera* hybrids of European origin found in the USA. Also, no significant correlation was detected between the titres of these hormones and grooming behaviour of the African honeybee towards the mite. Taken together, our results suggest that these hormones may not regulate *Varroa* mite-infestation rates and grooming behaviour of honeybees towards the ecto-parasite.

Highlights:

- The study confirmed the presence of MF in the haemolymph of African and European honeybees.
- Titres of MF and JH III vary across seasons in the haemolymph of the African honeybee.
- These hormones did not correlate with mite-infestation levels on adult workers in both subspecies.
- These hormones did not correlate with adult grooming behaviour of *A. m. scutellata* against the mite.

Keywords

Hormones, *Varroa destructor*, honeybees, behaviour

Introduction

Honeybees vary substantially in the degree of their defensive behaviours towards the ecto-parasitic mite *Varroa destructor* (Peng et al. 1987, Moritz and Mautz 1990, Fries et al.1996, Arechavaleta-Velasco and Guzman-Novoa 2001, Spivak and Reuter 2001, Invernizzi et al. 2015, Strauss et al. 2016, Nganso et al. 2017, 2018). The mite is currently the most severe threat to the health of honeybee, *Apis mellifera* L. particularly in Europe and North America (Le Conte et al. 2010, Neumann and Carreck 2010, Francis et al. 2013). These defensive behaviours expressed by honeybees against the

ecto-parasite include: hygienic and grooming behaviours, reduced mite reproductive success and population growth. Previously-conducted studies have reported that environmental, genetic and in-hive factors influence the levels of expression of these behavioural traits among honeybee species, subspecies, colonies within a population and individual honeybees within a colony towards the mite (Boecking et al. 2000, Currie and Tahmasbi 2008, Alaux et al. 2009, Rosenkranz et al. 2010, Arechavaleta-Velasco et al. 2012, Kirrane et al. 2015, Hamiduzzaman et al. 2017). However, there is limited information on the physiological mechanisms that regulate the intensity of these behavioural traits in honeybees towards the mite.

The insect juvenile hormone III (JH III) (methyl (2E,6E)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate) is the most common isoform of juvenile hormones found in several insects including honeybees (reviewed in Noriega 2014). This sesquiterpene epoxide has been reported to regulate several physiological processes in honeybees including caste differentiation during larval development, age polyethism, plasticity in age polyethism and aggression behaviours (Breed 1983, Breed et al. 1992, Huang et al. 1994, Huang and Robinson 1992, 1995, 1996, Pearce et al. 2001, Lin et al. 2004). It is biosynthesized from methyl farnesoate (MF) (methyl(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoate), its immediate precursor via the action of an epoxidase in the corpora allata, which is a pair of endocrine glands connected to the brain, through the classical mevalonate (MVA) pathway (reviewed in Noriega 2014). It is the major hormone that is produced and released in the haemolymph of crustaceans from the mandibular organs which function similarly to the corpora allata of insects that synthesises and secretes JH III (Laufer et al. 1987, Homola and Chang 1997). In crustaceans, MF has been reported

to perform analogue functions to the insect juvenile hormone III including: regulation of reproduction, metamorphosis, larval development, molting and behaviour; although JH III has not yet been reported in crustaceans (Borst et al. 1987, Laufer et al. 1987, Homola and Chang 1997).

The earliest discovery and quantification of MF in the haemolymph of insects including honeybees *Apis mellifera L.* of European origin in Gainesville, Florida, USA was made by Teal et al. (2014). This study suggested that MF could constitute an important addition to JH III as a circulating hormone in insects. Here, we undertook a study to determine if seasonal differences in *Varroa* mite-infestation rates in colonies of the African savannah honeybee *Apis mellifera scutellata* in Kenya are correlated with their titres of JH III, MF, MF + JH III and/or ratio of JH III to MF. We also determined the mite-infestation levels in colonies of *A. mellifera* hybrids of European origin found in the USA and their association with levels of these hormones during winter. Based on our understanding of the relationship between these parameters, we further assessed inter-colonial differences in grooming behaviour of *A. m. scutellata* towards the mite under natural conditions and their associations with levels of these hormones. The results from this study may offer opportunities for understanding the physiological mechanisms that regulate the behaviours of honeybees towards the ecto-parasite.

Materials and methods

Study sites

In Kenya, the study was conducted at an apiary in Kithimani (1°8' S, 37°25 E) located within the county of Machakos in January 2018 (hot dry season) and July 2018 (cooler dry season). The cooler dry season is charactarised by moderate foraging due to moderate availability of flowering plants while the hot dry season is charactarised by drastic reduction in foraging due to very limited availability of flowering plants in African honeybee colonies (Raina and Kimbu 2005). In Gainesville, Florida, USA, the study was conducted at the apiary of the United States Department of Agriculture/Agricultural Research Service-Centre for Medical, Agricultural and Veterinary Entomology (USDA/ARS-CMAVE) (29.63°38'N, 82.36°21'W) in January 2018 (winter). The winter period is charactarised by a drastic reduction or cessation in brood rearing due to very limited availability or absence of flowering plants in European honeybee colonies (Hood 2000, Martin 2001). The study was carried out for one month during a particular season at both study sites because activities within the honeybee colonies such as brood rearing and environmental conditions (e.g. floral availability) which are known to exert significant effects on the titres of these hormones are the same within a particular season (Raina and Kimbu 2005).

In Kenya, nine (9) queen right colonies, hosting *A. m. scutellata* colonies that originated from locally captured swarms (Hepburn and Radloff 1988, Raina and Kimbu 2005, Muli et al. 2014), were randomly selected at the apiary in Kithimani during the study periods. Whilst in USA, ten (10) queen right colonies, hosting hybrids of different

European subspecies that were bred from honeybee stocks purchased from local commercial beekeepers (Ellis, personal communication), were randomly selected at the USDA/ARS-CMAVE apiary. All the colonies were housed in standard Langstroth hives containing 3 to 4 brood combs and were never treated with acaricides or miticides. All colonies were subjected to standard beekeeping practices. To determine average air temperatures during each sampling period in each of the apiaries, air temperatures were measured thrice (3) using climate data loggers (iButtons Hygrochron, Maxim Integrated, San Jose, USA). These were 16 °C and 21°C during the cooler and hot dry seasons respectively at the apiary in Kithimani in Kenya and 15 °C during winter at the USDA/ARS-CMAVE apiary in the USA.

Assessment of levels of infestations with *Varroa* mites in African and European honeybee colonies

During each study period, *Varroa* mite-infestation levels on approximately hundred (100) adult worker honeybees and the percentage of mite infestation rates on adult honeybees were determined in each experimental colony of the African and European honeybees as previously described in (Nganso et al. 2017, chapter 2).

Assessment of grooming behaviour in the African honeybee colonies

During the cooler dry season, grooming behaviour was assessed in the selected colonies of the African savannah honeybee using the screen bottom board method as previously described (Nganso et al. 2017, chapter 2).

Nurse honeybees sampling

We sampled nurse honeybees, identified as those honeybees with their heads in cells containing larvae (Pearce et al. 2001, Robinson, 1987), from each experimental colony of both honeybee subspecies during each study period. Twenty (20) individual nurse honeybees were then collected from frames of opened brood in each colony using locally made aspirators into 50 mL Falcon™ conical centrifuge tubes (Corning, New York) (Pearce et al. 2001). The Falcon™ tubes containing the sampled nurse honeybees were placed immediately on ice to anaesthetise the nurse honeybees for five (5) min prior to haemolymph collection.

Haemolymph collection

The haemolymph collection was performed as previously described (Lin et al. 2004) with slight modifications. Briefly, a calibrated 5 µL disposable glass micro-pipette was used to collect 1µL of haemolymph from nine (9) and fifteen (15) individuals of African and European nurse honeybees respectively by piercing a hole on the inter-segmental membrane between the 2nd and 3rd abdominal segment from the tip of the abdomen. Only clear and slightly yellow haemolymph sample was collected whereas, cloudy yellow intestinal contents collected were discarded. The haemolymph samples were collected within twenty (20) min from all individual nurse honeybees kept on ice. The haemolymph that flowed into the micro-pipette (by capillary action) was then transferred directly into a 1.5 mL screw top vial capped with Teflon-lined crimp cap held in ice. The vial contained 150 µL of LC/MS grade water and 300 µL of LC/MS grade methanol (Sigma-Aldrich, St. Louis, MO) to denature any enzymes that could

affect JH III or MF. Capped samples were vortexed for thirty (30) s before storage at – 80 °C until processed.

Extraction and analysis of hormones from haemolymph

The extraction of JH III and MF from the haemolymph was done as previously described (Teal et al. 2014) but with modifications as follows. The methanol extract containing the haemolymph in each vial was transferred into a 1.5 mL micro-centrifuge tube to which 450 µL of GC grade pentane (Sigma-Aldrich, St. Louis, MO) was added. The sample was then vortexed for thirty (30) s before centrifugation at 8000 rpm for five (5) min at 5 °C. After centrifugation, the pentane extracts collected from three (3) samples were pooled and transferred to a new 1.5 mL micro-centrifuge tube. Samples were then centrifuged as described above to separate any water before transferring to a new 1.5 mL screw thread autosampler vial. The extracts were then dried completely under a gentle stream of nitrogen and 100 µL of a solution containing 90% acetonitrile + 10% acetone LC/MS grade (Merck, Billerica, MA) was added to each vial. Capped samples were vortexed further for thirty (30) s prior to chemical analysis by liquid chromatography–quadrupole time of flight–mass spectrometry (LC-QTOF-MS).

Chromatographic separation was achieved on an ultra-performance liquid chromatography (UPLC) Waters Acquity I-class system (Waters Corp., Milford, MA) fitted with an ACE C-18 column (250 × 4.6 mm internal diameter, 5µm particle size; Advance Chromatography Technologies, Aberdeen, Scotland), with a heater turned off and an autosampler tray cooled to 5 °C. Mobile phases consisted of water (solvent A) and acetonitrile (solvent B), each containing 0.01 % formic acid. The isocratic solvent

system used was 20-80 % A-B, run time 20 min. The flow rate was held constant at 0.2 mL/min. The injection volume was one (1) µL.

The UPLC system was interfaced by electrospray ionization to a Synapt G2-Si QTOF-MS (Waters) operated in the multiple reaction mode (Tof-mrm) in positive mode using the following transition ions *m/z* 123.0770, 191.1086, 219.1364, 233.1581 and 251.2277 for MF and *m/z* 189.1667, 217.1570, 235.1718, 249.1939 and 267.2139 for JH III (Teal et al. 2014). The target window for mrm was set at 7.2-7.45 min for MF and 2.74 -2.84 min for JH III. Data were acquired in resolution mode over an *m*/z range of 100 - 700 with a scan time of 1 s using a capillary voltage of 0.5 kV, a sampling cone voltage of 40 V, a source temperature of 100 °C and a desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/h. Methyl farnesoate differs structurally from the insect juvenile hormone III by the absence of an epoxide group (Fig 4.1).

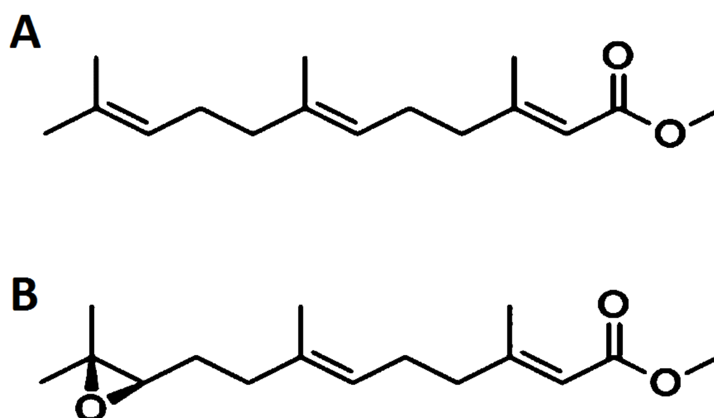

Fig 4.1. Structures of methyl farnesoate (MF) (A) and juvenile hormone III (JH III) (B) reported by Teal et al. (2014).

A continuous lock spray reference compound (leucine enkephalin; $[M + H]^+ = 556.2766$) was sampled at ten (10) s intervals for centroid data mass correction. The mass spectrometer was calibrated across the 50 - 1200 Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 propan-2-ol: water (90; 10 v/v). MassLynx version 4.1 SCN 712 (Waters Corp., Milford, MA) was used for data acquisition and processing. The identities of MF and JH III were confirmed by comparison of their retention times and mass spectral fragmentation patterns with those of authentic standards. The JH III and MF standards were a gift from the USDA/ARS-CMAVE laboratory in Gainesville, Florida, USA. Quantitative analysis to determine the amounts of JH III and MF in haemolymph samples of both honeybee subspecies was based on calibration curves generated from their standards as shown in Fig 4.2.

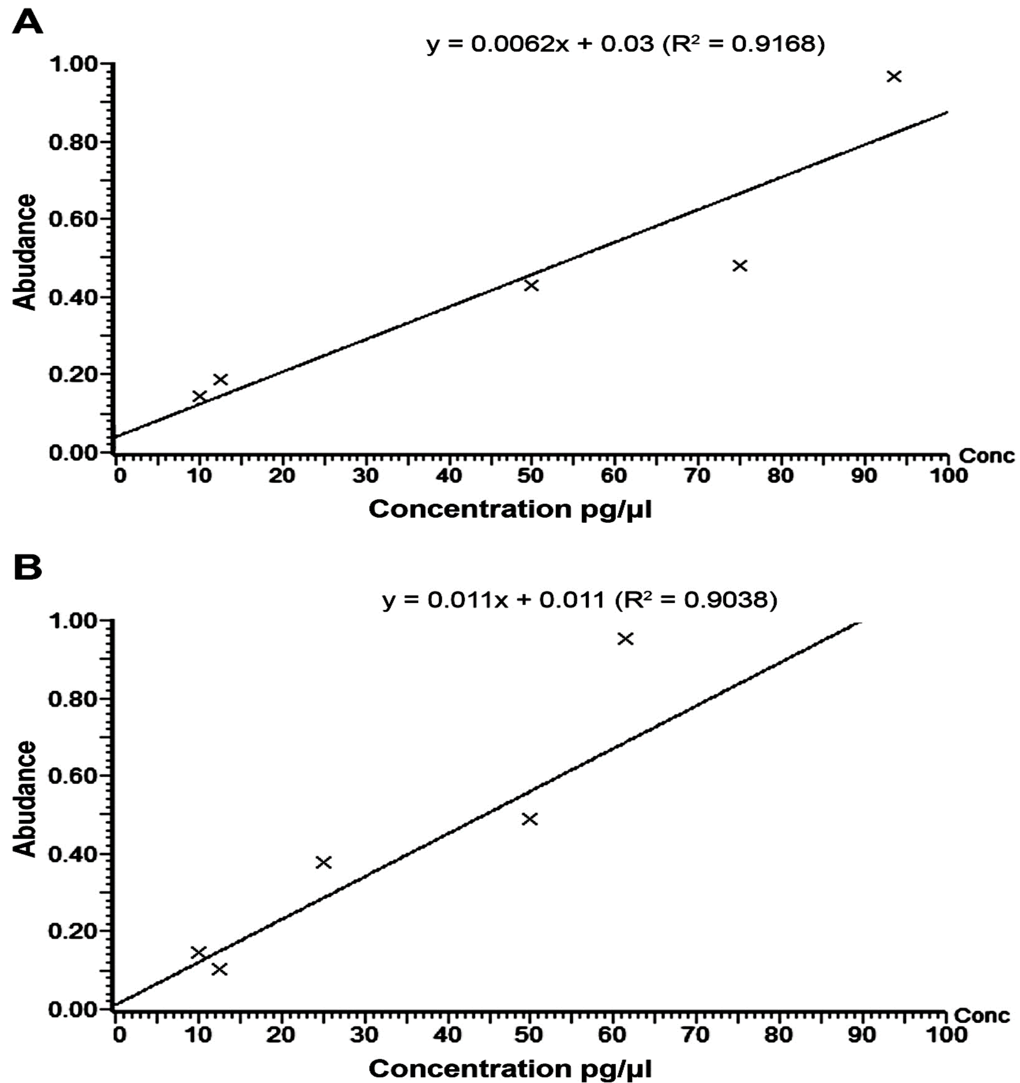

Fig 4.2 Calibration curves of synthetic juvenile hormone III (JH III) (A) and methyl farnesoate (MF) (B) used for external quantification of these hormones in the haemolymph of African and European honeybees.

Statistical analyses

All statistical analyses were carried out using R-Software version 3.2.5 (R Development Core Team 2015) and the alpha level was set at 0.05. To compare *Varroa* mite-infestation levels on adult workers, titres of JH III, MF, MF + JH III and JH III to MF

ratio between the cooler and hot dry seasons in the haemolymph of nurse honeybees collected from African honeybee colonies, the Mann-Whitney-Wilcoxon test was used. A Kruskal-Wallis rank sum test was used to examine differences in the adult mite-infestation level and titres of JH III, MF, MF + JH III or ratio JH III to MF among the African and European honeybee colonies during each study period. This statistical test was also used to examine differences in the percentage of damaged mites and ratio of total natural fallen mite to mite-infestation level on adult workers among the African honeybee colonies during the cooler dry season. The ratio of total natural fallen mite/ mite-infestation level on adult workers is the fraction of the mites removed by honeybees off their bodies relative to the total mite population present in their colonies (Nganso et al. 2017). Spearman's rank order correlation analysis was then conducted to establish the existence of a relationship between the percentage of mite damage and infestation levels with *Varroa* mite in the African honeybee colonies during the cooler dry season. This statistical test was also used to determine if there are correlation between the percentage of damaged mite, adult mite-infestation levels, ratio of total natural fallen mite to mite-infestation levels on adult workers to titres of JH III, MF, MF + JH III and ratio JH III to MF per colony in the African honeybee during each season. To establish the existence of a relationship between the mite-infestation levels on adult worker honeybee to titres of these hormones per colony in the European honeybee during winter, the Spearman's rank order correlation analysis was also conducted.

Results

The presence of JH III in the haemolymph from nurse workers of African and European honeybees was supported by the presence of a prominent base peak $[M + H]^+$ at *m/z* 267.3264 and 267.1948 respectively having a molecular formula of $C_{16}H_{26}O_3$ (Fig 4.3). Other diagnostic characteristic fragments for ions *m/z* 189.2708, 235.2027 and 235.0626 confirmed the presence of this hormone in the haemolymph of both honeybee subspecies (Fig 4.3).

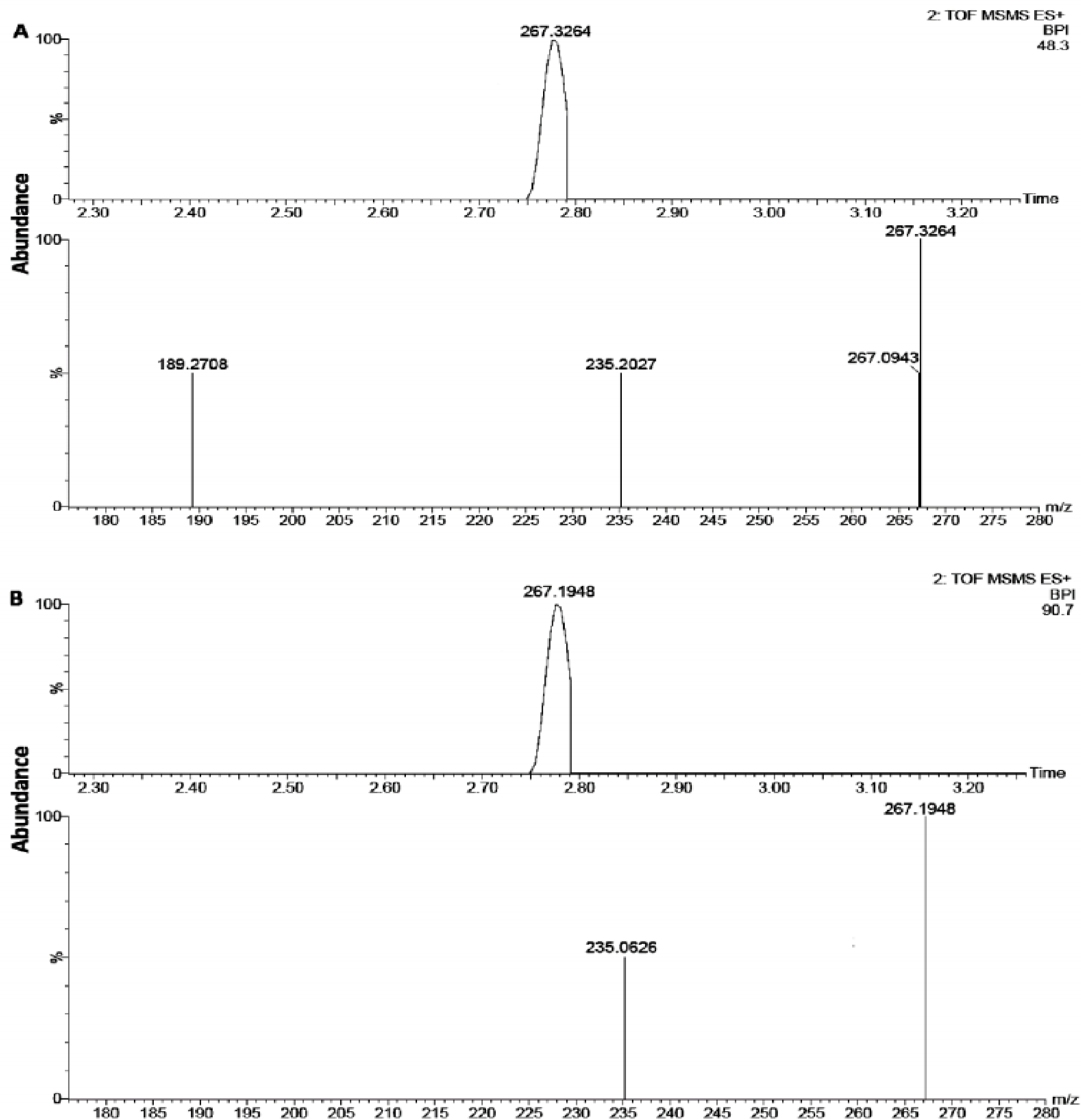

Fig 4.3. LC-QTOF-MS representative total ion chromatogram and mass spectrum showing juvenile hormone (JH III) in the haemolymph samples of African (A) and European (B) honeybees.

Likewise, the presence of MF in the haemolymph samples from nurses of African and European honeybees was supported by the presence of a prominent base peak $[M + H]^+$ at *m/z* 251.2238 and 251.1174 respectively having a molecular formula of $C_{16}H_{26}O_2$

(Fig 4.4). Other diagnostic characteristic fragments for ions *m/z* 191.0042, 191.2004 and 233.2019 confirmed the presence of this hormone in the haemolymph of both honeybee subspecies (Fig 4.4).

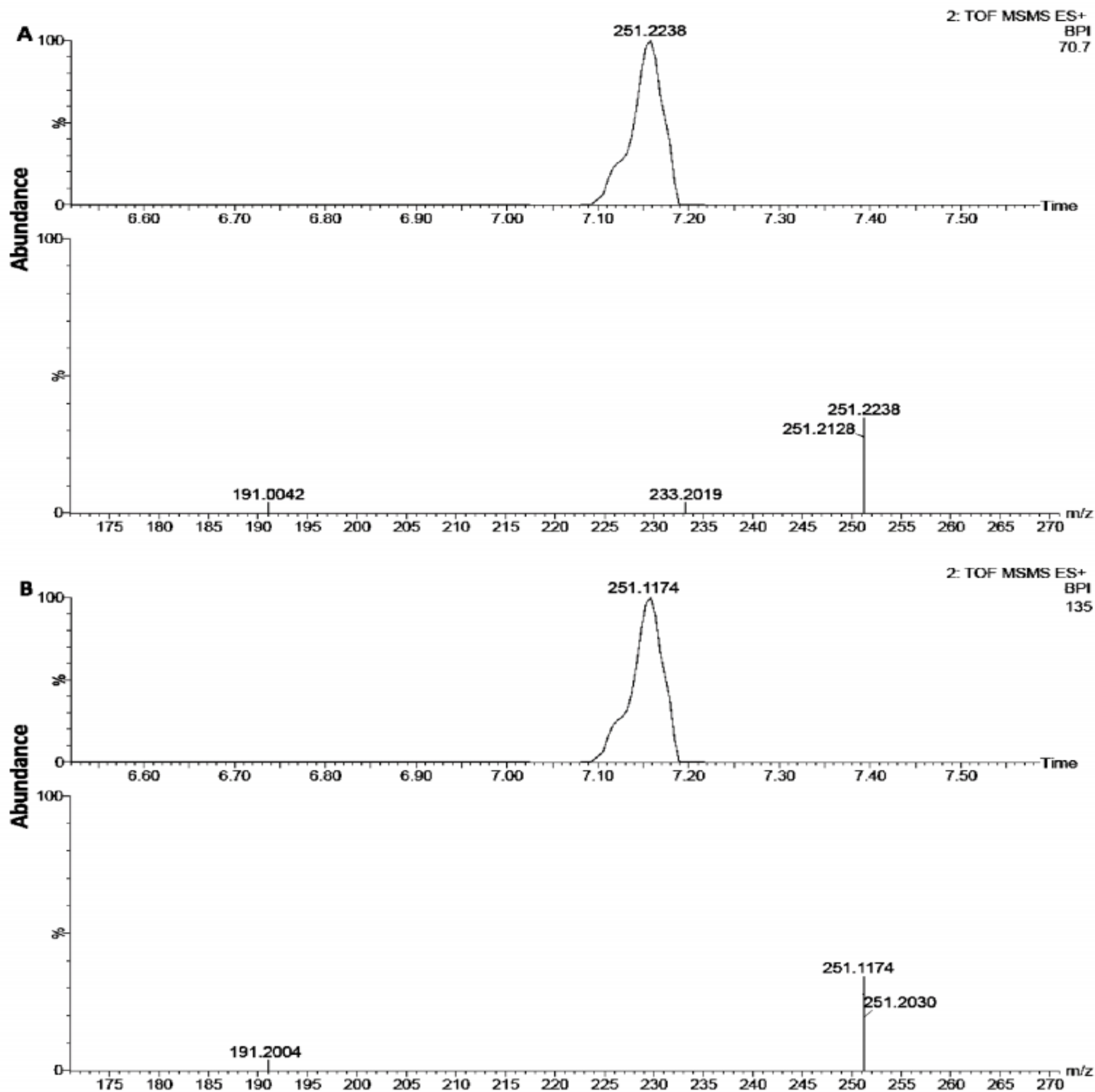

Fig 4.4. LC-QTOF-MS representative total ion chromatogram and mass spectrum showing methyl farnesoate (MF) in the haemolymph samples of African (A) and European (B) honeybees.

An infestation rate of 8 ± 1.8 mites/100 adult workers was recorded during the cooler dry season which was significantly lower (~ four-fold less) than the infestation rate during the hot dry season at 30 ± 6.0 mites/100 adult worker in the African honeybee colonies (W = 74, P = 0.002). The titres of JH III (W = 509, P = 0.01), MF (W = 548, P = 0.001) and MF + JH III (W = 564, P = 0.0004) were significantly higher during the cooler than the hot dry season in the haemolymph of African nurse honeybees (Fig 4.5). In contrast, the titre of the ratio of JH III to MF (W = 311, P = 0.36) was not significantly different between both seasons (Fig 4.5).

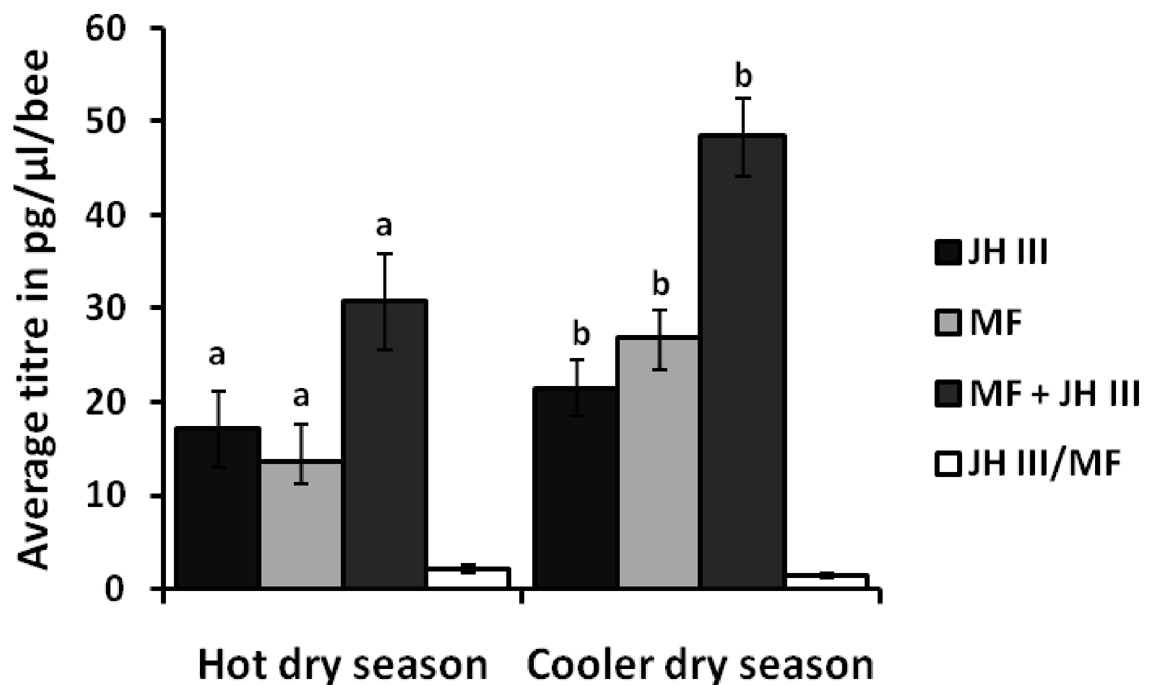

Fig 4.5. Comparison of the average titres (mean ± S. E.) of juvenile hormone III (JH III), methyl farnesoate (MF), MF + JH III and ratio of JH III to MF between the hot and cooler dry seasons in the haemolymph of nurse honeybees of *A. m. scutellata*. Pairs of Bars with letters indicate significant differences effect for each category.

There was no significant difference in the mite-infestation levels on adult worker honeybees during the cooler ($H = 9.64$, d.f. = 8, $P = 0.29$) and hot ($H = 13.01$, d.f. = 8, $P = 0.11$) dry seasons among the African honeybee colonies. In contrast, a significant difference in the mite-infestation levels on adult worker honeybees was detected among the European honeybee colonies in winter ($H = 25.32$, d.f. = 9, $P = 0.003$). The percentage of mite damage ($H = 10.90$, d.f. = 8, $P = 0.21$) or ratio of total natural mite fall to mite-infestation levels on adult workers ($H = 12.59$, d.f. = 8, $P = 0.13$) were not significantly different among the African honeybee colonies during the cooler dry season.

During the cooler dry season, there was no significant difference in the titres of JH III ($H = 9.42$, d.f. = 8, $P = 0.31$), MF ($H = 2.98$, d.f. = 8, $P = 0.94$), MF + JH III ($H = 9.05$, d.f. = 8, $P = 0.34$) or ratio of JH III to MF ($H = 5.80$, d.f. = 8, $P = 0.67$) among the African honeybee colonies. During the hot dry season, there was also no significant difference in the titres of MF ($H = 9.88$, d.f. = 8, $P = 0.27$) and ratio of JH III to MF ($H = 8$, d.f. = 8, $P = 0.43$). Though a significant difference was detected in the JH III ($H = 16.61$, d.f. = 8, $P = 0.03$) and MF + JH III ($H = 18.80$, d.f. = 8, $P = 0.02$) among the African honeybee colonies. During winter, there was no significant difference in the titres of JH III ($H = 2.07$, d.f. = 9, $P = 0.99$), MF ($H = 6.69$, d.f. = 9, $P = 0.67$), MF + JH III ($H = 5.30$, d.f. = 9, $P = 0.81$) or ratio of JH III to MF ($H = 8.92$, d.f. = 9, $P = 0.44$) among the European honeybee colonies.

During the cooler dry season, we found no significant correlation between percentage of damaged mites and the infestation levels with the mites in the savannah honeybee (Spearman's rank correlation: $r = 0.59$, $P = 0.21$) (Appendix 1A). No correlation was

detected between the *Varroa* mite-infestation level on adult workers/colony and titres of JH III (Spearman's rank correlation: r = -0.26, P = 0.50), MF (Spearman's rank correlation: r = 0.23, P = 0.55), MF + JH III (Spearman's rank correlation: r = 0.04, P = 0.91) or ratio of JH III to MF (Spearman's rank correlation: r = -0.07, P = 0.85) in African honeybee during this season (Appendix 1B-E). During the hot dry season, we also found no significant correlation between *Varroa* mite-infestation level/colony and titres of JH III (Spearman's rank correlation: r = -0.34, P = 0.37), MF (Spearman's rank correlation: r = -0.32, P = 0.40), MF + JH III (Spearman's rank correlation: r = -0.38, P = 0.32) or ratio of JH III to MF (Spearman's rank correlation: r = -0.13, P = 0.74) in the African honeybee (Appendix 1F-I). In the European honeybee, there was no significant correlation between *Varroa* mite-infestation level/colony and titres of JH III (Spearman's rank correlation: r = -0.38, P = 0.28), MF (Spearman's rank correlation: r = 0.01, P = 0.97), MF + JH III (Spearman's rank correlation: r = 0.06, P = 0.86) or ratio of JH III to MF (Spearman's rank correlation: r = 0.39, P = 0.27) during winter (Appendix 1J-M).

During the cooler dry season, there was no significant correlation between the percentage of damaged mites/colony and titres of JH III (Spearman's rank correlation: r = -0.40, P = 0.29), MF (Spearman's rank correlation: r = 0.03, P = 0.93), MF + JH III (Spearman's rank correlation: r = -0.09, P = 0.81) or ratio of JH III to MF (Spearman's rank correlation: r = -0.50, P = 0.18) in the African honeybee (Appendix 1N-G). There was also no significant correlation between the ratio of total natural mite fall to mite infestation level/colony and titres of JH III (Spearman's rank correlation: r = 0.31, P = 0.42), MF (Spearman's rank correlation: r = -0.11, P = 0.79), MF + JH III (Spearman's

rank correlation: r = 0.14, P = 0.72) or ratio of JH III to MF (Spearman's rank correlation: r = 0.41, P = 0.28) in the African honeybee (Appendix 1R-U).

Discussion

In this study, we confirmed the presence of MF as an additional circulating hormone to JH III in the haemolymph of nurse honeybees of *A. m. scutellata* in Kenya and *A. mellifera* hybrids of European origin found in USA. The titres of both hormones obtained herein were within the ranges, 0.03–45.5 pg/µl for JH III and 0.08–118.7 pg/µl for MF, as previously reported in the haemolymph of insects including honeybees of European origin (Teal et al. 2014, Montes et al. 2017).

Our results demonstrate that the titres of MF and JH III fluctuate significantly across seasons, with resultant significant effects on the total concentrations of MF + JH III in the haemolymph of the African savannah honeybees (Fig 4.5). These results are consistent with previous findings that JH III titres also vary significantly across seasons in the haemolymph of honeybees under natural conditions, thereby mediating plasticity in their behaviour in response to changes in environmental and colony conditions (Fluri et al. 1982, Bühler et al. 1983, Winston, 1987, Huang and Robinson 1992, 1995).

The observed seasonal changes in the titres of these hormones in the African honeybee could be influenced by colony age demography (Huang and Robinson 1992, 1995). Huang and Robinson (1992) have shown that foragers could actually exert inhibitory effects on nurse honeybees and on each other at distinct times of the year. For instance, during autumn or winter when brood rearing reduces in European honeybee colonies

due to the disappearance of flowers that leads to reduced foraging, JH III titres have been shown to decrease in foragers and consequently in nurse honeybees because foragers spend most of their times in the hive (Fluri et al. 1982, Bühler et al. 1983, Huang and Robinson 1992, 1995). However, the titres of this hormone increased both in foragers and nurse honeybees due to the onset of foraging that leads to increased brood rearing in European honeybee colonies. Moreover, JH III has been reported to regulate the rates of metabolism in honeybees because low levels of this hormone in winter nurse honeybees correlated with higher levels of fat reserves relative to nurse honeybees in spring and summer (Fluri et al. 1982, Huang et al. 1994). We therefore expect that the titres of JH III, MF and MF + JH III will increase in nurse honeybees during floral availability which leads to increased foraging and consequently brood rearing and vice-versa during floral disappearance as foraging and brood rearing decrease in African honeybee colonies.

We found that the titres of JH III, MF, MF + JH III and ratio of JH III to MF did not correlate with *Varroa* mite-infestation levels and did not significantly differ among the African and European honeybees. Given obvious differences in the adult mite-infestation rates between the cooler and hot dry seasons in the African honeybee colonies and among the colonies of the European honeybee, our findings suggest that these hormones may not regulate the variability in the mite-infestation levels recorded in both honeybee subspecies. It thus appears that the titres of these hormones are likely triggered by environmental and colony conditions such as temperature, food availability, amount of brood and colony age structure as previously demonstrated other than the presence of the mite (Fluri et al. 1982, Bühler et al. 1983, Winston, 1987,

Huang and Robinson 1992, 1995). However, further studies are warranted to validate these findings.

In the current study, we also did not find a relationship between the titres of these hormones and colony grooming behaviour of the African honeybee towards the mite. The lack of a correlation between these parameters could be due to the non-differential expression of grooming behaviour towards the ecto-parasite among the African honeybee colonies. If each colony expresses different levels of aggressive behaviour towards the mite, the overall amounts of these hormones may vary among colonies. However, the results obtained herein should be interpreted with caution because we found no relationship between grooming behaviour and *Varroa* mite-infestations rates in colonies of *A. m. scutellata* as previously demonstrated also in our study (Nganso et al. 2017). Given that the mite-infestation levels on adult workers appear not to be regulated by the titres of these hormones in colonies of both honeybee subspecies, it is also possible that they may not influence the grooming rate of honeybees towards the mites. Previous studies have reported that JH III levels did not regulate inter-colonial differences in aggressiveness in honeybees (Pearce et al. 2001). Also, Robinson et al. (1987) reported no significant differences in the titres of JH III between European and Africanized honeybees despite the fact that Africanized honeybees are known to be extremely aggressive compared with European honeybees. Hence, further investigations are warranted as no report exists at the moment linking the amounts of these hormones to the grooming behaviour of honeybees towards *Varroa* mite.

In summary, our study provides further support that natural environmental changes are related to endocrine changes that are known to play a role in honeybee behaviour and

ecology. The findings obtained herein suggest that the titres of JH III, MF, MF + JH III and ratio of JH III to MF do not influence *Varroa* mite-infestation rates in colonies of the African and European honeybees. These titres may not regulate the grooming behaviour of honeybees towards the mite. However, further studies are recommended to validate these results. This is important in the context of generating new knowledge about the physiological mechanisms that might regulate the behaviour of honeybees against this serious parasite.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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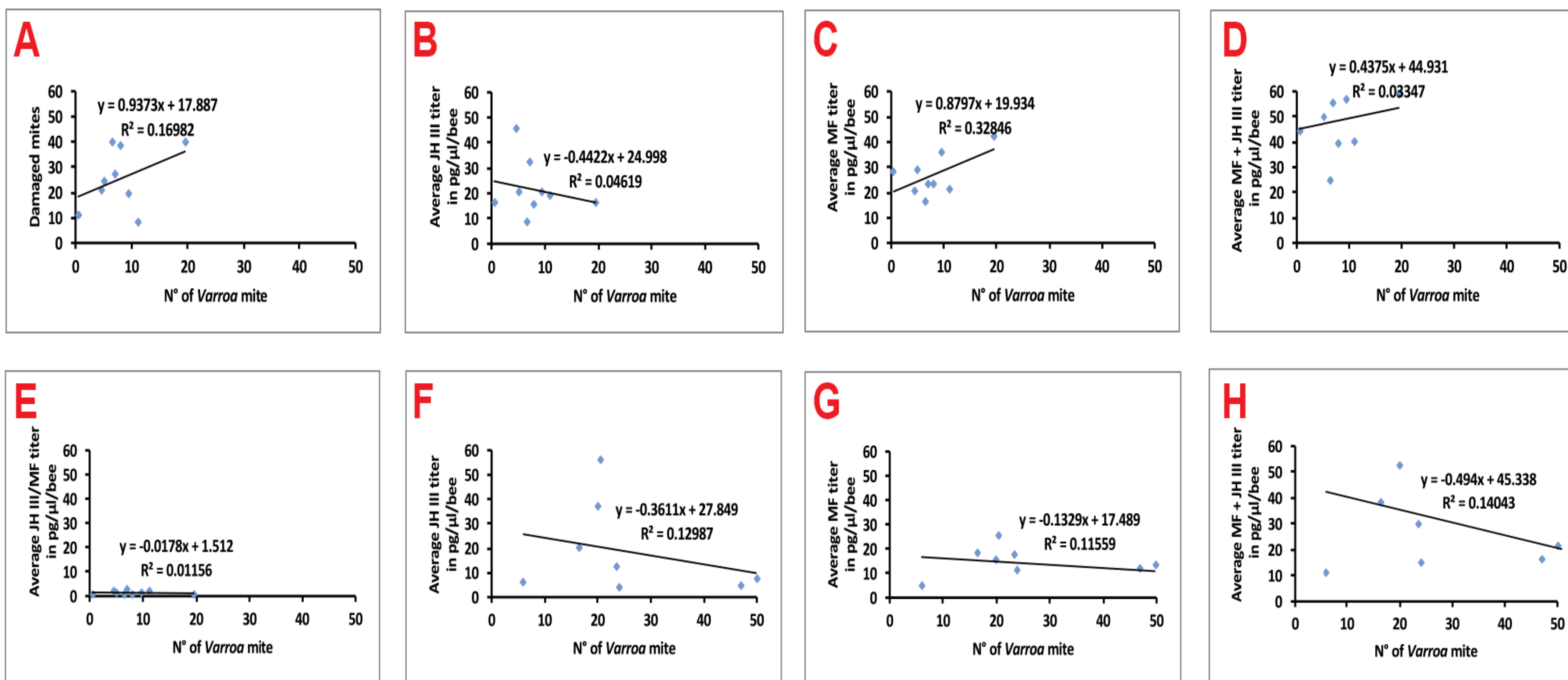
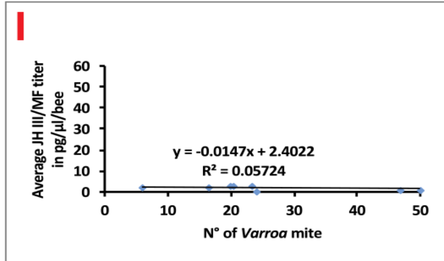
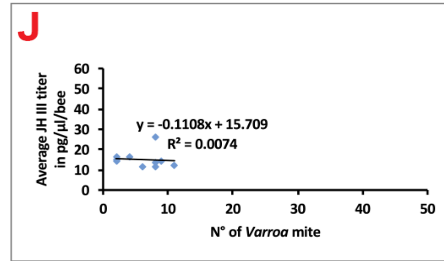
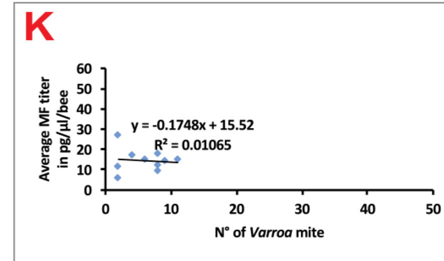
Appendix

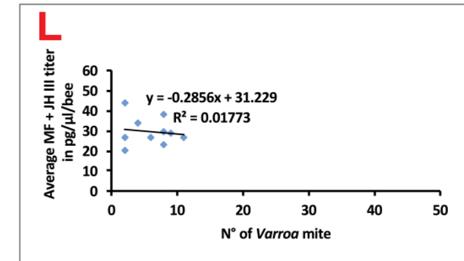
Appendix 1: Correlations between *Varroa* mite-infestation level and percentage of damaged mites per colony in African honeybee; *Varroa* mite-infestation level, percentage of damaged mites, ratio of total natural mite fall to mite infestation level and titres of juvenile hormone III (JH III), methyl farnesoate (MF), MF + JH III or ratio of JH III to MF per colony in African and European honeybees.

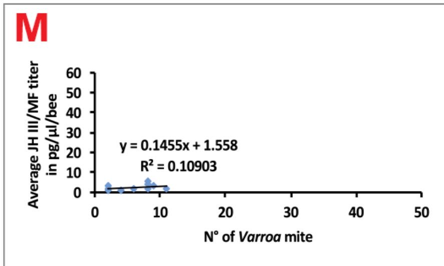


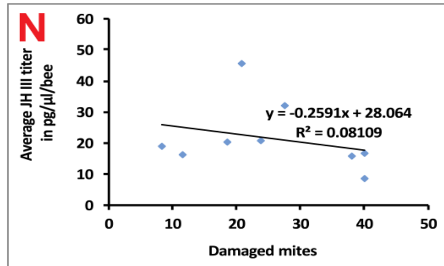

I
Average JH III/MF titer in pg/µl/bee
y = -0.0147x + 2.4022
R² = 0.05724
N° of *Varroa* mite

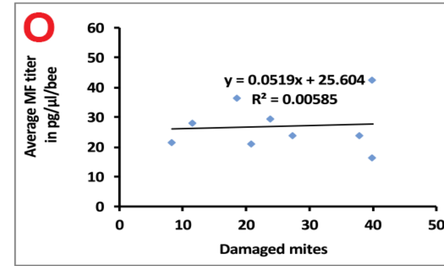

J
Average JH III titer in pg/µl/bee
y = -0.1108x + 15.709
R² = 0.0074
N° of *Varroa* mite

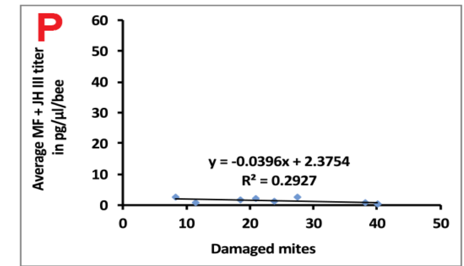

K
Average MF titer in pg/µl/bee
y = -0.1748x + 15.52
R² = 0.01065
N° of *Varroa* mite

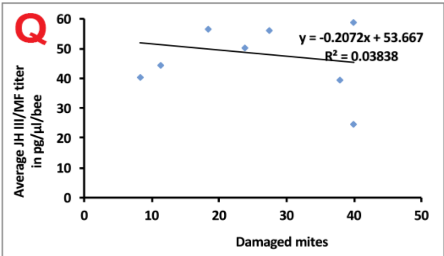

L
Average MF + JH III titer in pg/µl/bee
y = -0.2856x + 31.229
R² = 0.01773
N° of *Varroa* mite

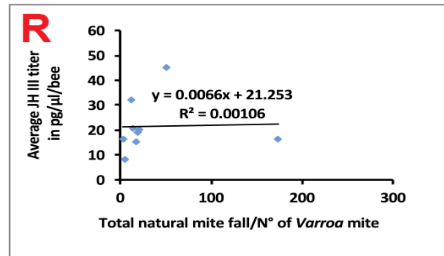

M
Average JH III/MF titer in pg/µl/bee
y = 0.1455x + 1.558
R² = 0.10903
N° of *Varroa* mite

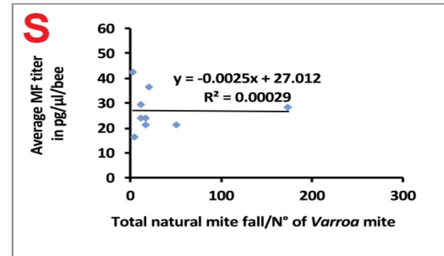

N
Average JH III titer in pg/µl/bee
y = -0.2591x + 28.064
R² = 0.08109
Damaged mites

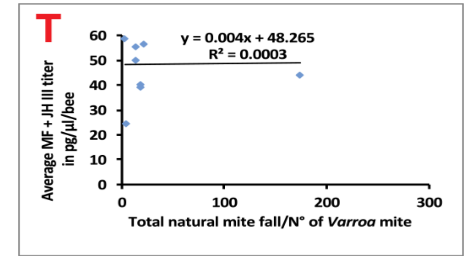

O
Average MF titer in pg/µl/bee
y = 0.0519x + 25.604
R² = 0.00585
Damaged mites

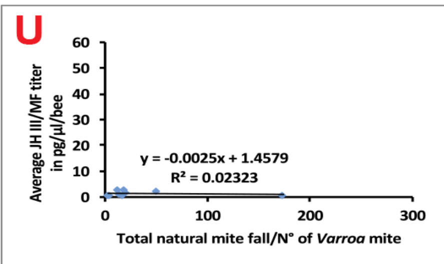

P
Average MF + JH III titer in pg/µl/bee
y = -0.0396x + 2.3754
R² = 0.2927
Damaged mites


Q
Average JH III/MF titer in pg/µl/bee
y = -0.2072x + 53.667
R² = 0.03838
Damaged mites


R
Average JH III titer in pg/µl/bee
y = 0.0066x + 21.253
R² = 0.00106
Total natural mite fall/N° of *Varroa* mite


S
Average MF titer in pg/µl/bee
y = -0.0025x + 27.012
R² = 0.00029
Total natural mite fall/N° of *Varroa* mite


T
Average MF + JH III titer in pg/µl/bee
y = 0.004x + 48.265
R² = 0.0003
Total natural mite fall/N° of *Varroa* mite


U
Average JH III/MF titer in pg/µl/bee
y = -0.0025x + 1.4579
R² = 0.02323
Total natural mite fall/N° of *Varroa* mite

CHAPTER FIVE

General conclusion

Africa is the only area in the world that engorges a rich genetically diverse and abundant wild honeybee, *Apis mellifera* L. populations which provide natural pollination services to agricultural crops and income for rural communities (reviewed in Dietemann et al. 2009, Pirk et al. 2017). African honeybee populations are currently reported to be resistant to the most serious threat, *Varroa destructor* and its associated pathogens when compared to their European counterparts (Dietemann et al. 2009, Muli et al. 2014, Pirk et al. 2014). However, it is critically important to take preventive measures to safeguard the current health status of these keystone species for sustainable food security, poverty alleviation and biodiversity conservation. In this regard, research efforts have focused mainly on identifying behavioural mechanisms of resistance and/or tolerance that contribute to the survival of African honeybee populations against *Varroa* mite (Allsopp 2006, Strauss et al. 2013, Strauss et al. 2015, Strauss et al. 2016). Also, candidate genes that influence the behavioural traits of honeybees against the mites have been identified and suggested as excellent biomarkers that may be useful for breeding programmes aimed at increasing the survival of susceptible honeybee populations (Arechavaleta-Velasco et al. 2012, Hamiduzzaman et al. 2017). In the same vein, the goal of this thesis was to unravel the potential tolerance and resistance mechanisms that contribute to the survival of the African savannah honeybee, *A. m. scutellata* in Kenya as they interact with *Varroa* mites. Additionally, this study further

attempted to search for possible hormonal bio-markers specific to any resistance or tolerance behaviour that could be used for *Varroa* mite-infestation diagnosis.

To understand the resistant behavioural defense mechanisms that are responsible for the survival of *A. m. scutellata*, chapter two of this thesis compared hygienic and grooming behaviours in this honeybee subspecies and *A. mellifera* hybrids of European origin found in the United States of America against the mite. The findings showed that two additional undescribed damage patterns inflicted on mites by honeybees were present in both African and European honeybee colonies. In addition, the African savannah honeybee which maintains lower mite colony infestations (~ three-fold lower) removed significantly more mites off their bodies relative to the total mite population present in their colonies than their European counterpart. They also inflicted significantly more damage categories to the mites than their European counterparts though the expression levels of hygienic behaviours were similar in both honeybee subspecies. The findings in chapter two supports the conclusion that grooming behaviour could be a potential tolerant mechanism displayed by the African savannah honeybees towards mite infestation. However, both hygienic and grooming behaviours could not explain the lower mite-infestation levels recorded in *A. m. scutellata* colonies; suggesting that other adaptive resistant mechanisms such as reduced mite reproductive success, lower viral prevalence within honeybees and mites might play a key role in suppressing the mite population growth in colonies of *A. m. scutellata*.

Chapter three of this study investigated the involvement of other potential resistant mechanisms including suppression of mite reproduction in worker brood cells of *A. m. scutellata* to explain the low mite infestation levels recorded in colonies of this

honeybee subspecies. High mite infertility rates (26 – 27 %), high percentage of unmated daughter mites (39 – 58 %) and low mite fecundity (1.7 – 2.2, mean number of eggs laid) were identified as adaptive resistance processes of reduced *Varroa* mite reproductive success in *A. m. scutellata* colonies. Offspring mortality in both sexes and absence of male offspring were also identified as key factors accounting for the high percentage of unmated daughter mites produced in *A. m. scutellata* colonies. Taken together, the results obtained herein provide additional insights into the key resistant defense mechanisms including suppression of mite reproductive success that confer survival in this specific population of *A. m. scutellata* against the mite. The consistency of results regarding mite reproduction in two geographically distinct *A. m. scutellata* populations (South Africa, Strauss et al. 2016 and Kenya, this study) suggests general adaptations towards *V. destructor* within African honeybees, most likely due to the higher number of wild colonies and lack of miticide use in their colonies (Pirk et al. 2017).

Chapter four of this study sought to understand the physiological mechanisms that regulate the behaviours of honeybees towards the mites. To achieve this, the titres of the insect juvenile hormone III (JH III) and its immediate biosynthetic precursor methyl farnesoate (MF) were quantified in the haemolymph of nurse honeybees of *A. m. scutellata* in Kenya across two seasons and in the haemolymph of nurse honeybees of *A. mellifera* hybrids European origin found in the USA in one season. The titres of these hormones were then correlated with *Varroa* mite-infestation levels on adult worker honeybees in colonies of both honeybee subspecies and colony grooming behaviour of the African honeybee towards the mite. The results obtained herein indicate that the

titres of JH III and MF vary significantly between seasons with resultant significant effects on the total concentration of MF + JH III in the haemolymph of the African nurse honeybees. These results confirm previous findings that seasonally related changes in JH III titres occur in the haemolymph of honeybees under natural conditions and mediate plasticity in their behaviours in response to changes in environmental and colony conditions (Fluri et al. 1982, Bühler et al. 1983, Winston, 1987, Huang and Robinson 1992, 1995). The findings in chapter four further indicate that the titres of JH III, MF, MF + JH III and ratio of JH III to MF did not correlate with adult mite-infestation levels in both honeybee subspecies despite significant differences in the infestation levels with the mites recorded between seasons in the African honeybee and among colonies of the European honeybee. These findings suggest that these hormones may not regulate the variability in the mite-infestation rates on adult workers in African and European honeybee colonies. No correlation was also detected between colony grooming behaviour of the African honeybee towards the mite and titres of these hormones which could be due to the non-differential expression of this trait among colonies of this honeybee subspecies. However, it is possible that these hormones may not influence the intensity of honeybee's grooming behaviour towards *Varroa* mite because no relationship was found between the titres of these hormones and adult mite-infestation levels in colonies of *A. m. scutellata*. Nevertheless, further studies are warranted to validate the results obtained herein as no report exists at the moment linking the levels of these hormones in the haemolymph of honeybees to mite-infestation levels and grooming behaviour.

In conclusion, this study identified resistant and tolerant behavioural defense mechanisms that partly explain the ability of the African savannah honeybees in Kenya to survive the mite infestations. This study also identified a new index that could be used to assess honeybee's grooming behaviour across species and subspecies. This index was the ratio of total natural mite fall to adult worker bee mite infestation level, which represents a fraction of the total mite removed by honeybees off their bodies relative to the total mite population present in their colonies. Its use alongside damage levels and patterns in fallen mites provides a new approach to compare grooming behaviour across species or subspecies. Furthermore, the results obtained herein suggest that JH III and MF cannot be considered as potential hormonal biomarkers which could be exploited in mite-infestation diagnosis. Further studies are recommended in this interesting area to help improve our understanding of the physiological mechanisms that regulate honeybee's behaviour towards the mite. It is important to note that other resistant mechanisms such as low viral prevalence and high expression of *Varroa*-specific hygienic (VSH) behaviour (which is the selective ability of nurse honeybees to detect, uncap and remove mite-infested brood (Harbo and Harris 2005, Ibrahim and Spivak 2006) have also been shown to contribute to the survival of *A. m. scutellata* in Kenya (Muli et al. 2014, Cheruiyot et al. 2018). Hence, this and previous studies suggest that the ability of the African savannah honeybee in Kenya to survive the mite's attack is dependent on host factors rather than parasite virulence (Allsopp 2006, Dietemann et al. 2009, Strauss et al. 2016, Pirk et al. 2016). This study also supports the popularly held view that the naturally untreated and not genetically bred African honeybee populations may have evolved natural behavioural defence mechanisms to

counteract mite-infestations without requiring any human interference through a natural selection process (Dietemann et al. 2009, Locke 2015, Pirk et al. 2016). Unlike European honeybees, African honeybees are genetically diverse (Hepburn and Radloff 1988, Dietemann et al. 2009). This high genetic diversity could offer rich potential resistance loci in a host-colony queen which interact epistatically between them to influence the same mite resistant phenotype (Conlon et al. 2018). The presence of these multiple resistance loci, with epistatic interactions between them, could then hinder the rapidly evolving *Varroa* mite to evolve resistance to a single host resistance pathway thereby providing an evolutionary benefit to the host (Gonzalez-Cabrera et al. 2016, Beaurepaire et al. 2017, Conlon et al. 2018). Hence, currently, beekeepers in Kenya do not have to treat the mite infestations with miticide in honeybee colonies. Despite these results, it is important to understand how the individual and social immune systems of honeybees as well as nutrition counteract *Varroa* mite and its associated pathogens without human intervention in African honeybees. It is also crucial to understand the molecular, genetic and physiological basis of desirable traits of African and European honeybees as they interact with the mites in order to identify unique and/or shared pathways involved in response towards the mite infestations in the nearest future. Such knowledge may help researchers to understand better the honeybee-parasite interactions, thus assisting with the sustainable management of this important economic parasite to help restore the global health of honeybees which are indispensable to human well-being.

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