Molecular Characterization, Ecology and Pathogens of Honeybees in Newly Established Colonies in Kitui County, a Semi-Arid Ecosystem in Kenya

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A thesis submitted in partial fulfilment for the degree of Masters of Science in Genetics in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

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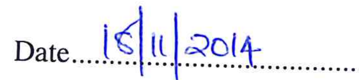
Signature Date 

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DEDICATION

To Humforce and Margret Mumoki. Papa, you worked so hard to educate me. Mommy, you never let me settle for less. Papa, mommy; Cheers to you! You gave me the courage and the wings to fly!

To my siblings; Mike, Linda, Gloria and Maria; thank you for being there for me....you gave me good laughs when all I wanted to do (sometimes) was not laugh!

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CCD	Colony Collapse Disorder
df	Degrees of Freedom
dNTPs	Deoxyribonucleotide triphosphates
FAO	Food and Agriculture Organisation of the United Nations
icipe	International Centre of Insect Physiology and Ecology
KTBH	Kenyan Top Bar Hive
MUSCLE	Multiple Sequence Comparison by Log- Expectation
MGB	Minor Grove Binder
MSA	Multiple Sequence Alignment
NCBI	National Centre for Biotechnology Information
PCIA	Phenol Chloroform Isoamyl Alcohol
PSU	Pennsylvania State University
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
SEKU	South Eastern Kenya University

ABSTRACT

Colony Collapse Disorder, CCD, has led to losses of up to 33% of adult worker bees in Europe and the USA. Its predictors include *Nosema*, honeybee viruses, *Varroa*, and overuse of pesticides. The goal of this project is to monitor the CCD predictors in a dry land ecosystem such as Kitui district, taking into account the apicultural practices unique to this continent. Sampling was done from three apiaries in Kitui located 2KM apart. Each apiary had seven of three types of hives; Kenyan Top Bar, Langstroth and log. Thirty bees were sampled from each colony, 10 each for sub-species, virus and *Nosema* analysis. $tRNA^{ILE}$-ND2 and intron of EF1α was analysed for sub-species analysis. A portion of the small sub-unit of *Nosema* rRNA was utilised for *Nosema* diagnosis. Primers specific for Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV) and Israeli Acute Paralysis virus (IAPV) were used in virus detection. Results showed that the predominant subspecies of honeybees in Kitui is *Apis mellifera scutellata* but there is presence of Scutellata-Monticola and Scutellata-Litorea hybrids. Pathogen diagnosis revealed absence of *Nosema*, BQCV and IAPV. Presence of DWV was confirmed in 39.5% of honeybee colonies but absent in *Varroa*. Colony growth was shown to be influenced by seasons, type of hive used and forage diversity. The pollen diet was identified as polyfloral, with family-level plant diversity potentially exerting great influence on colony growth. This work demonstrates the key role that the environment plays in honeybee health and growth.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

Honey bees are social insects which together with ants and wasps, belong to the order hymentoptera (Michener 2007). The genus *Apis* has about 11 known species the four most common of which are *Apis mellifera* (the western honeybee), *Apis cerana* (the eastern honeybee), *Apis dorsata* (the giant honeybee) and *Apis florae*-the dwarf honeybee (Crane 2009). Of them all, *Apis mellifera* is the most important generalist pollinator of agricultural (Allsopp *et al.* 2008) and natural ecosystems, with subspecies and geographical races found globally.

Through their pollination activities, honeybees provide crucial ecosystem services (Kremen *et al.* 2007), the quantification of which has always been controversial (Allsopp *et al.* 2008, Ollerton *et al.* 2012). The Food and Agriculture Organisation of the United Nations (FAO) estimates that about a third of all plants or plant products consumed by man are either directly or indirectly dependant on honeybee pollination (FAO 2010). These services are carried out by both managed and wild honeybees, with the managed honeybees being responsible for pollination of 80% -85% of all pollinated commercial hectares, and include 80% of the 300 crops grown worldwide (Allsopp *et al.* 2008). Honeybee pollination is valued at $14 billion in the United States (Morse and Calderone 2000) and USD 212 billion globally (Gallai *et al.* 2009).

With the backdrop of the direct and indirect benefits that honeybees confer to ecosystems, honeybee colonies in many parts of the world have experienced gross population declines. This trend that seems to have been experienced before (Underwood and vanEngelsdorp 2007), with among the earliest reports being that of the “May” disease in 1986 (Aikin 1897). However, whereas single organisms or events could be named as the cause of previous honeybee losses, this is not the case with Colony Collapse Disorder, CCD (Cox-Foster *et al.* 2007, Dainat *et al.* 2012a) a phenomenon marked by among others, an abrupt loss of adult worker bees populations in apparently healthy honeybee colonies, leaving behind the queen, brood and food stores in terms of honey and pollen (vanEngelsdorp *et al.* 2009). In the USA, about a third of the total 2.4 million honeybee colonies were lost during the winter of 2006-2007, marking the start of CCD (Stokstad 2007). This trend has continued in the years since, leading to highly elevated colony losses, especially following the winter seasons. While many other countries have reported serious declines in honeybee populations, the only other place outside of the USA to document cases of CCD is Switzerland where 49% of all colonies analysed were found to have collapsed due to CCD. This is also the first report of the phenomenon in Europe (Dainat *et al.* 2012b).

A pilot metagenomic survey to identify the causes of colony collapse disorder was carried out in 2007 led by researchers from Pennsylvania State University (PSU) and the United States Department of Agriculture (USDA). The result of this study pinpointed significant markers for use in monitoring and assessment of CCD

including the presence of seven viruses, a mite and microsporadian parasites (Cox-Foster *et al.* 2007). These, in addition to poor beekeeping practices such as movement of beehives from place to place, over exposure to pesticides (Desneux *et al.* 2007) as well as poor honeybee nutrition (Brodschneider and Crailsheim 2010) are now known to be factors strongly linked to CCD. In a bid to monitor and predict cases of CCD in the global apicultural scope, four main predictive markers have been highlighted; *Nosema cerenae*, *Varroa destructor*, Deformed Wing Virus and changes in levels of vitellogenin. The disadvantage of these markers is that their predictive ability is strongly dependant on the season (Dainat *et al.* 2012a).

While colony collapse disorder has not been reported in Africa, many apiculturists have described progressive declines in wild honeybee populations, a trend that so far has mostly been attributed to destruction of habitats (Brown and Paxton 2009).

1.1.1 Global honeybee landscape

Honey bees are social insects belonging to the Kingdom Animalia, phylum Arthropoda, class Insecta, order Hymenoptera, family Apidae, genus *Apis* and species *Apis mellifera* (Michener 2007). Based on morphometrical studies, at least 29 sub-species of *Apis mellifera* have been described (Ruttner 1988) and these have been classified into four mitochondrial haplotype lineages A, M, O and C (Ruttner *et al.* 1978). The A haplotype is found throughout Africa, M group found in western and northern Europe, C in eastern Europe and the O group in Turkey and the Middle East. A fifth haplotype Y has recently been described as belonging to the honeybees

of northern Africa (Franck *et al.* 2001). In Kenya, there are four identified sub-species. These include *Apis mellifera litorea* also referred to as the coastal honeybee, *A.m. monticola*-the mountain honeybee, *A. m. scutellata* the Savannah honeybee (Meixner *et al.* 2000) and *Apis mellifera yemeintica*, also referred to as the desert honeybee indigenous to arid areas such as Saudi Arabia (Alqarni 2006).

1.1.2 Identification of subspecies

1.1.2.1 Morphometric analysis

Honeybees have been identified using two main types of morphometrical analyses. Wing morphometrics has been used extensively in the classification of honeybees from different parts of the world (Ruttner *et al.* 1978). Currently, digital wing morphometric analyses such as the Automatic Bee Identification System (ABIS) have been developed (Schröder *et al.* 1995) and tested (Francoy *et al.* 2006) and found to be faster and more efficient than conventional wing morphometric techniques (Francoy *et al.* 2008).

The second type of morphometrical analysis is by studying the abdominal banding patterns. This analysis was conducted for the description of Kenyan honeybees, the result of which characterised three sub-species of honeybees native to Kenya. *A. m. monticola* was found to have no bands on the abdomen and thus seen to be a plain black bee; *A. m. litorea* was identified to have two yellow bands on the abdomen while *A. m. scutellata* was identified to have three yellow abdominal bands (Raina and Kimbu 2005), as shown on Figure 1.

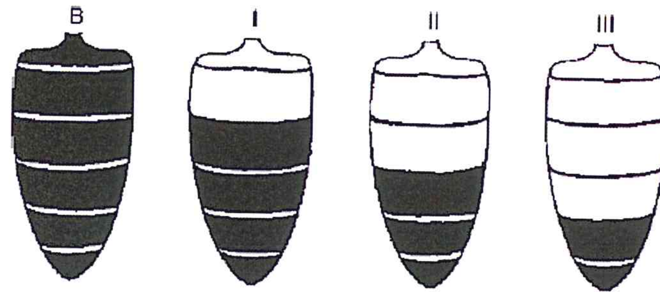

Figure 1: Abdominal colour bands showing the plain black bee (B), one yellow band (I), two yellow bands (II) and three yellow bands (III). Image adopted from Raina and Kimbu 2005.

1.1.2.2 Molecular analysis

Various genes have been employed in the molecular characterisation of honeybee sub-species. These include mitochondrial genes such as Cytochrome C Oxidase subunit I (COI) and the Isoleucine transfer RNA to the NADH Dehydrogenase subunit II ($tRNA^{ILE}$-ND2) gene region (Arias and Sheppard 1996) as shown on Figure 2. The intron of elongation factor 1α (Arias and Sheppard 2005) is a nuclear gene that has been used in establishing the phylogenetic relationship within the genus *Apis*. Single Nuclear Polymorphisms (SNPs) have been employed in the identification of honeybee sub-species (Whitfield *et al.* 2006) where 1500 SNPs were scored across multiple subspecies. *Dra*I Restriction Fragment Length Polymorphism (RFLP) of the COI-COII mitochondrial gene region has been used extensively in identification of the different mitotypes circulating in the A, Y and O lineages found in different parts of Africa (Franck *et al.* 2001).

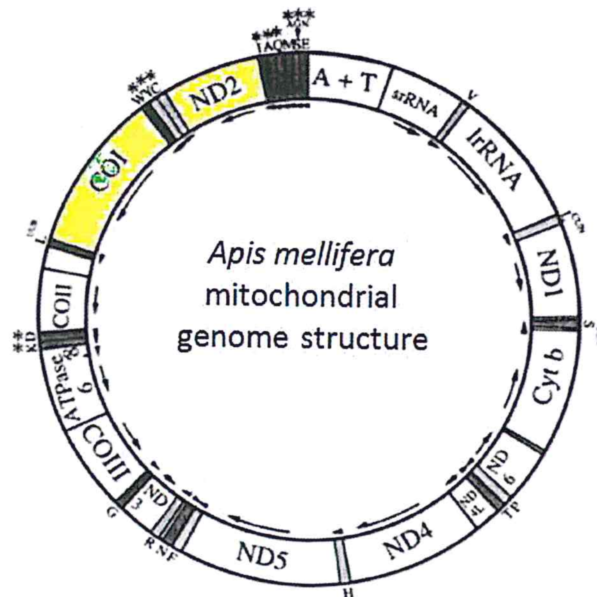

Figure 2: Structure of the honeybee mitochondrial genome. The regions shaded yellow show the loci used for subspecies identification in this work

1.1.3 Drastic Decline in Honeybee Populations

The first report of the honeybee Colony Collapse Disorder, CCD was made in November 2006 by David Hackenberg, an American apiculturist from Florida, USA. Hackenberg realized that 368 of the 400 hives he was inspecting were empty of bees, despite being fully occupied three weeks earlier. By the end of the 2006-2007 winter season, Hackenberg had lost 2,550 of his 3000 hives, an economic loss of about USD 450, 000 (Stokstad 2007). This experience that was shared by many more farmers around the country and currently CCD is thought to contribute about 33% of all colony losses experienced during winter in the USA (USDA 2012).

The main symptoms of CCD include; rapid loss of the adult honeybee worker population leaving the beehive filled with brood and food (Oldroyd 2007) absence of dead workers in the affected colony or apiary sites, delayed invasion of the affected hives by pests such as *Athenia tumida* (Cox-Foster *et al.* 2007), ample food stores in the collapsed colonies and few honeybee pathogens such as *Varroa destructor*, at the time of the collapse (vanEngelsdorp *et al.* 2010). Simply put, CCD is usually a 'case of empty hives' (Stokstad 2007)! In the 2007 metagenomic survey of honeybee pathogens conducted in order to identify the microbes involved in CCD (Cox-Foster *et al.* 2007), identified seven viruses: Acute Bee Paralysis virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus, CBPV (Ribière *et al.* 2010), Deformed Wing Virus, DWV (Miranda and Genersch 2010), Israeli Acute Paralysis Virus (IAPV), Kashmir Bee Virus (KBV) and Sacbrood Virus (SBV). Some of these viruses such as DWV are believed to be vectored by the honeybee mite *Varroa destructor* (Tentcheva *et al.* 2004, Yue and Genersch 2005, Chen *et al.* 2009), with current studies showing that virus replication also occurs in the mite (Ongus *et al.* 2004, Gisder *et al.* 2009). Also highlighted in the survey was the microsporadian parasites *Nosema apis* and *N. ceranae* with the latter being more virulent (Paxton *et al.* 2007).

Varroa destructor (Anderson and Trueman 2000) is an ectoparasitic mite of honeybees said to cause altered physiological responses in the developing bees such as lowering the level of vitellogenin, a hemolymph protein in honeybees with functions in honeybee immunity and development (Amdam *et al.* 2004). Differential

gene expression has also been noted in parasitized and non-parasitized pupae (Navajas *et al.* 2008). While the *Varroa* mite identified in almost all parts of the world (Anderson and Trueman 2000, Rosenkranz *et al.* 2010), its presence in Kenya was documented (Fazier *et al.* 2010) from a sampling exercise done in 2009 in the three East African countries; Kenya, Uganda and Tanzania.

Investigations to the epidemiological causes of Colony Collapse Disorder/ Drastic decline in honeybee populations has led to the unveiling of viruses and microsporadia as the main culprits (Tentcheva *et al.* 2004, Cox-Foster *et al.* 2007, Oldroyd 2007, Dainat *et al.* 2012a).

While there are about 21 known honeybee viruses, only seven of these viruses have been implicated in causing the drastic decline of honeybee colonies (Tentcheva *et al.* 2004, Cox-Foster *et al.* 2007). These include black Queen Cell Virus (BQCV), Deformed Wing Virus (DWV), Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Sacbrood virus (SBV), Kashmir Bee Paralysis Virus (KBV) and Israeli Bee Paralysis Virus (IAPV). These affect honeybees at different stages of the honeybee life cycle (Allen and Ball 1996, Chen and Siede 2007). The viruses mostly belong to the order Picornavirales which contains five families, among which Iflaviridae and Dicistroviridae families contain common honeybee viruses (ICTV 2013). Recent research has shown that several new viruses have been reported to attack honeybees from non-*Apis* sources. An example of this is the Tobacco Ring Spot Virus (TRSV); a pollen-borne virus that has now been shown to infect honeybees and their mites, *V. destructor* (Li *et al.* 2014) RNA viruses such as TRSV

have very high mutation rates and have been a significant source of infectious diseases both in man an arthropods of economic importance such as the honeybee (Flenniken 2014). In addition, many honeybee diseases have been identified in non-*Apis* insects such as the recent reports of the ability of DWV to replicate and transmit in Bumble bees (Bombus spp.) (Furst *et al.* 2014). In Kenya, presence of BQCV, DWV and ABPV have reported (Muli *et al.* 2014). The only other East African country where honeybee virus investigation has been conducted is Uganda, where the presence of BQCV has been shown Kajobe *et al.* 2010).

There are three main fungal diseases of honeybees: Chalkbrood, Stonebrood and Nosema diseases of honeybees, with Nosema being the most common of the mycoses. Many species from the genus *Nosema* are parasites of the Apidae family. The three main parasites from this genus are *N. apis*, *N. ceranae* and *N. bombi*.

In honeybees, *Apis mellifera*, Nosema disease is caused by two pathogens; of the genus *Nosema* which belong to the Kingdom Fungi and Phylum Microspora. This phylum is made up of spore-forming unicellular parasites whose main mode of reproduction is through germination of the heat-resistant microspores. Examples of microsporadian parasites that infect honeybees are *Nosema apis* and *N. ceranae*, with the latter being more virulent and has been labelled as one of the diagnostic pathogens of colony collapse disorder in the USA and Europe (Dainat *et al.* 2012a).

Nosema ceranae is mostly prevalent in the warmer climates (Martín-Hernández *et al.* 2007, Tapaszti *et al.* 2009), while *N. apis* is more prevalent in cooler areas (Fries 2010). The infective stage of the pathogen is the non-germinated spore which is

ingested in contaminated pollen or honey (Higes *et al.* 2008b). Transmission of the parasite has been reported to occur horizontally by the worker bees to brood (Smith 2012) and to the queen (Higes *et al.* 2009) during feeding, and from infected drones to the rest of the colony *via* mating with the queen (Traver and Fell 2011a). This last route of transmission is a potential source of spread of *N. ceranae* from colony to colony because drones from different colonies congregate to mate with a virgin queen. *Nosema* can also be transmitted vertically from an infected queen to the eggs laid (Traver and Fell 2012). Heavy infection by *N. apis* can be detected by faecal marks of diarrhoea on the combs and the sides of the hive (Hertig 1923). This contamination on the hive is a great source of infection to honeybees as the spores contained in honeybee faecal waste are viable and capable of staying so for over a year (Bailey 1962). Unlike *N. apis*, *N. ceranae* infection has not been observed to be associated with diarrhoea (Higes *et al.* 2007)

1.1.4 Beekeeping in Kenya

While in Europe and the USA where beekeepers mainly use the Langstroth type of bee hive (Graham *et al.* 1992), beekeepers in Kenya also make use of Log hives (Plate 1), Kenyan Top Bar Hive, KTBH (Platt 1983) and mud hives (Hussein 2000, 2001).

In Kitui County, the log hives are simple and consist of hollowed out logs made from well identified trees known locally as "Itula" in the Kamba dialect. These logs are cut and curved to suitable lengths, coated on the inside by wax to attract bees and placed

atop very tall trees (about 20M tall). Log hives have two main advantages; they require a lower financial investment at the start of the commercial venture and yield high amounts of propolis and wax. The main disadvantage of the log hive is that due to lack of movable combs and location of the bee hive up the tree, hive management and maintenance is very difficult (Wilson 2006). In addition, these types of bee hives have lower colonisation and higher absconding rates (Okwee-Acai *et al.* 2010). It is for this reason, over the past 30 years, bee keepers have been encouraged to adopt the Kenyan Top Bar Hive (KTBH) and the Langstroth hive (Sande *et al.* 2009, Okwee-Acai *et al.* 2010). This is because the KTBH and the Langstroth hives are easier to handle during the normal beehive maintenance practices due to the presence of movable frames. Another advantage is that, using a queen excluder, one is able to separate the brood combs from the honey combs. This single advantage usually leads to increased honey production in terms of honey quality and quantity (Sande *et al.* 2009), and this ensures ability to maintain brood even during honey harvest. This guarantees that there is production of the next generation of honeybees (Graham *et al.* 1992).

This project aims at monitoring the health of wild honeybee colonies in Kitui district, in the different honeybee hive types that are common in the region. The information generated will provide us with insight on the state of health of the wild honeybees, and some recommendations on the most appropriate beekeeping practices for Kenyan apiculturists in Kitui.

Plate 1: A traditional log hive belonging to Mr. Joseph Mbuvi Mulwa, from Nguuni division in Mwingi district, Kenya. (Photo taken by Fiona Nelima Mumoki)

As far back as the 1700s the global honeybee sphere has been experiencing times of decreases in colony populations. The causes to some of these population decreases have been accounted although this is not the case for all (vanEngelsdorp and Meixner 2010). Various factors have been reported to affect the growth and productivity of the global honeybee population. These include nutrition (Brodschneider and Crailsheim 2010), honeybee diseases and pests (Neumann and Carreck 2010), climatic factors (Potts *et al.* 2010), pesticides (Frazier *et al.* 2008) and most recently described; the type of hive used to house honeybees (Ande *et al.* 2008).

There are many pests of the honeybees and bee products, the most common of which are mites, large and small hive beetles, bee lice (*Braula sp* and *Megabraula sp*), frogs, honey badgers (*Mellivora capensis*), wax moths (*Galleria melonella* and *Achroia grisella*) among others. The most common group are the mites with examples of parasitic mites such as *Acarapis woodi* (the honeybee tracheal mite),

Varroa destructor, *Tropilaelaps clareae* and *Euvarroa* spp. Non-parasitic pests include *Melittiphis alvearius* which feeds on stored pollen *and Neocypholaelaps and Afrocypholaelaps* genera which feed on flowers but are phoretic on bees. Other non-parasitic mites include *Forcellina faini*, *Pseudacarapis indoapis, Melichares dentriticus* (Sammataro *et al.* 2000). Of the named pests, *Varroa* has been named as a key cause in the decline of the global honeybee populations both in North America (Cox-Foster *et al.* 2007) and Europe (Dainat *et al.* 2012a).

Red-brown in colour, the mite has two phases: a phoretic phase where it attaches itself to an adult honeybee and a reproductive phase that takes place inside a sealed brood cell (Rosenkranz *et al.* 2010). Therefore, mite is dependent on the honeybee for its whole life cycle. *Varroa* preferentially attach to brood over adults bees and drone brood over worker brood, a phenomenon called Drone Cell Preference, DCP (Fuchs 1990). The reason for this could be that drone cells are larger than the worker cells. In addition, drone brood contains higher quantities of fatty acid esters which are strong attractants for the Varroa mite (Le Conte *et al.* 1989). In this capped cell, the mite also undergoes reproduction giving rise to a four daughter mites and a male. During the phoretic phase, the mite is able to move from bee to bee, colony to colony (during robbing) and apiary to apiary. In this way, pathogens that are vectored by *Varroa* are transmitted between individuals and colonies. The Varroa mite has been implicated in transmission and replication of various honeybee pathogens such as DWV (Bowen-Walker *et al.* 1999), ABPV (Martin 2001, Tentcheva *et al.* 2004),

SBV (Shen *et al.* 2005a), Varroa destructor virus 1 (VDV 1), IAPV (Di Prisco *et al.* 2011) and KBV (Shen *et al.* 2005b).

There are different types of honeybee hives in use in Africa today. These include traditional hives such as bucket hives, basket hives, log hives, bark hives, mud hives and pot hives (Ande *et al.* 2008). Modern hives in common use in Africa include the Langstroth hive and the Kenyan Top Bar Hive, KTBH (Hussein 2000, 2001, Okwee-Acai *et al.* 2010). Majority of the apiculturists in Kenya keep bees in traditional log hives as compared to the modern frame hives such as KTBH and Langstroth hives. The different types of hives differ in the productivity of wax, honey and propolis (Kebede and Lemma 2007, Sande *et al.* 2009, Yirga and Ftwi 2010) and also create varying microclimates in the hives leading to varying degrees in honeybee strategies such as swarming, absconding and colonisation (Ande *et al.* 2008, Okwee-Acai *et al.* 2010). Finally, these different types of hives have been noted to attract pests of different types, in different rates and in different proportions (Ande *et al.* 2008).

The aim of this project is to assess the health of honeybees in three different types of hives popular in Kenya and it is the first study to assess the effect that the type of beehive may have on the health of the honeybee.

1.1.5 Honey bee nutrition

In Africa, native honey bees are reportedly responsible for pollinating 70% of all agricultural crops (Allsopp *et al.* 2008), many of which are essential commercial

crops. These plants produce flowers with nectar; which is a source of carbohydrates for the honeybees, but also oils and pollen, with the latter serving as the principal source of protein for honeybee larvae (Winston 1991).While a lot of attention has focused on the epidemiological causes of the global honeybee population decline, many environmental factors with a direct bearing on honeybee nutrition are thought to be major contributors. These include habitat destruction and fragmentation, pesticide use (Desneux *et al.* 2007) and other forms of landscape enhancement methods such as mono-cropping which reduce floral diversity necessary for honeybee proper nutrition (Richards 2001, Decourtye *et al.* 2010).

Wind and bees are the world's most important pollinating agents, with the honeybee, *Apis mellifera* L., being the most important generalist pollinator (Potts *et al.* 2010). In Africa, native honey bees are reportedly responsible for pollinating 70% of all agricultural crops, some of which are essential commercial crops. Honeybee pollination is valued at $14 billion in the United States (Morse and Calderone 2000) and USD 212 billion globally (Gallai *et al.* 2009). Pollination is beneficial for some plants such as the sunflower, while very essential for others such as the orchid, watermelon and apple. These plants produce flowers with nectar and pollen. Nectar is the principle source of carbohydrates for the honeybee colony while pollen is their principle source of protein and lipids (Brodschneider and Crailsheim 2010).

Nectar is collected by honeybees and stored in the colony combs and capped. This is then converted into honey. Honey is the storage source of colony carbohydrates that supports the honeybee colony during periods of dearth. Pollen is the main source of

protein and lipids for both the honeybee adults and brood (Hrassnigg and Crailsheim 2005) and shortage of this key commodity seems to have greater consequences for colony health and survival as compared to lack of nectar (Schulz *et al.* 1998, Schmickl and Crailsheim 2001). Pollen from the flower is collected by forager bees and stored in pollen baskets on the legs of the honeybees. These are then taken and deposited in comb cells in the bee hives. The stored pollen is then mixed with regurgitated nectar, honey and honeybee glandular secretions (containing microorganisms such as *Lactobacillus sp.*) to give rise to a slightly fermented mixture known as beebread. Due to the fermentation process and processing due to the mandibular secretions, beebread possesses a higher nutritional composition compared to freshly collected pollen grains. This also includes addition of vitamins by the microorganisms (Herbert and Shimanuki 1978).

Figure 3 shows just how dependent the adult honeybee is on the colony stores of pollen grains (beebread). Adult nutrition on the other hand determines the level with which adult bees invest on the quality of larval development (Schmickl *et al.* 2003) and how many larvae are reared to pupae. In a colony, the number of larvae making it to the pupal stage can be reduced through cannibalism, a technique that also enables the adult bees to receive their protein supply during periods of very low protein availability (Webster *et al.* 1987, Schmickl and Crailsheim 2001). Larval quality and quantity greatly impacts the next generation of adult bees (Archer *et al.* 2014), and herein lays the link between honeybee colony health and nutrition.

The current continuous decline in honeybee populations around the world has prompted extensive research into the world of the honeybee in terms of its health and ecology. Recent studies have focused on the role of pollen grains in transmission of various honeybee pathogen including viral (Singh *et al.* 2010), microsporadian (Higes *et al.* 2008a) and fungal (Flores *et al.* 2005) pathogens.

Examples of plants that form the bulk of the pollen diet of East African bees include *Acacia, Zea mays, Leonotis, Baleria, Agave, Commelina* and *Sansevieria* (Villanueva and Roubik 2004).

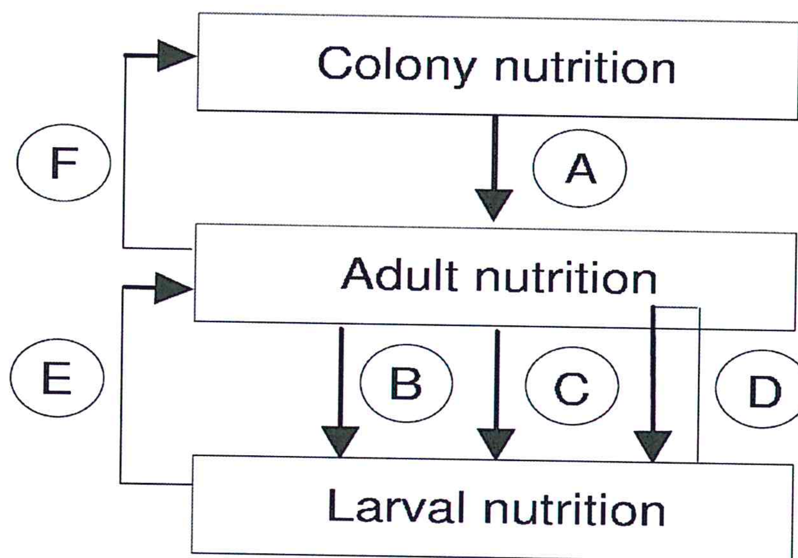

Figure 3: schematic representation of the three levels of honey bee nutrition, dependencies, and possible effects of protein malnutrition. A: dependency of adults on colony food stores; B: investment in larval quality; C: regulation of larval number; D: cannibalism; E: impact of larval nutrition on next adult generation; F: impact of adults on colony nutrition. Adapted from (Brodschneider and Crailsheim 2010).

1.2 STATEMENT OF THE PROBLEM

Europe and North America have suffered significant losses of honeybee colonies to colony collapse disorder. Presently, researchers have developed various predictors for CCD and among them are viral pathogens, the microsporadian parasite *Nosema* and the destructive parasitic mite *Varroa*. Although CCD has not yet been reported in Africa, several of the aforementioned predictors have already been identified in various parts of the continent, including the East African countries, South Africa and Northern Africa. This discordance in the occurrence of CCD despite the presence of its predictors could in part be due to the significant variation in beekeeping practices around the world, such as the types of honeybee hives popular in different parts of the globe and differences in honeybee nutrition. There is a lot of literature outlining the pathogens that affect the honeybees in the developed world, but very limited information on the pathogens affecting honeybees of Africa and how beekeeping practices unique to this continent affects the health of African honeybees. This project aims at monitoring the presence of CCD predictors in the honeybees of Kitui County, while taking into account the different hive types popular in Kenya, and the floral resources available in this semiarid ecosystem. The data generated will enable the honeybee stakeholders here in Kenya to adjust their beekeeping practices for healthier and more productive bee colonies.

1.3 JUSTIFICATION

Kitui County is a semi-arid agro-ecological zone in Kenya and it is the country's second largest honey producer, second to Baringo County in the Rift Valley (Raina 2013). Unlike the bee farmers in Baringo County where arable farming is incorporated into beekeeping, most apiculturists in Kitui County depend on beekeeping as their sole income earner and this is done mainly by small-scale farmers with immediate family members as the main workforce. These households keep bees in hundreds of (mostly) traditional log hives, and rely on traditional beekeeping skills passed on from generation to generation. Such households would lose a very important component of their income, should their honeybee colonies disappear due to CCD. This project aims at using tools in molecular genetics to monitor the health of feral (wild) honeybee colonies in Kitui County, taking into account the traditional beekeeping methods practiced by apiculturists in this area and comparing them to modern beekeeping practices in use in the developed world, where Colony Collapse Disorder has been widely reported. The information generated will provide us with insight on the state of health of the wild honeybees, and some recommendations on the most appropriate beekeeping practices for Kenyan apiculturists in Kitui.

1.4 NULL HYPOTHESIS

There is no relationship between factors that affect colony health and ecology, in the honeybees of Kitui.

1.5 OBJECTIVES

1.5.1 General Objective

To contribute to the health and productivity of pollinators in Kenya by investigating the effect that different types of hives may have on the health and ecology of honeybees

1.5.2 Specific Objectives

1. To identify the subspecies of honeybees in Kitui County using morphological and molecular markers
2. To determine the presence of disease-causing microorganisms in the honeybees of Kitui county
3. To determine the relationship between type of beehive and honeybee health and ecology by comparatively investigating various indicators of colony health and growth
4. To identify the main food sources for honeybees in Kitui through pollen identification

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 STUDY AREA

The work was conducted in South Eastern Kenya University (SEKU) in Kitui County located in the eastern part of Kenya, Yatta Division, Kwa-vonza Location. Kitui County is a semi-arid region that receives an annual rainfall of about 500 mm-1050 mm of rainfall. The region experiences high temperatures throughout the year at a range of 16°C-32°C and has an altitude of 400M-1800M above sea level. Three apiaries separated by an average distance of 2.34 KM were established in SEKU (Figure 4).

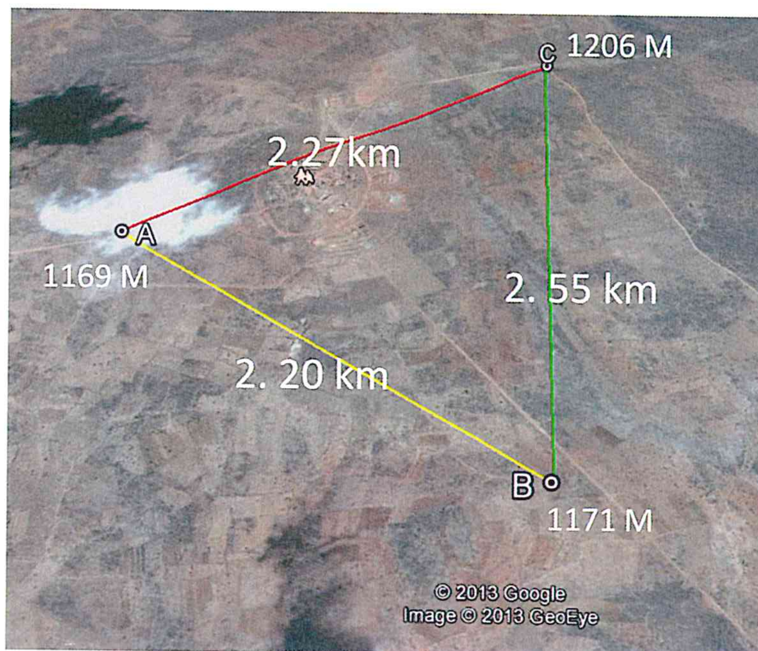

Figure 4: A map of SEKU showing the locations of the three apiaries A, B and C.

2.2 EXPERIMENTAL DESIGN

Three apiaries were set up in SEKU. Each apiary had three types of hives-Log hives, KTBH and Langstroth hives. Each apiary had seven of each type of hive and each hive is recognised as an individual colony.

2.3 SAMPLING STRATEGY

All colonies (hives with bees) were sampled. From each colony, 30 foragers were collected and put into individual cryotubes containing 95% ethanol. Ten of the sampled bees were used for virus analysis, 10 for sub-species identification and 10 samples for *Nosema* investigation. These sample size per colony have been widely used in virus and *Nosema* investigations using both conventional and Real time PCR (Chen *et al.* 2006, Klee *et al.* 2007, Chen *et al.* 2008).

2.3.1 Inclusion and Exclusion Criteria

Queens, brood, drones, injured or clearly sick bees were excluded from the study, as were nurse bees and guard bees. Only forager bees were collected for analysis.

2.4 MORPHOLOGICAL AND MOLECULAR IDENTIFICATION

2.4.1 Morphological identification of honeybee subspecies

The abdominal banding pattern as used by (Raina and Kimbu 2005) was used in the morphological identification of the honeybees of Kitui where the number of yellow bands on the abdomen of the honeybee were counted for a group of 440 honeybees

collected in June 2013 from three types of hives and 43 colonies in three apiaries in SEKU, Kitui County.

2.4.2 Molecular Identification of Honeybee Subspecies

The middle left leg of the honeybees was removed and inserted into a 1.5mL eppendorf™ tube. Total DNA isolation was then performed using Phenol Chloroform Isoamyl Alcohol (PCIA) 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA, from Sigma-Aldrich® (Taufkirchen, Germany). Honeybee tissue homogenisation was carried out using Phosphate Buffered Saline (PBS) after which an equal amount of PCIA was added to allow for the phase separation of the cellular constituents. DNA precipitation was done using ice-cold absolute ethanol. The resultant DNA was washed using 70% ethanol and resuspended in 40 μL of nuclease free water. Spectrophotometric quantification of the DNA was then carried out using the BioSpec-mini DNA/RNA/Protein Analyzer from Shimadzu, Japan.

The total DNA was used in PCR amplification using the following regimen in a 20μL reaction: 1X Phusion polymerase buffer, 0.5mM dNTPs, 0.5 μM primers, 1.25mM $MgCl_2$, 1 μL of template cDNA, 0.5U Phusion High Fidelity DNA polymerase and water to top up the volume. The thermocycler program consisted of an initial denaturation step at 98°C for 30 seconds, 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 20 seconds. The cyclic steps were followed by a final extension at 72°C for 10 minutes. The primary locus for subspecies identification was the partial Cytochrome C Oxidase Subunit I (COI) gene fragment, although other loci such as the intron of

Elongation Factor 1 α (EF 1α) and the tRNA-Isoleucine-NADH dehydrogenase sub-unit 2 (tRNA-ND2) gene fragments were also amplified. The primers for use in amplification of these loci are outlined in Table 1.

Table 1: Primer names and sequences for honeybee sub-species identification

Primer	Forward sequence	Reverse sequence	Size (bp)	References
COX	GGTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAAATCA	658	(Folmer *et al.* 1994)
EF 1α	AAGATCGGTGGTATCGGTAC	TGGTGAGCGCTGCTGGAG	470 - 500	(Arias and Sheppard 2005)
ND2	TGATAAAAGAAATATTTT	TGAAACTATTATATAAATTG	1000	(Arias and Sheppard 2005)

Amplicons were resolved by electrophoresis on a 1.5% agarose gel with 0.5μg/ml of ethidium bromide and analysed under UV light in a UV transilluminator. Target DNA bands were then excised from the gel using sterile blades. The excised DNA bands were then purified from the agarose gel by use of the Quick Clean II Gel Extraction Kit from GenScript Inc., (Piscataway, NJ, USA) following the manufacturer's instructions, and sequenced using the dideoxy sequencing technique by use of an ABI3730XL DNA analyser from Applied Biosystems. This was carried out in Macrogen Inc. in Seoul, South Korea.

2.4.3 Sequence editing and phylogenetic analysis

Editing was done using the sequence editing software BioEdit version 7.0.5.3 (Hall 1999) where forward and reverse sequences were aligned in a pairwise alignment to create a consensus sequence. Base correction was done using the base calling technique, by comparing the called base in the forward electropherogram against that in the same position in the reverse electropherogram. Primer sequences were then removed from the consensus, resulting in a gene fragment containing only the sequence of interest.

Edited sequences were compared to those in the GenBank DNA repository using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990) whereby the sequences that showed highest similarity were downloaded and combined with the query sequences in analysis. Open reading frames were detected using the Translate tool in the European Molecular Biology Open Suit Software (EMBOSS). Multiple sequence alignment (MSA) and phylogenetic trees were constructed using MUSCLE software version 3.8.31 (Edgar 2004). Bootstrapping was done 1000 times. Visualisation of the resultant Nexus tree was done using FigTree software version 1.3.1 (Rambaut 2007). Heat maps were generated using R software (R Core Team 2014). The edited sequences were later deposited into the GenBank repository and currently have the accession numbers: KF833377-KF833397 for the COI sequences, KF824771-KF824791 for the ILE-ND2 genes.

2.5 DIAGNOSIS OF HONEYBEE VIRUSES AND *NOSEMA*

2.5.1 Detection of honeybee viruses

2.5.1.1 RNA isolation, cDNA synthesis and qPCR

Each honeybee abdomen was dissected using sterile pairs of forceps, and five abdomens per colony were pooled for nucleic acid extraction. Total RNA isolation was performed using the acid guanidium thiocyanate-Phenol-Chloroform-Isoamyl alcohol technique (Chomczynski and Sacchi 1987) and spectrophotometric readings of the resultant RNA recorded. Complementary DNA synthesis was performed using random hexamer primers from the RevertAid™ First Strand cDNA synthesis Kit (ThermoFischer Scientific, Waltham, Massachusetts, USA). The resultant cDNA was utilized as template in PCR amplification using various virus specific primers (Table 2). Virus identification was done using virus-specific primers as shown in Table 2 using the following regimen in a 20 μL reaction 1X Phusion polymerase buffer, 0.5mM dNTPs, 0.5μM virus specific primers, 1.25mM $MgCl_2$, 1 μL of template cDNA, 0.5U Phusion High Fidelity DNA polymerase and water to top up the volume. The thermocycler program consisted of an initial denaturation step at 98°C for 30 seconds, 35 cycles of denaturation at 98 °C for 10 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 20 seconds. The cyclic steps were be followed by a final extension at 72°C for 10 minutes.

Amplicons were loaded on a 2% agarose gel with 0.5µg/ml for 1.5 hours at 80V. The results were viewed in a Kodak Gel Logic Transilluminator. The positive samples

were re-amplified using the DWV primers. These were separated on a 1.5% agarose gel. Purified and sent for sequencing in Macrogen Inc., South Korea.

Pathogen diagnosis was also confirmed using qPCR using SYBR Green (Ponchel *et al.* 2003) chemistry. Amplicons were loaded on a 2% agarose gel with 0.5µg/ml for 1.5 hours at 80V. The results were viewed in a Kodak Gel Logic Transilluminator. The positive samples were re-amplified using the DWV primers. These were separated on a 1.5% agarose gel. Purified and sent for sequencing in Macrogen Inc., South Korea.

2.5.2 Detection of *Nosema* microsporadia

2.5.2.1 DNA isolation and qPCR

Honeybee abdomens were dissected using sterile pairs of forceps, where ten abdomens per colony were pooled for nucleic acid isolation. Total DNA was isolated from the pools of ten bees per colony, using the CTAB technique (Doyle and Doyle 1987). 10 µL of the CTAB isolation buffer (100 mM Tris HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 2% (w/v) cetyltrimethylammonium bromide; 0.2% (v/v) 2-mercaptoethanol) was added to the pooled abdomens, and using a micropestle, crushed into a slurry. After addition of Proteinase K, the mixture was then be incubated overnight at 55 °C. DNA isolation proceeded using the Phenol: Chloroform: Isoamyl alcohol (25:24:1) and the resultant DNA resuspended in 50 µL of nuclease-free water.

Custom made *Taq*Man MGB probes (Applied Biosystems, Foster City, CA) were used to detect the presence of *Nosema apis* and *N. cerenae* and all reactions done in duplicate. The PCR thermo profile consisted of 40 cycles at 95°C for 10 minutes, 95°C for 15 seconds and 60 °C for 1 minute. Non-template negative controls were included to ensure primer/probe specificity and lack of contamination. The positive control samples were used to aid in the identification.

2.5.3 Visualisation, gel purification and sequencing of amplicons for disease diagnosis

Amplicons were resolved by electrophoresis on a 2% agarose gel with 0.5μg/ml of ethidium bromide and analysed under UV light in a UV transilluminator. Target DNA bands were excised from the gel using sterile blades. DNA was then purified from the agarose gel by use of the Quick Clean II Gel Extraction Kit from GenScript Inc., (Piscataway, NJ, USA) following the manufacturer's instructions. The resultant DNA fragments were then sent for sequencing to Macrogen Inc. in Seoul, South Korea. Sequencing was done through the dideoxy sequencing technique by use of an ABI3730XL DNA analyser from Applied Biosystems.

2.6 EFFECT OF TYPE OF BEEHIVE ON HONEYBEE HEALTH AND ECOLOGY

2.6.1 Assessing the effect that the type of honeybee hive may have on colony health

Samples of nurse bees were collected from honeybee combs with evidence of brood on them, mostly found at the centre of the hives. For log hives, samples were collected from clusters of honeybees found at either openings of the traditional hive by scooping honeybees using the standard sugar-shake half-cup and putting the bees into a Mason jar (Macedo *et al.* 2002). Care was taken not to shake the combs that contained the queen bee. For the KTB and Langstroth hives, combs were shaken into a large basin, the bees gathered and collected using the standard sugar-shake cup into a mason jar for shaking. The reddish-brown (Rosenkranz *et al.* 2010) coloured mites were then shaken against a white background and counted as outlined by (Macedo *et al.* 2002). Monitoring of *Varroa* loads were done monthly for a year from December 2012 to December 2013.

2.6.2 Effect of type of bee hive on colony weight as an indicator of colony growth

Honeybee colonies were weighed to determine the growth of honeybee colonies. Growth here was indicated by increase in weight of honeybee colonies either due to increase in the numbers of honeybees (adults and brood) or increase in hive products

such as wax or honey. Monitoring of colony weights was done monthly for a year from December 2012 to December 2013.

2.6.3 Number of frames occupied as an indicator of colony growth

All frame hives were opened and the number of frames colonised i.e., the number of frames in which honeybees had constructed combs, were counted. Counting of frames was only possible for the Frame hives (Langstroth and KTB hives) as and not for the Log hives as the latter do not contain movable frames. This data was collected from December 2012 to February 2014.

2.7 IDENTIFICATION OF THE KEY POLLEN SOURCES FOR HONEYBEES

2.7.1 Sample collection and preservation

Samples of honeybee foragers were collected using a bee vac machine as was done for foragers for the sub-species identification and disease diagnosis experiments. A total of ten (10) honeybee foragers were collected from each colony. Samples were collected from all three types of hives that were colonised, and in all the three apiaries. The bees were stored in 95% ethanol and transported back to the lab in Duduville campus, *icipe*, Nairobi.

2.7.2 Pollen extraction and identification

Samples of pollen for identification were taken from the legs of the bees using sterile blades. The pollen was then inserted into 1.5 mL eppendorf tubes into which 700 uL

of 10% sodium hydroxide was added. The tubes were then vortexed to mix and centrifuged at 15 300 rcf for five minutes. After this, the supernatant was discarded leaving the wet pollen pellet at the bottom of the tube. To this, 700 uL of acetic acid was added and the tubes vortexed and centrifuged at 15 300 rcf for 5 minutes and the supernatant was then discarded. Pollen extraction was then carried out using the Erdtman acetolysis technique (Erdtman 1960) where 700 uL of acetolysis mixture (9:1 acetic anhydride: Sulphiric acid) added. The tubes were incubated for 20 minutes in a boiling water bath, then span as outlined before. Again, the supernatant was discarded and the pellets washed using 700 uL of acetic anhydride. The wash step was repeated using distilled water after which the pellets were resuspended in 50 uL of neat glycerol. The extracted pollen grains were then mounted on microscope slides and covered with a cover slip.

Pollen identification was done by cross-referencing against reference samples at the Pollen Repository found at the Palynology laboratory of the National Museums of Kenya. Characteristics such as pollen size, shape, aperture number, aperture type, surface sculpting and exine thickness were studied for classification.

This information was recorded in Microsoft Excel Spreadsheets for data analysis.

Table 2: Primer sequences for virus and *Nosema* diagnostics, showing also the expected product size

Primer	Fwd sequence	Reverse sequence	Size (bp)	Reference
BQCV	TGGTCAGCTCCCACTACCTTAAAC	GCAACAAGAAGAAACGTAAACCAC	700	(Benjeddou *et al.* 2001)
IAPV	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG	586	(Di Prisco *et al.* 2011)
DWV	ATCAGCGCTTAGTGGAGGAA	TCGACAATTTTCGGACATCA	701	(Chen *et al.* 2004)
CBPV	AGTTGTCATGGTTAACAGGATACGAG	TCTAATCTTAGCACGAAAGCCGAG	455	(Ribière *et al.* 2002)
KBV	GATGAACGTCGACCTATTGA	TGTGGGTTGGCTATGAGTCA	417	(Stoltz *et al.* 1995)
SBV	GCTGAGGTAGGATCTTTGCGT	TCATCATCTTCACCATCCGA	824	(Chen *et al.* 2004)
ABPV	TTATGTGTCCAGAGACTGTATCCA	GCTCCTATTGCTCGGTTTTTCGGT	900	(Benjeddou *et al.* 2001)
N. apis	GGAACACCTTTTCTCCTACAAGCAA	CCAAAAACTCCCAAGAGAAAAACAAAAC	92	(Traver and Fell 2011b)
***N. apis* Probe**	ACGCCAGCATACCTTT			
N. cerana	CTTGCCAAACCCTCCC	ACCTGATCCAACGCAAATGCTA	103	(Traver and Fell 2011b)
***N. cerana* Probe**	GGTTGGGAGAAGCCGTTACC			

CHAPTER THREE

3.0 RESULTS

3.1 IDENTIFICATION OF HONEYBEE SUBSPECIES IN KITUI

3.1.1 Morphological identification of honeybee sub-species by use of abdominal banding patterns

Variation was seen in the abdominal banding patterns of the honeybee samples. The apiary data showed that majority of the samples in all the three apiaries had two or three yellow bands as shown on Figures 5 and 6. The proportion in colour banding patterns was examined across all three apiaries. All apiaries have all five possibilities (no yellow band, one, two three and four bands). Pearson's Chi-square test revealed that the five proportions (possibilities) were significantly different when the proportions were compared against the three apiaries (χ^2= 18.2705, df = 8, α=0.05, P = 0.01929)

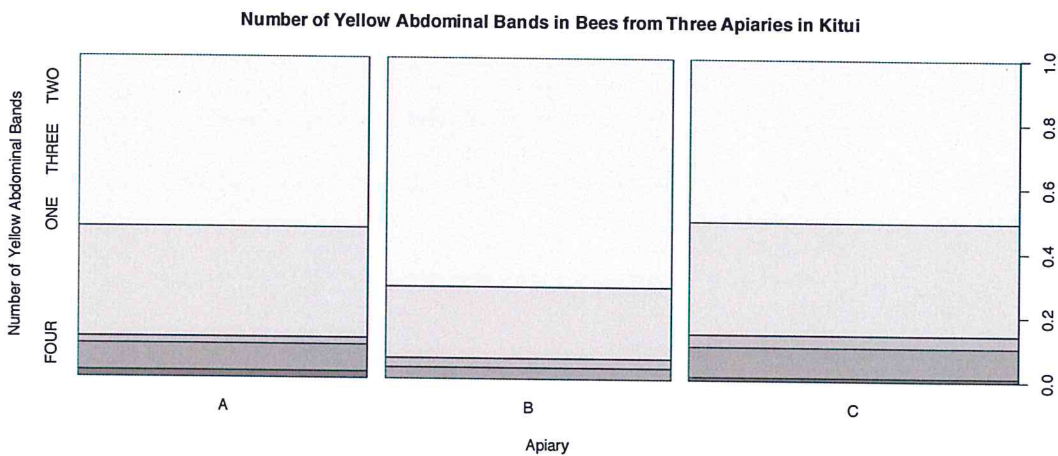

Figure 5: Proportions of diversity in abdominal banding patterns in the 440 honeybee samples collected from 43 colonies in June 2013

The Chi-square goodness-of-fit test revealed that the observed proportions of abdominal bands significantly deviated from expected proportions (1/5) which would signify equal proportions of all abdominal bands in the three apiaries (χ^2= 519.8636, df = 4, $P < 0.00001$).

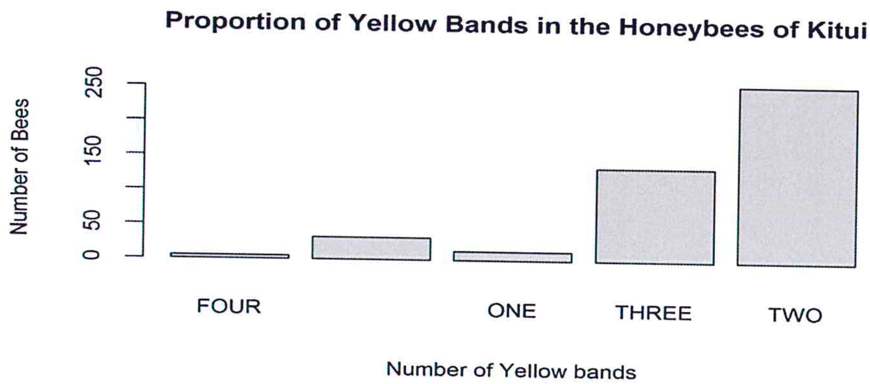

Figure 6: Proportions of yellow bands in the honeybees of Kitui County.

Majority of the bees examined had two yellow bands which may characterise *Apis mellifera litorea* or a Litorea-Scutellata hybrid as shown on Figure 6 below (χ^2= 143.59, df = 2, P < 0.0001)

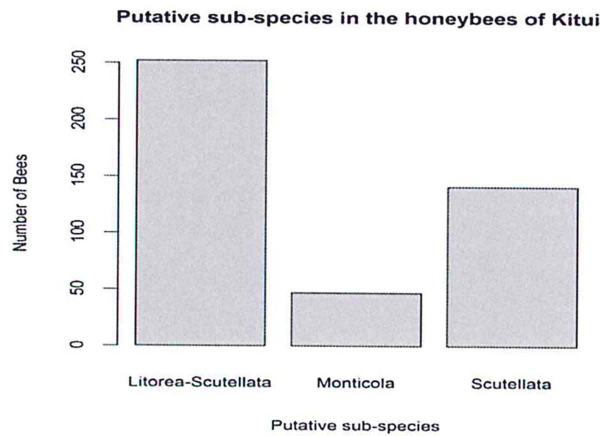

Figure 7: Subspecies identified based on abdominal banding patterns. The chart shows that majority of the honeybees were hybrids of *Apis mellifera litorea* and *A. m. scutellata*. This trend was consistent in all the three apiaries

3.1.2 Results on Molecular Identification of Sub-species

3.1.2.1 Cytochrome C oxidase Sub-Unit One (COI)

Amplification of the partial COI gene was carried out using the Folmer primers (Folmer *et al*. 1994) as described above. The amplicons were run on a 1.5 % agarose gel containing 0.5µg/mL of ethidium bromide, for one and half hours. The COI gene was amplified in all the samples, and showed a band size of 700bp (Plate 2). The amplicons were excised from the gel, purified and sequenced.

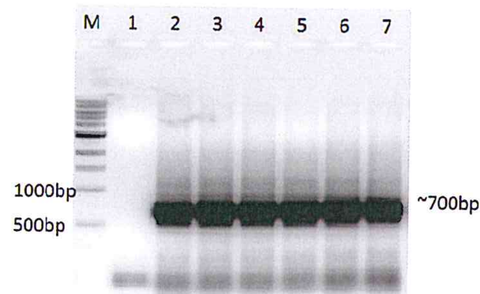

Plate 2: A representative gel image showing positive amplification of COI gene using the Folmer primers; M= 1 kb DNA marker from New England Biolabs, Lane 1= Non-template negative control, Lane 2-7= amplification of COI gene from the honeybees of Kitui

3.1.2.2 High Resolution Melt (HRM) Analysis

The results of the HRM analysis were displayed on a graph as shown on Plate 3. This technique was explored due to its potential as a fast, effective and inexpensive diagnostic tool for molecular diagnosis. However, the results as depicted on Plate 3 show that the melting curves from the different honeybee sub-species form very similar patterns, and as such, this locus cannot be used for sub-species identification using HRM.

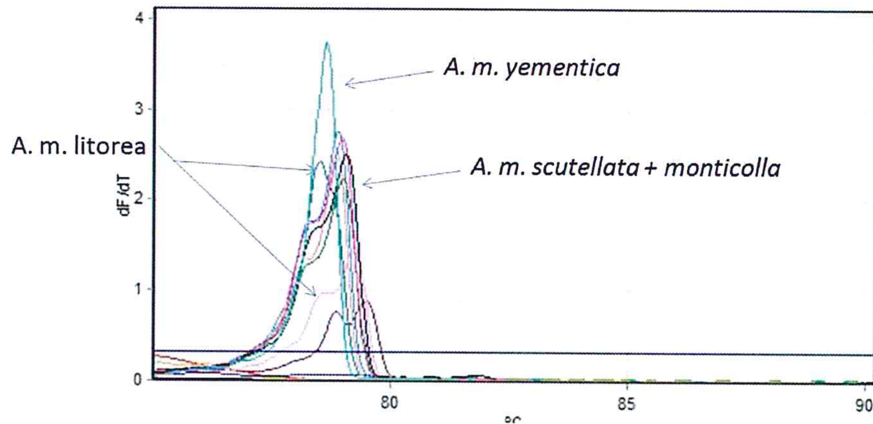

Plate 3: HRM curves of positive control samples used to type the species to which the honeybees of Kitui belong. COI primers were used. The curves of the known subspecies are highly similar to each other and hence unsuitable for sub-species differentiation.

3.1.3 tRNA^{ILE}-ND2 gene fragment amplification

The second gene explored for use in identification of honeybee sub-species was the tRNA^{ILE}-ND2 gene fragment. The amplicons were resolved on a 2% agarose gel. Plate 4 shows the successful amplification of the tRNAILE-ND2 gene which gave a fragment size of 700 bp.

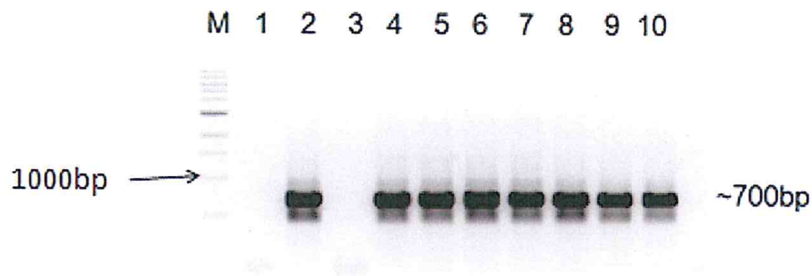

Plate 4: A representative gel image showing amplification of tRNA^{ILE}-ND2 gene fragment using the ILE and L1 primers. Amplicons of about 700 bp were observed.

3.1.4 Intron of elongation Factor 1α

The final locus explored for its potential in resolving the subspecies of honeybees in Kitui County was the intron of Elongation 1 α. Successful amplification resulted in amplicons about 300 bp in size, which were separated electrophoresis on a 2% agarose gel. These amplicons were purified and sequenced as done for the aforementioned genes.

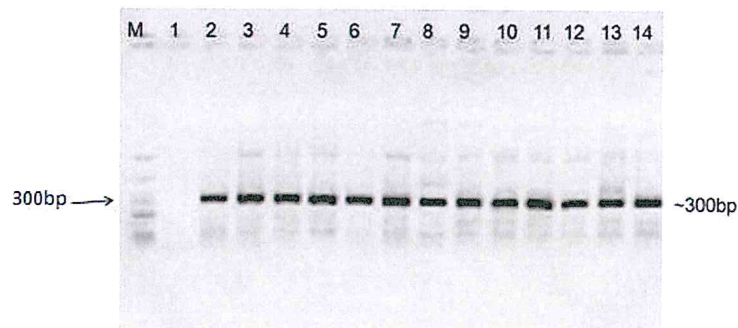

Plate 5: A gel image showing amplification of the intron of the Elongation factor 1α on a 2.5% agarose gel, where M=GeneRuler™Low Range DNA ladder, Lane 1= Non-Template Control, Lane 2-14= amplification of EF 1α gene fragment from honeybees in Kitui

3.1.5 Resolution on Heat map and dendrograms

The dendrogram created using the partial COI locus (Figure 7) resolved the honeybees of Kitui into two main groups. The first group clustered with other identified Scutellata bees and the highland bees sampled from high altitude areas including, Nadasa (2549M), Ngeta (2627M) Mt. Elgon 3000M above sea level. The second group clustered closer to the honeybees of the coastal region including those sampled from the South coast Kaya Mukawa region (68M) and Ocean side (15M)

above sea level. The tree generated using the tRNA^{ILE}-ND2 gene fragment (Figure 8) also created two main clusters for the honeybees of Kitui. The first cluster contains majority of the honeybee samples from Kitui and a reference sequence AY712682 that was identified as *A. m. scutellata* sampled from Gatimbi, in Kenya (Arias and Sheppard 2006). The second cluster formed around sequences generated from identified *A. m. monticola, A. m. scutellata* and the Africanised honeybee.

3.1.5.1 Dendrogram created using the partial COI gene

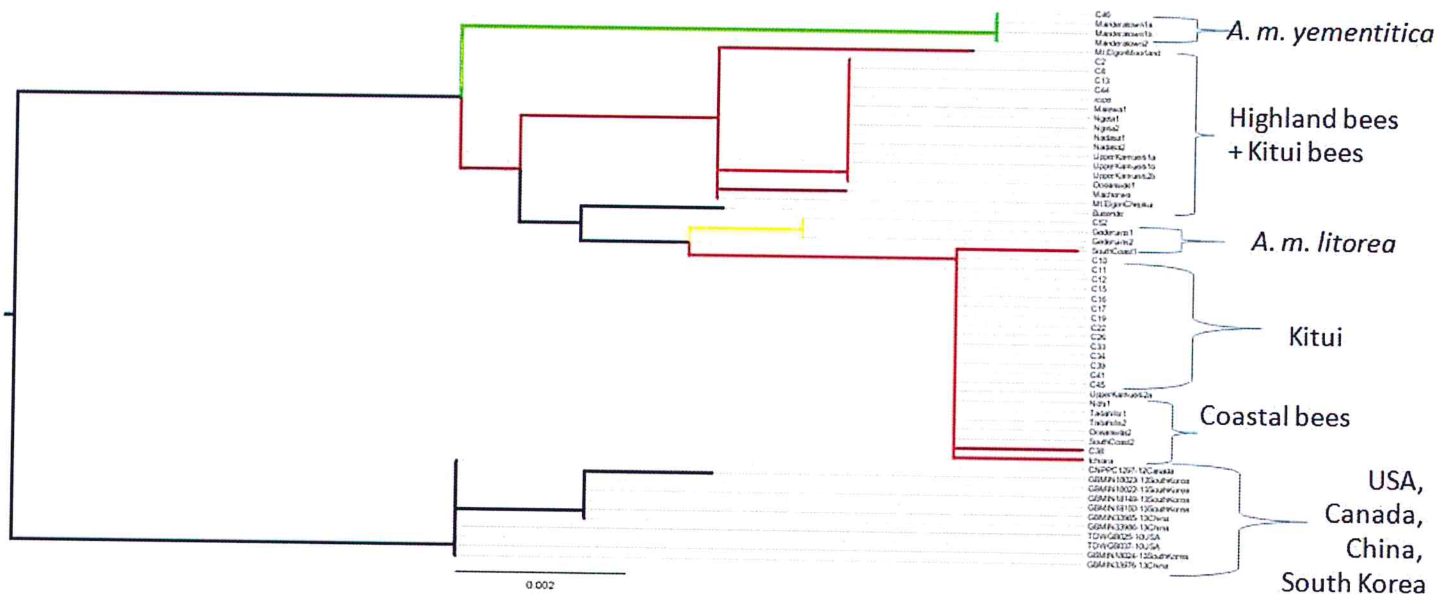

Figure 8: A dendrogram created using MUSCLE software Version 3.8.31 (Edgar 2004) using the default parameters. The tree shows that the honeybees of Kitui clustered into two main groups, one clustering with the highland honeybees thought to compose of *A. m. monticola* and its hybrids with *A. m. scutellata*. The second group clustered with the coastal honeybees known to consist of *A. m. litorea* and its hybrids.

3.1.5.2 Heat-map and dendrogram created using the tRNA ^{ILE}-ND2 gene fragment

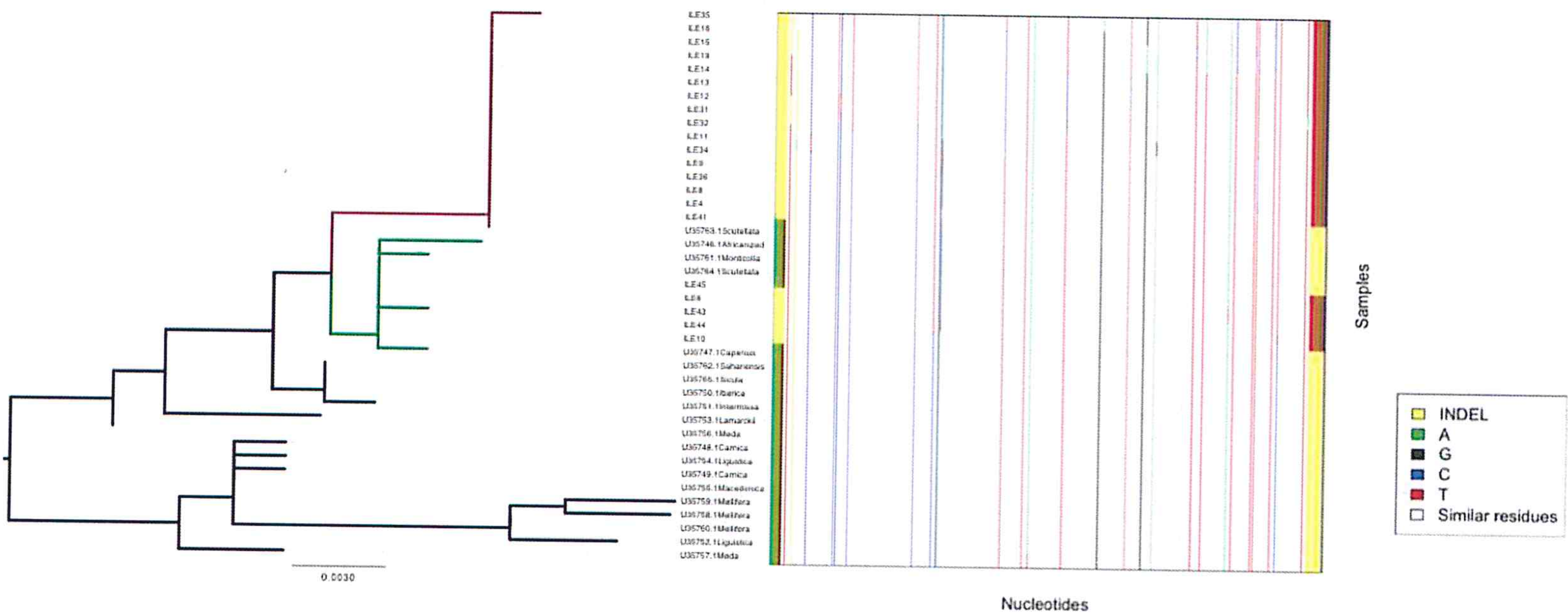

Figure 9: The above diagram consists of a dendrogram and its corresponding Heat map. multiple sequence alignment (MSA) and resultant dendrogram was created using MUSCLE software version 3.8.31 (Edgar 2004) using the default software settings The resultant tree was visualised using FigTree Software Version 1.3.1 (Rambaut 2007). .

3.2 IDENTIFICATION OF VIRAL AND MICROSPORADIAN PATHOGENS

3.2.1 Detection of virus in the honeybees of Kitui

The qPCR technique was utilised in the diagnosis of various viruses in honeybees and their parasitic mite *Varroa destructor*. While seven honeybees were investigated, the three main viruses focused on were DWV, BQCV and IAPV. Amplification of positive controls for these viruses can be seen on Plate 6. The other 4 viruses (SBV, KBV, ABPV and CBPV) were investigated although positive controls for these viruses were not available.

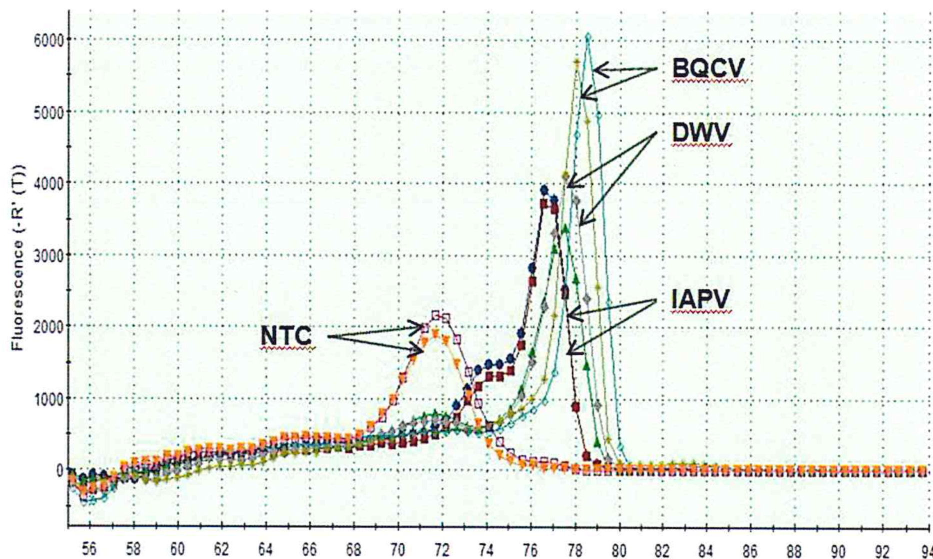

Plate 6: Gel image showing amplification of virus positive control samples the dissociation temperature of the amplicons in relations to the non-template control.

Of the seven viruses investigated, only deformed wing virus (DWV) was detected in the honeybees of Kitui. This result was confirmed using convectional PCR and real time PCR. The amplification plots for the real time PCR are as shown on Plate 7.

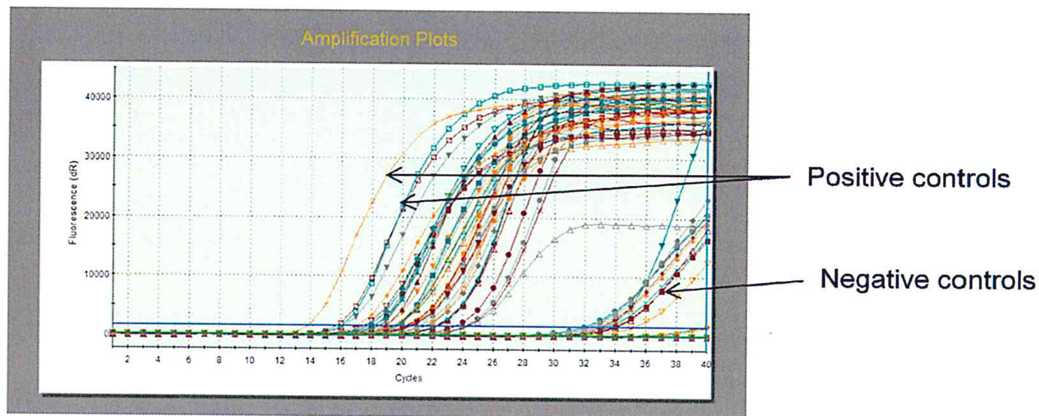

Plate 7: qPCR amplification plot for Deformed Wing Virus (DWV). Thirty nine colonies were analysed for the disease diagnosis and 14 of these were positive for DWV.

The identity of the amplification products were determined by examining the dissociation curves following the qPCR experiment. The curve of the amplicons was compared to that of the positive control samples to determine the dissociation of the target gene from that of non-specific amplification (Plate 8). From the dissociation curves of the non-template negative control samples, the dissociation curves of the primer dimmers and other non-specific amplicons was also determined.

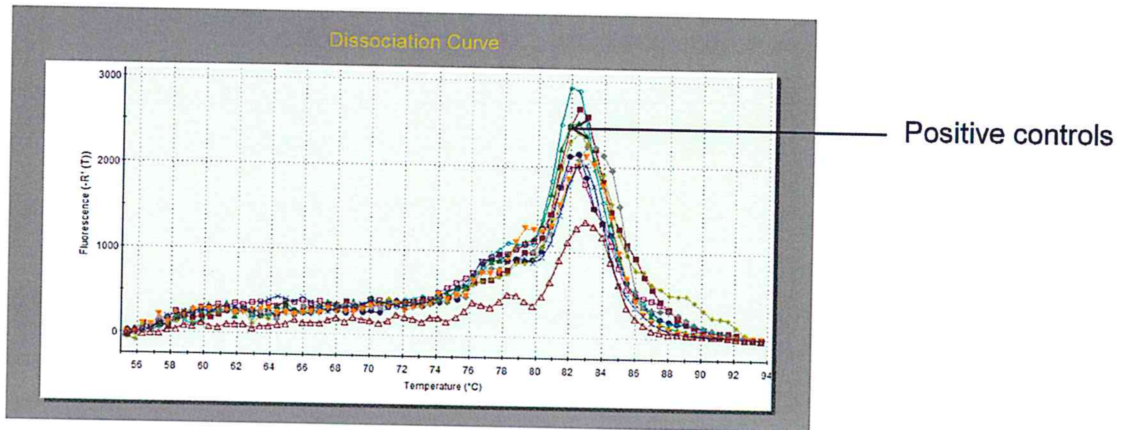

Plate 8: qPCR dissociation curve showing dissociation of positively amplified DWV in honeybees collected from three apiaries in Kitui, Kenya.

The products of amplification using both the conventional PCR and real time PCR techniques were separated on a 2% agarose gel with ethidium bromide. The result of the resolution was viewed using a uv transilluminator and captured using an attached Kodak camera (Plate 9).

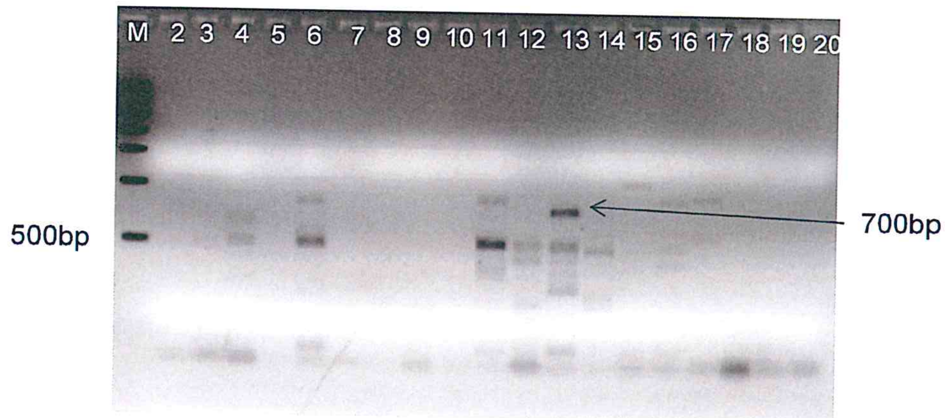

Plate 9: A representative gel image showing amplification of DWV from the honeybee samples of Kitui. M= 1kb DNA ladder from New England Biolabs (NEB). Lanes 2-20 represent samples amplified. The target gene is 700 bp.

Virus investigation was also carried out in *Varroa*, a known vector of the honeybee deformed wing virus, DWV. The results show that DWV was not detected in any of the pools of mites examined either by use of convectional PCR or by real time PCR.

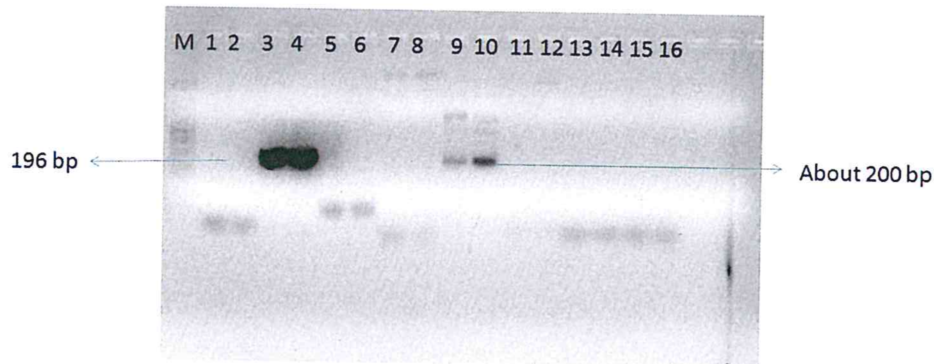

Plate 10: A representative gel image showing virus investigation in *Varroa destructor;* M= Low Range DNA ladder (Fermentas), 1,2= Negative control, 3,4= *V. destructor* Housekeeping genes, 5,6= ABPV, 7,8=BQCV, 9,10=CBPV, 11,12=DWV, 13,14=IAPV, 17,18=SBV virus investigation. There was amplification of the housekeeping gene but not for any of the viruses investigated.

A single-sample proportion Chi-square test was carried out to find out if there was any significant different between the colonies where DWV was identified against those where DWV was not present. The results revealed that there was a significant difference in proportion in the DWV-present against the DWV-absent colonies, where χ^2=10.0833, df = 1 and P= 0.001496 (Figure 10).

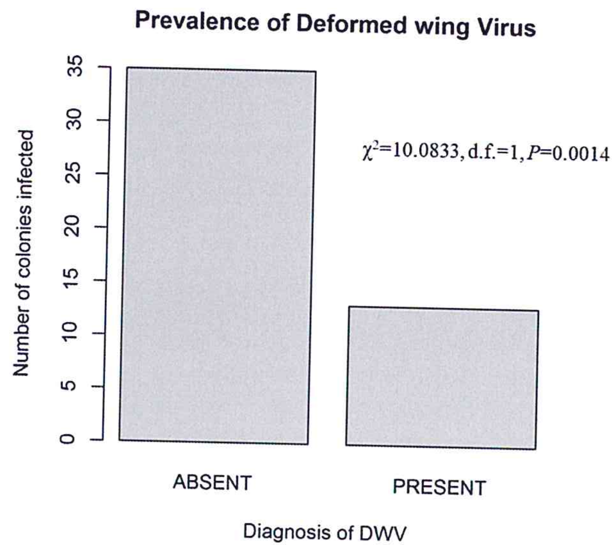

Figure 10: Prevalence of DWV in the honeybees from three apiaries in Kitui. Thirty nine colonies were analysed for the presence of DWV. 14 of the 39 colonies were found to be positive for the virus. A Chi square test for single proportions was used to determine whether the proportions of colonies positive for DWV were significantly different from those negative for the virus.

Further analyses were carried out to find out whether there was any significant difference in presence of DWV when compared to the type of hive used (χ^2=5.4505, df = 2 and P= 0.006553) and the apiary (χ^2=1.3689, df = 2 and P= 0.5) .In both cases, there was no significant difference in the prevalence of DWV. This is represented on figures 11 and 12, respectively.

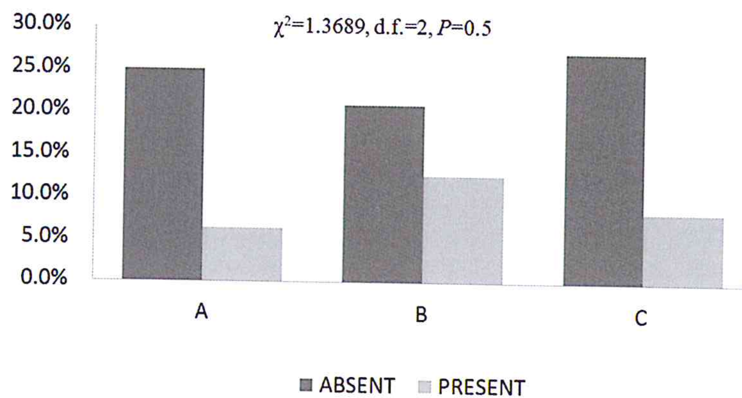

Figure 11: Prevalence of DWV in the three apiaries A, B, C. There was no significant difference in the prevalence of the pathogen in any of the apiaries.

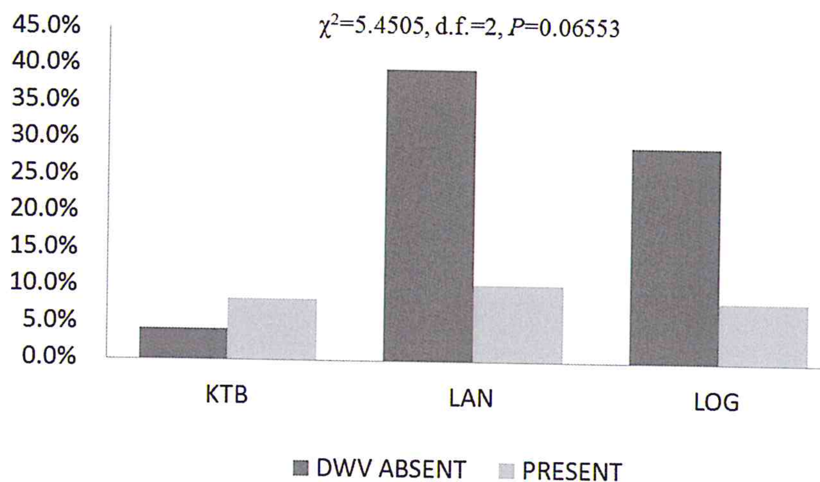

Figure 12: Prevalence of DWV in the three types of hives; KTB, Langstroth and traditional Log hive. There was no significant difference in the prevalence of DWV when compared against the three hive types.

3.2.2 Results for *Nosema* diagnosis

Nosema ceranae is one of the named predictors of colony collapse disorder. Diagnosis of this pathogen was carried out by real time PCR using *Taq*Man probes with Minor Grove Binder (MGB). *Nosema apis* and *N. ceranae* Positive controls for this experiment were obtained from Pennsylvania State University, from the lab of Prof. Christina Grozinger. The real time PCR results show the detection of *Nosema apis* and *N. ceranae* in the positive controls but not in the samples from Kitui. This is shown in Plate 11.

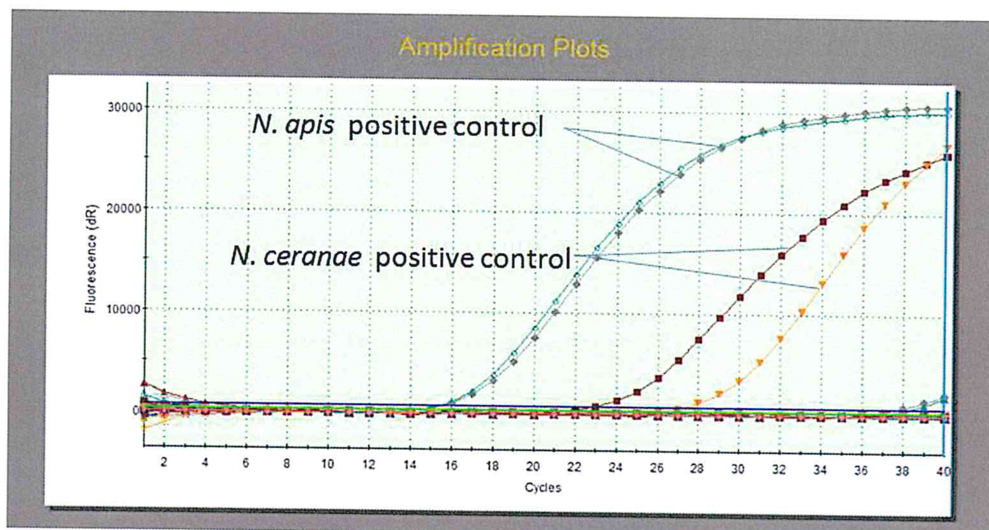

Plate 11: A qPCR amplification plot showing detection of *N. apis* and *N. ceranae* positive control samples but no detection of the pathogens in any of the samples from Kitui

3.3 ANALYSIS OF THE EFFECT OF HIVE TYPE ON COLONY GROWTH AND HEALTH

3.3.1 Results of prevalence of Varroa mites as an indicator of colony health

The prevalence of Varroa mites was examined using the standard sugar roll technique where a fine white powder (in this case icing sugar) was used to dislodge mites attached on the adult nurse honeybees. The number of mites dislodged was then counted, recorded and analysed.An analysis of the prevalence of Varroa was then examined in the three types of hives. The results showed that there was no difference in the prevalence of *Varroa* when comparatively examined across the Langstroth, traditional log hive and the Kenyan Top Bar Hive (Figure 13).

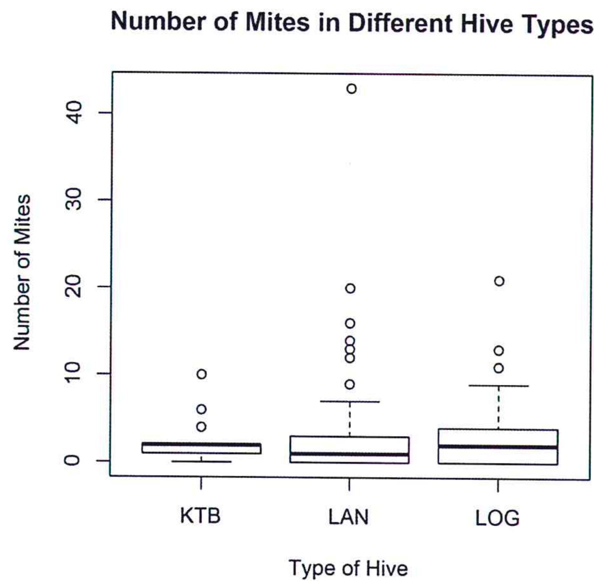

Figure 13: A Bar graph showing variation of *Varroa* numbers by type of hive. The highest numbers of *Varroa* were recorded in the Langstroth hive followed by the log hive. The Kenyan Top Bar hive recorded the lowest number of mites. Using the negative binomial distribution error model, χ^2= 0.025 df =2, α=0.05 P=0.987.

When the number of mites was examined across the three apiaries, the results also showed that there was no significant evidence to show that the number of Varroa mites varies across the different apiaries (Figure 14).

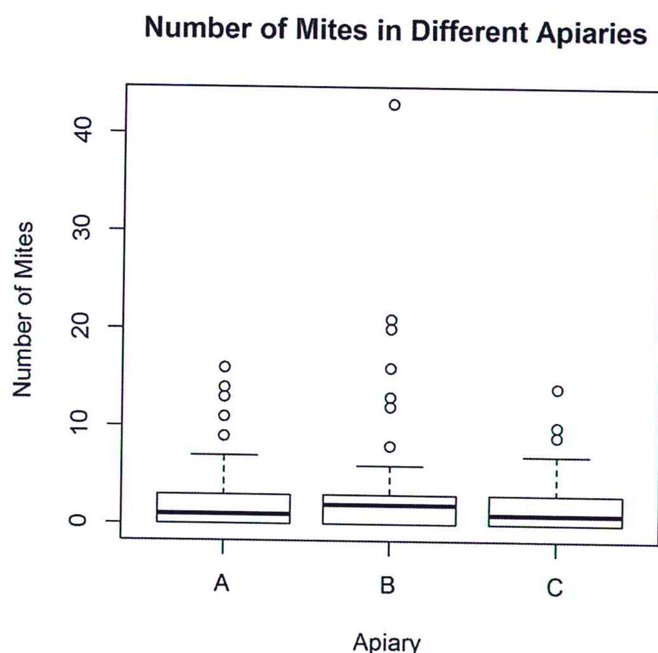

Figure 14: A boxplot showing variation in the number of *Varroa* in the three apiaries assessed. Apiary B recorded the highest number of mites. Apiary A and C recorded almost equal numbers of mites. Statistical analysis was carried out using the generalized linear model which showed that there was no significant difference in the number of mites per apiary. χ^2=4.2079, df =2, α=0.05, *P*=0.122.

The prevalence of *Varroa destructor* was compared against that of DWV, for samples collected in the month of June 2013. The results showed that there was no significant difference in the mean number of mites against that of the prevalence of DWV, where t=-0.2134, df=20, α=0.05 and P=0.8331 (Figure 15). This analysis was

done using the Welch two-sample t-test with non-equal variances, were the non-equality of the variances was confirmed using the Two Variances F test (F=0.9311, numerator df= 34, denominator df =12 and *P*=0.8215). In addition, there was no correlation between the prevalence of mites and DWV. This analysis was carried out using the two-sided Spearman's Rank-Order (*P*<0.0001 and *rho*=0.0168)

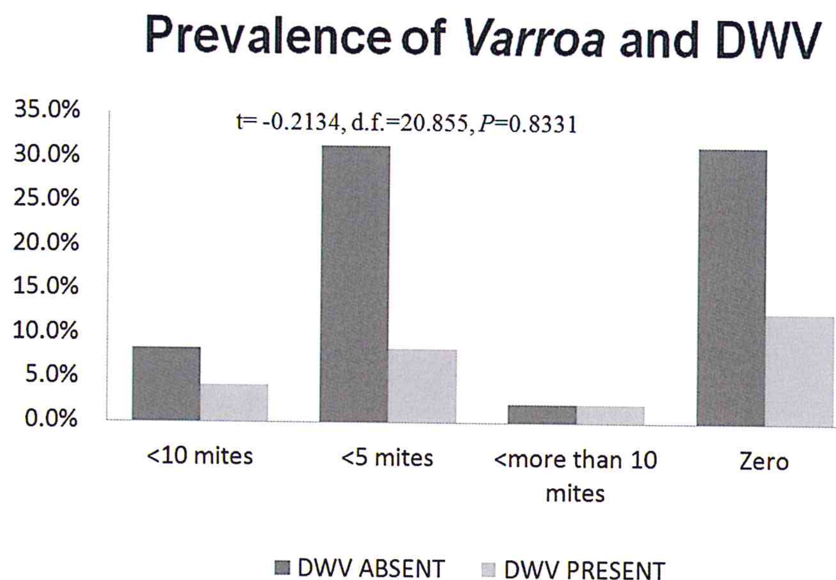

Figure 15: A bar graph showing how the means of the number of mites compares against the prevalence of DWV. The results show that there was no significant difference in the prevalence of DWV when compared against that of the number of mites. In, addition, the results of analysis carried out using the two-sided Spearman's Rank-Order test revealed that there was no correlation between the prevalence of mites and that of DWV.

The prevalence of *Varroa* was seen to significantly vary when examined across the different seasons of the year (Figure 16). Statistical analysis was carried out using the Chi square test, χ^2=23.024, df= 3, α=0.05 P<0.001. The results of these analysis

showed that there was significant difference in the prevalence of Varroa mites in the different seasons of the year. Means of the different months were separated using the adjusted Tukey test (HSD) which identified two main groups in the data as the short rains and dry season (a) and cold season and long rains (b)

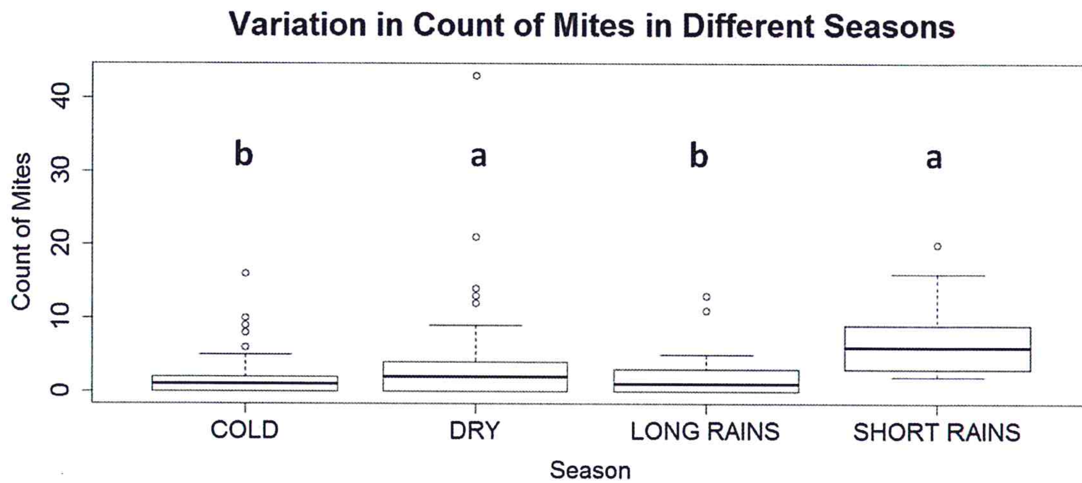

Figure 16: A Scatter plot showing seasonal variation in the number of Varroa mites. The months under the short rains category (October) recorded the highest number of mites while the drier months (February, January November and December recorded the highest range. The highest number of mites recorded was 43. The letters a and b represent the groups that were significantly different as identified using the Tukey HSD test.

Variation in the different months of the year showed that August 2013 recorded the highest number of mites while March 2013 had the lowest number of the same (Figure 17).

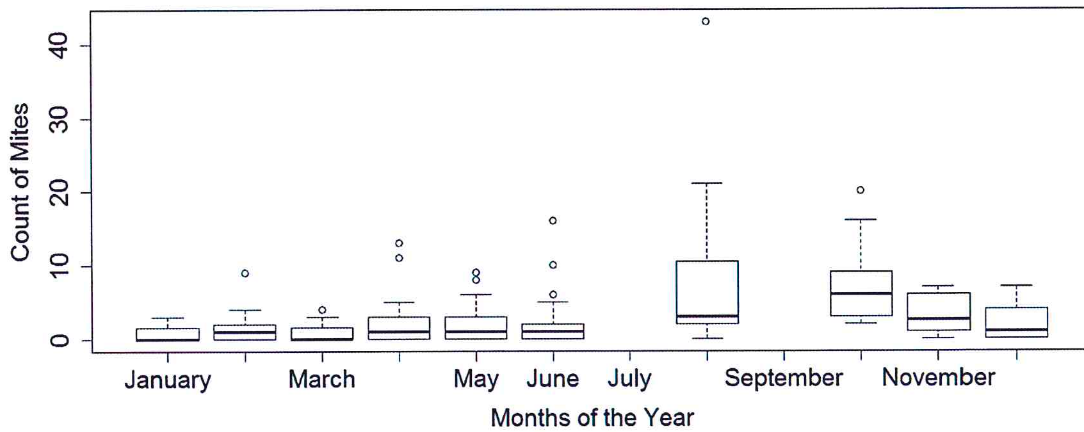

Figure 17: Analysis of mite numbers against the different months of the year

Finally, the number of honeybee viruses was found to be positively correlated to colony weights. This test for correlation was carried out using the Spearman's Rank-Order test which revealed that there was positive correlation when the prevalence of *Varroa destructor* was compared to colony weights (S=1178.196, $P<0.0001$, *rho*=0.9360.51). Therefore, with increase in colony weights, there was increase in the number of *Varroa* Figure 18).

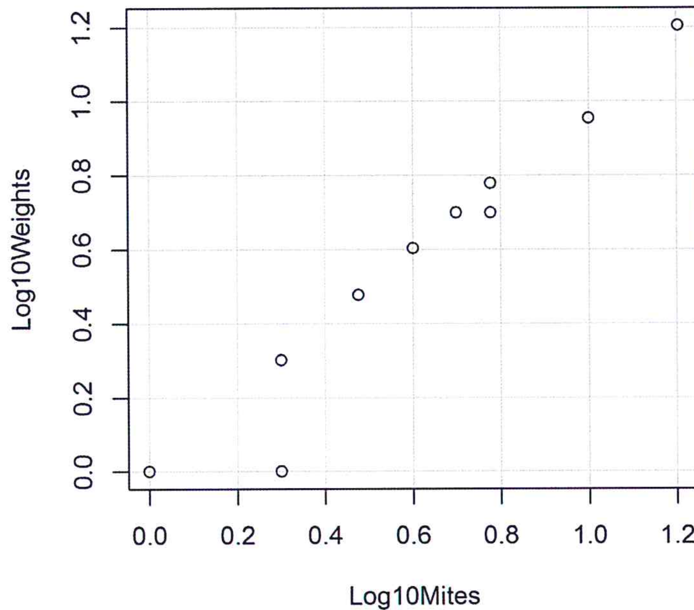

Figure 18: Correlation between the number of mites and colony weights. The graph shows that as the weight of colonies increases in the three apiaries, so does the number of mites.

3.3.2 Results showing weights of colonies as an indicator of honeybee colony growth

Honeybee colonies were weighed from December 2012 to January 2013. The results show that colony growth differed significantly in the three different apiaries with apiary B showing greater growth than both apiary A and C (Figure 19).

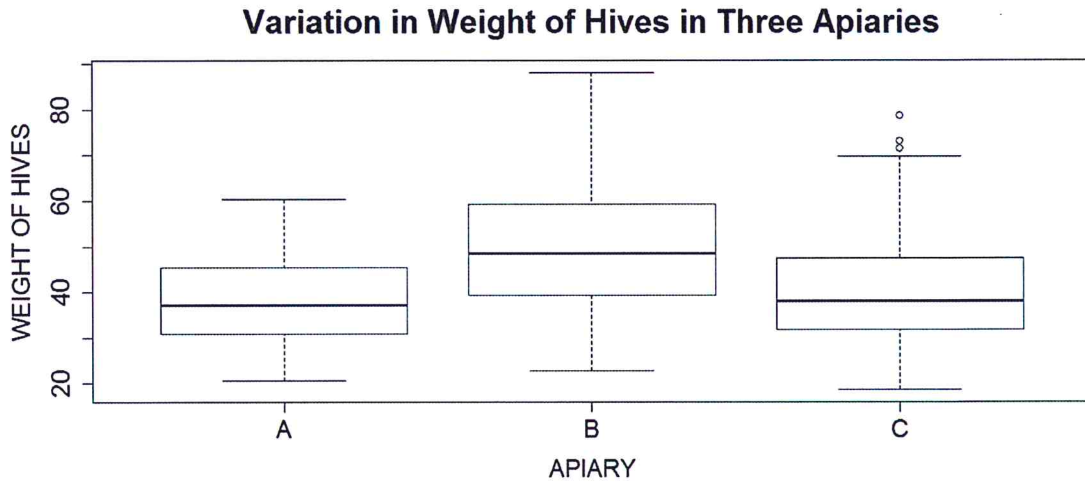

Figure 19: Comparative Colony Growth against three types of Apiaries (A, B and C) by assessing the weights of honeybee colonies. The general ANOVA statistical test was carried out to check for differences in weight of hives in the three apiaries F=14.34, α=0.05, df= 2, set d.f= 212, *P*=<0.001. Mean separation was carried out using the Tukey test. This revealed that there is no significant difference between apiaries C and A but there was significant difference between apiary B against both C and A

When colony weights were compared against the different seasons, the results showed that seasons do significantly affect the growth of colonies as indicated by colony weights. The cold season Cold season (May and June) had the greatest colony weights while the long rains season showed the least amount of growth (Figure 20). The least amount of growth is realized during the short rains which are preceded by a long dry spell. Statistical analyses were done using ANOVA. df= 3, 212, α=0.05, F=5.27, P=0.002. This showed that there is significant difference in the variation of colony growth in the different seasons. Separation of means was done using the LSD test due to unequal replication. Mean growth in the cold season was 47.40,

dry=42.82, long rains=39.23 while short rains was 42.52. This indicated that there were 3 groups the first two are cold season and long rains season with showed significant differences. The third group with short rains and dry season where there was no significant difference in growth between these two seasons.

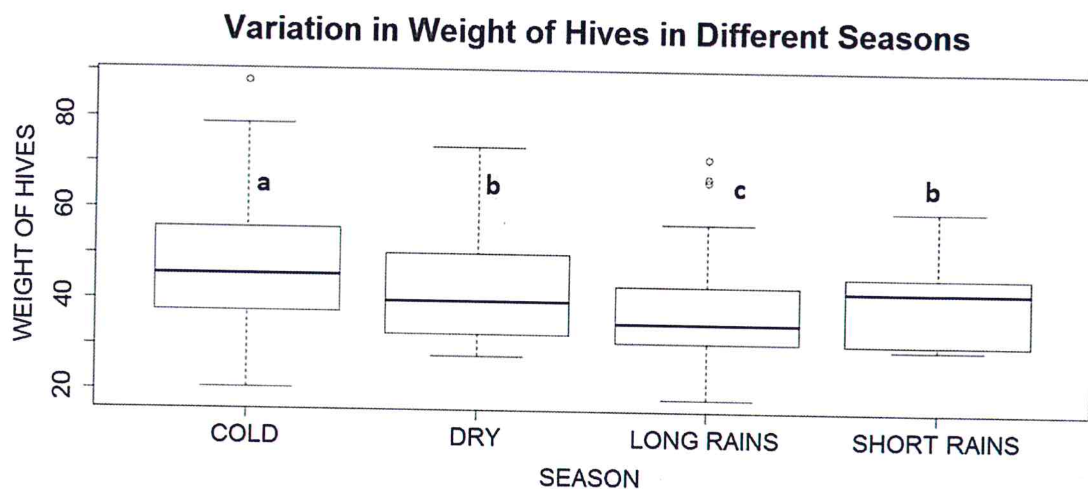

Figure 20: Seasonal Variation in colony growth. Most growth is seen to take place during the cold season right after the long rains. Cold season (May and June), Dry season (January, February, August, November and December), Long rains (March and April), Cold reason (May, June and July) and Short rains (October)

A monthly assessment of honeybee colony weights showed that the greatest honeybee growth was experienced in the months following the rainy seasons. The highest growth was experienced in November, the month immediately following the long rains in March (Figure 21).

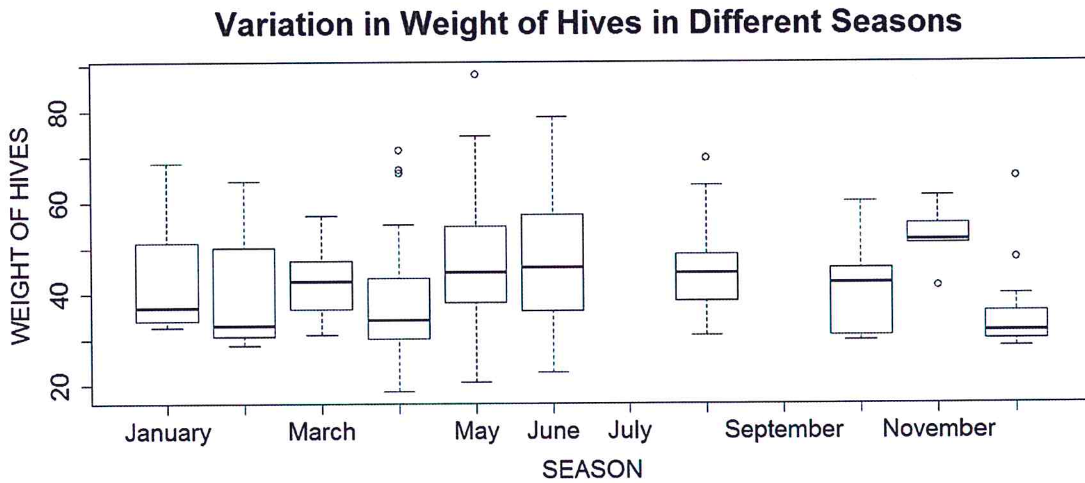

Figure 21: Analysis of colony growth in all three types of hives against different months of the year. The highest mean growth is recorded in November, after the rainy season in October.

Further, Analysis of Variance (ANOVA) revealed that there were significant differences in colony growth when examined against the three hive types (F=7.19, df= 2, group df= 212, α=0.05, *P*= <0.01). The highest level of colony weight was recorded for the Langstroth hives. There was no significant difference in colony weights between the KTB and Log hives (Figure 22). Separation of means was carried out using the Tukey test, revealing that there is no significant difference in KTB and Log hives but that there is a significant difference between growth in the Langstroth hive as compared to both the KTB and Log hives

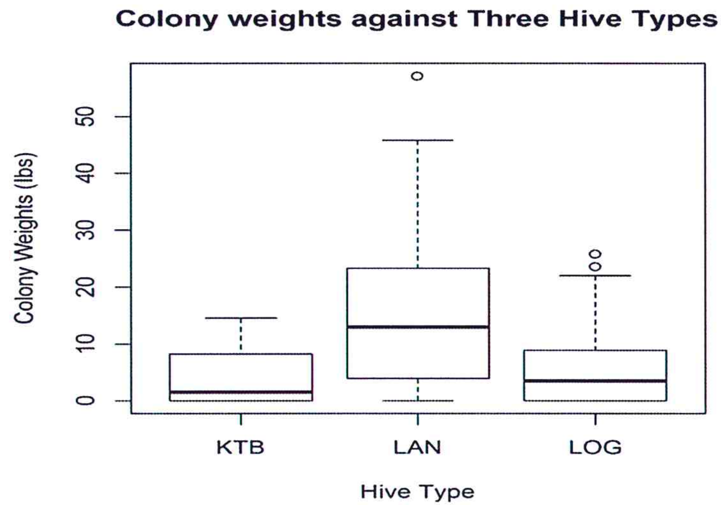

Figure 22: Analysis of colony growth indicated by hive weight measurements. The greatest growth is recorded in the Langstroth Hive followed by the the Log Hive, then the Kenyan Top Bar Hives F=7.19, df= 2, group df= 212, α=0.05, P= <0.01.

3.3.3 Results showing number of occupied frames as an indicator of colony growth

The second indicator of colony growth is the Number of Occupied Frames. An analysis of these data shows that the month of December had the lowest number of occupied frames while May, October and June recorded the highest scores (Figure 23).

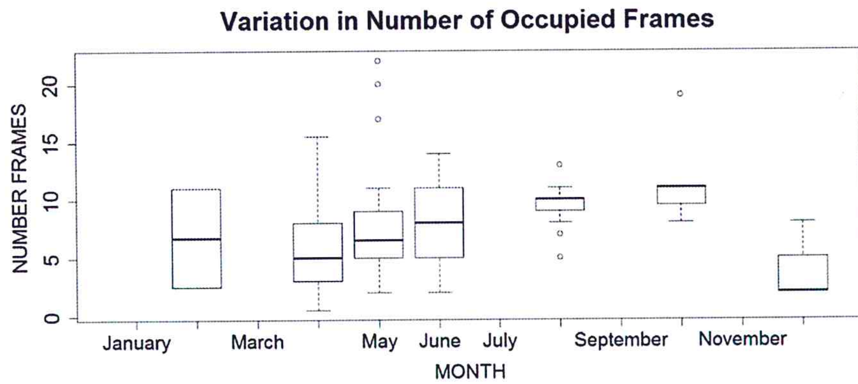

Figure 23: Number of Occupied Frames as an indicator of colony growth. Highest number of occupied frames was reported in April and May and October, while the least growth was realized in December.

There were significant differences in the number of occupied frames when compared against the different apiaries. Apiary B had greater growth as compared to both A and C (Figure 24). This showed that there was significant difference in number of occupied frames in the three apiaries. Separation of means was carried out using the Tukey (HSD) test. This showed that there was no significant difference between the number of occupied frames in apiary A and C but there was a significant difference in the number of occupied frames in apiary B in comparison to both A and C.

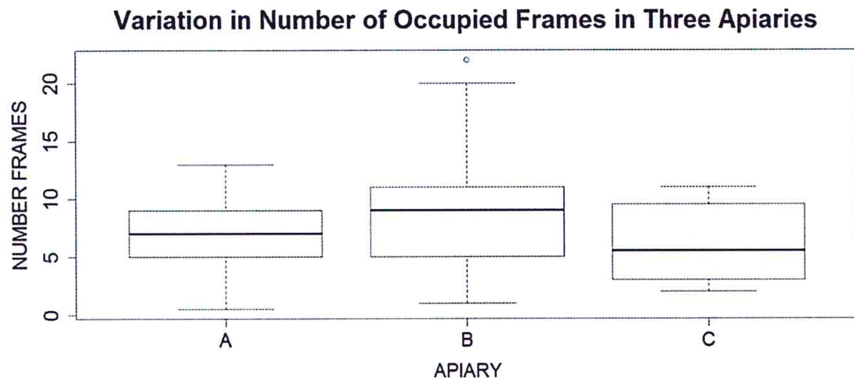

Figure 24: The number of occupied frames as an indicator of colony growth when compared against the three apiaries. Statistical test conducted was the ANOVA F= 6.029, df=2, group df= 118, α=0.05 and *P*=0.0032.

The Student's t-test was employed to compare the mean average growth in the KTB and Langstroth hives. The results showed that the Langstroth hive recorded significantly higher growth as compared to the Kenyan top Bar Hive (Figure 25). These results from this analysis are in line with those obtained when the weight of hives were compared against the different types of hives.

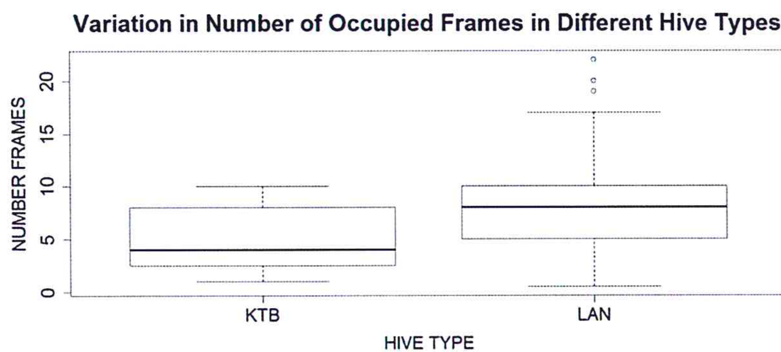

Figure 25: Colony Growth in different types of Frame Hives. Average growth is higher in the Langstroth Hive than in the Kenyan Top Bar

3.4 ANALYSIS OF THE KEY POLLEN SOURCES FOR HONEYBEES

The availability of pollen sources in the different seasons of the year was examined. Results show that Season Four which had high rainfall, low temperature and high humidity had the greatest availability of pollen grains while season Two (Low rainfall, high temperature and low humidity) had the lowest availability of pollen grains (Figure 26). For this analysis, the month of May was categorised under Season Four while February was Season Two.

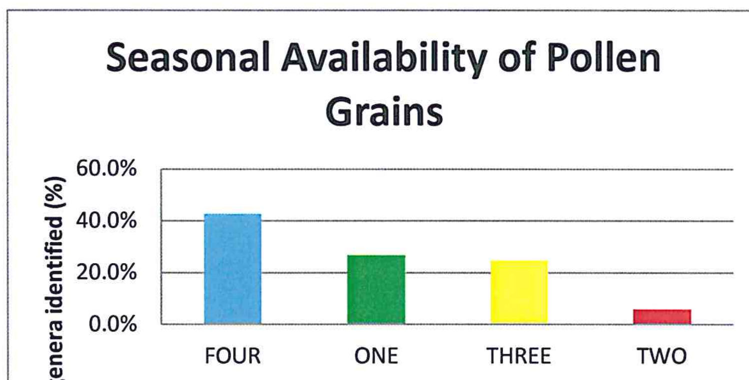

Figure 26: Variability in pollen availability in the three apiaries against the four seasons assessed. Season Four (May) had the highest level of availability of pollen grains while season Two (February) had the lowest level of pollen availability.

When the four seasons were examined across the different apiaries, the results showed that apiary B was the only apiary with availability of pollen in Season 2 (February). Apiary Four, three, and one had no pollen collected in February (Figure 27).

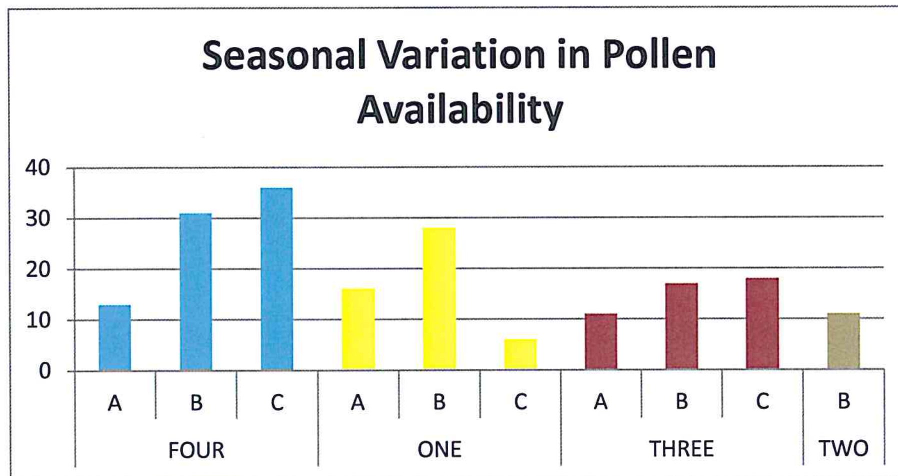

Figure 27: A bar graph showing variation in the amounts of pollen grains collected in Apiaries A, B and C, in Season I, II, III and IV.

Identification of pollen grains at the National Museums of Kenya revealed 18 plant families utilized by honeybees in the three apiaries set up in Kitui. Apiaries A and B had greater family level diversity than did apiary C (Figure 28).

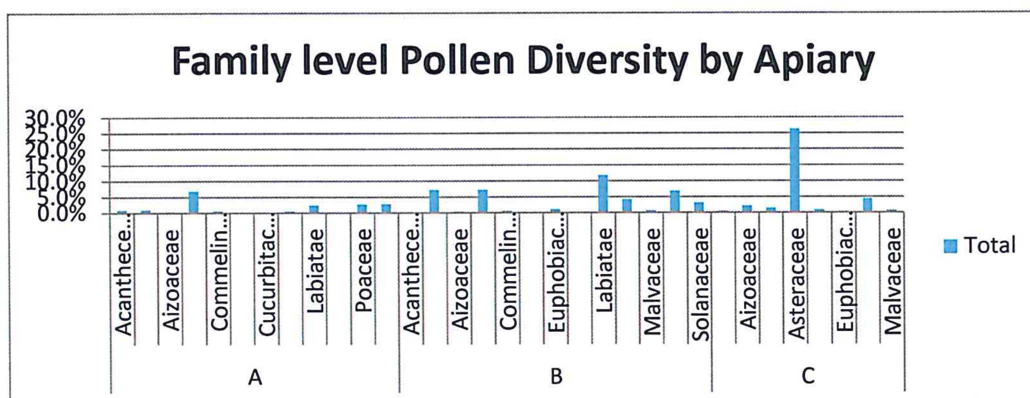

Figure 28: Family level diversity in the three different apiaries A, B and C in SEKU Kitui.

Genera level diversity showed that Apiary B had greater diversity as compared to both A and C (Figure 29)

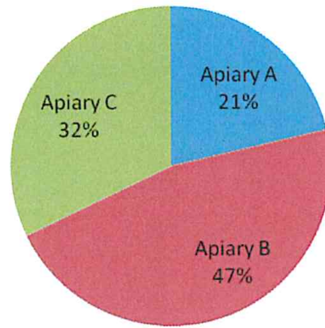

Figure 29: An apiary comparative assessment of plant genera foraged upon by honeybees. An analysis of the genus-level plant diversity revealed a total of 34 plant genera utilized by the honeybees of Kitui.

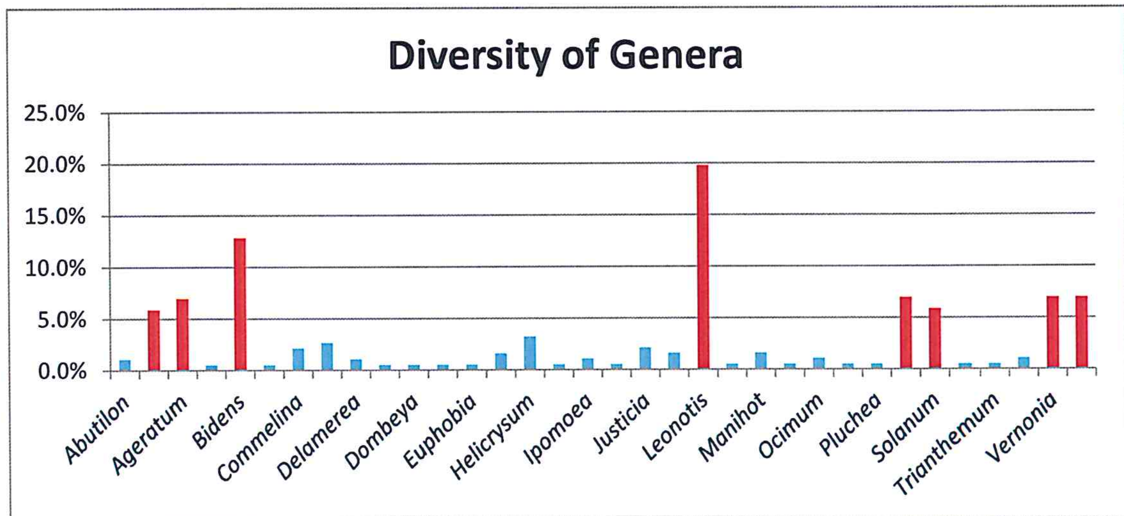

Figure 30: General Diversity of plant genera from all the three apiaries in SEKU, Kitui. The plant genera in red contain plants that were identified with the highest frequency.

Finally, a comparison of the plants identified was made in the three different apiaries in SEKU, with a bid to find out which plants were shared among the three colonies, which ones were shared between two colonies and which were unique to various apiaries. The results show that six plant genera were shared among all apiaries, six were shared between apiaries A and B and only two exclusively between apiaries B and C. There were no plant genera shared exclusively by apiary A and C (Figure 31).

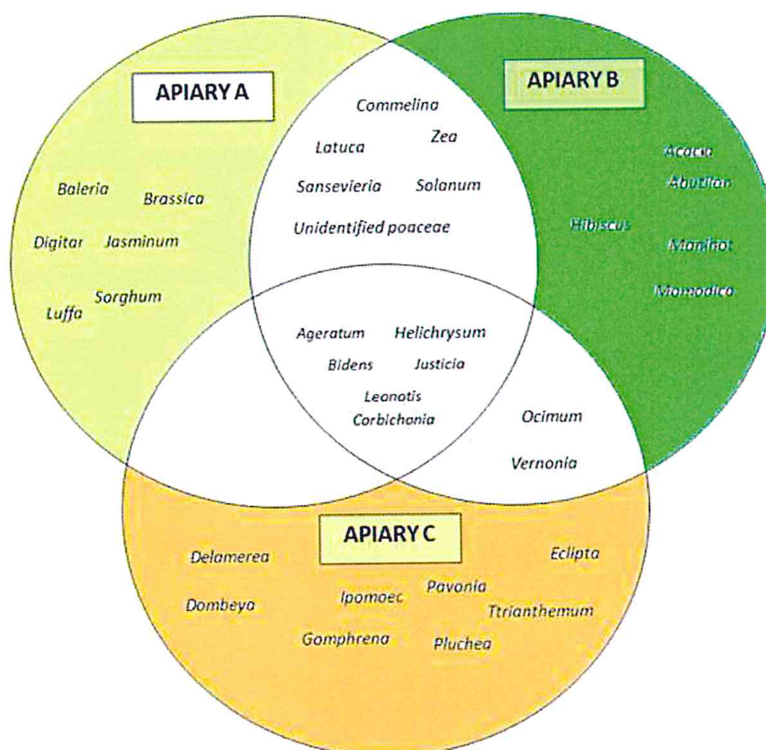

Figure 31: Comparative analysis of the plant genera identified in the three apiaries studied.

CHAPTER 4

4.0 DISCUSSION

4.1 MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF HONEYBEE SUBSPECIES

The outcome of the morphological analysis indicated that honeybees in the three apiaries in Kitui are of mixed genotypes, going by the differences in the colour of abdominal bands observed. There was the small plain black bee, a colour usually identifying the Monticola sub-species, honeybees with one yellow band perhaps indicating a Monticola-Scutellata hybrid, the two banded bees indicating the Scutellata-Litorea hybrid and the three and four banded bees; an identity of the Scutellata honeybee. In this morphological assessment, only one parameter (abdominal banding pattern) was examined. While this was successful in bringing out the diversity of the honeybee colonies, the technique alone was not sufficient for the accurate identification of hybrids from actual sub-species in these honeybees. For instance, a black bee could be *A. m. monticola* or a Scutellata-Monticolla hybrid with a black phenotype.

Three genes were analysed for their potential to resolve the identity of sub-species in Kitui County. Two of the gene fragments (COI and $tRNA^{ILE}$-ND2) are mitochondrial in origin while one of the genes (EF 1α) is of nuclear origin.

While the partial COI gene has been used extensively as a species BARCODE gene for many organisms (Hebert *et al.* 2003), the gene was also found to be adequate in the resolution of honeybee sub-species in Kenya. The two clusters that formed

(Figure 8) seem to separate the honeybees of the highland (mountainous) regions and their hybrids, from the honeybees of the coastal (lowlands) regions and their hybrids. Reference sequences to aid in the subspecies identification using the COI locus were obtained from the Barcode of Life Data Systems, BOLD (Ratnasingham and Hebert 2007). These sequences were very well curated and all were generated using the Folmer primers (Folmer *et al.* 1994), amplifying the partial COI locus. The High Resolution Melt (HRM) analysis technique was explored for its potential as a fast, inefficient and inexpensive way of identification of organisms without the constant need for sequencing (Reed *et al.* 2007). The results of this experiment (Plate 3) showed that the partial COI locus used was not able to generate profiles that could adequately resolve known sub-species from different ecological zones. A region with greater genotypic variation should be explored for this.

The Elongation Factor 1α gene primers amplified fragments varying from 275-300 bp in size. While this gene has the potential to identify the different sub-species of organisms, in honeybees, it has been noted to occur in duplicate (Danforth and Ji 1998) and this might interfere with the phylogenetic analysis. In addition, after performing a BLAST search against other sequences deposited in GenBank, the highest hits were recorded by *Apis mellifera* genes deposited in GenBank by the Honeybee Genome sequencing consortium (Weinstock *et al.* 2006) due to lack of sufficient sequence deposits in the GenBank nucleotide database. For this locus to be useful in subspecies identification there is need to generate more sequence data from all known honeybee subspecies.

The tRNA^{ILE}-ND2 gene fragment gave a 688bp amplicon, the nucleotide sequences which when resolved against sequences deposited in the GenBank DNA repository showed that the honeybees in Kitui County belong to the Scutellata group. This resolution is based on sequences deposited by Arias and Sheppard in 2005, using the same DNA loci used for identification in this work. However, these honeybees seem to be hybridizing with the mountain honeybee sub-species *A. m. monticola* and the coastal honeybee sub-species *A. m. litorea*, to give Scutellata-Monticola and Scutellata-Litorea hybrids. This result seems to be in agreement with suggestions by Raina and Kimbu in 2005 on the possibility of zones of honeybee hybridisation in Eastern province, among the honeybees of the mountain *A. m. monticola*, the bees of the savannah *A. m. scutellata* and the honeybees of the coast *A. m. litorea*. These honeybees are thought to migrate from their area of origin during the dry spells when there is little floral resource to be found. The heat map (Figure 9) revealed that the regions on the tRNA^{ILE}-ND2 gene fragment that led to the greatest subspecies variation in African bees is the 5′ and 3′ gene region. Perhaps more diagnostic assays, such as those utilizing the High Resolution Melt Analysis could focus on these regions.

When comparing between the results of the morphological and molecular data, two issues become important. Firstly, in honeybees, the colour trait is controlled not by one gene but by multiple genes linked in a quantitative trait loci (Mougel *et al.* 2012). These genes are found in the nucleic DNA as opposed to mitochondrial DNA. Secondly, mitochondrial DNA is maternally inherited. Therefore, the subspecies

identified shows the subspecies of orign of the queens. Thus in the case of a plain black hybrid bee where the Scutellata queen was fertilized by Monticola drones, the worker would be identified as Scutellata using the $tRNA^{ILE}$-ND2 gene fragment but as Monticola using the abdominal colour banding pattern. While phenotypic markers are essential in the identification of honeybees, they should ideally be paired up with genotypic markers as the phenotypic changes are results of a variety of genotypic variations. Nuclear DNA however undergoes recombination (unlike mitochondrial DNA) and would be the perfect tool to use to study hybridisation and the population genetics of the honeybees.

The fact that Kitui County is a major zone of hybridisation for the honeybees of Kenya (Figure 32) has implications on its importance as when monitoring honeybees for disease and pest diagnosis. This is the first study to examine the subspecies in Kitui county using molecular tools.

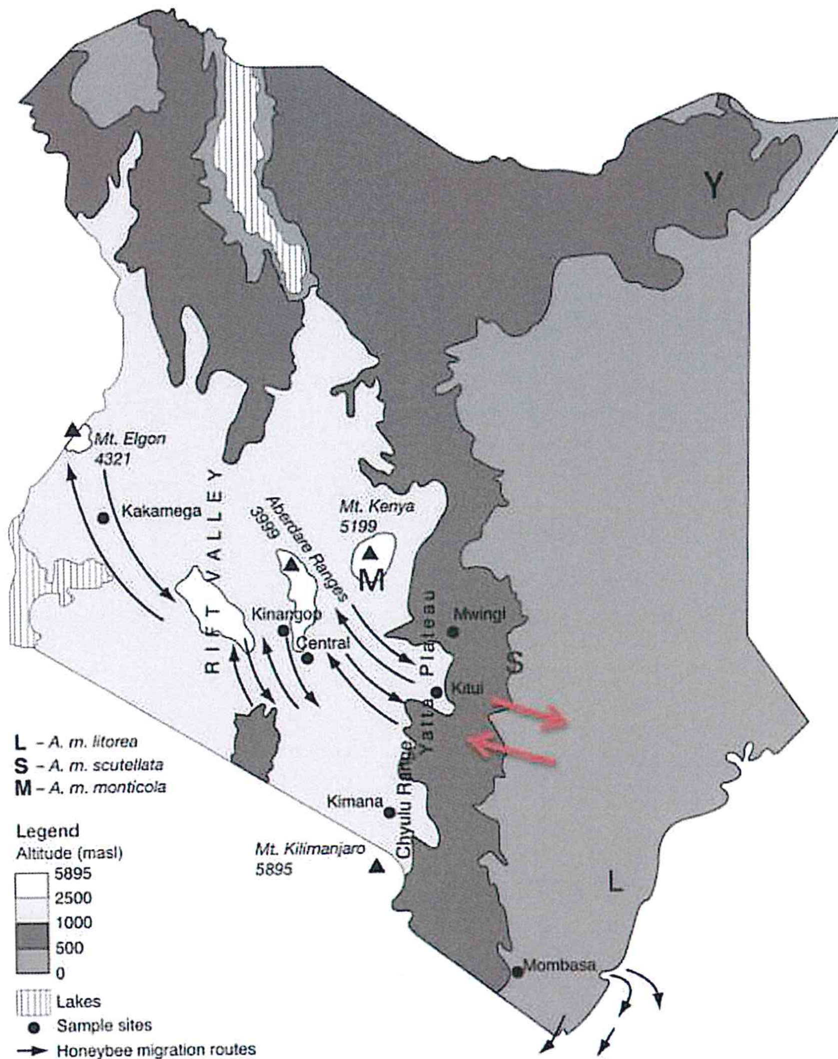

Figure 32: Map of Kenya showing three of the four known sub-species of honeybees in Kenya. The black arrows indicate known swarming and migration routes of *Apis mellifera* races (Raina and Kimbu 2005) while the red lines indicate a putative route of migration of and swarming of the Scutellata and Litorea honeybees. The point Y on the map represents the desert honeybee *Apis mellifera yemenitica* which has so far been identified in Mandera.

4.2 DISEASE DIAGNOSIS IN THE HONEYBEES OF KITUI

The outcome of the virus detection showed the presence of Deformed Wing Virus (DWV) in the honeybees of Kitui. This detection was first done by qPCR using SYBR Green (Ponchel *et al.* 2003) where data on the amplification plots (Plate 7) were verified using the dissociation curves (Plates 6 and 8). Amplification was identified as positive if its melting temperature and dissociation curve was found to match that of the positive control sample. The prevalence of DWV infection was 14 out of 39 (35.9%). These amplicons were loaded on an agarose gel where the same prevalence was shown (Plate 9).

The investigation of viruses in *Varroa destructor* revealed that either the mites had no viruses at all, or that the viral titres in the mites was too low to be diagnosed using real time PCR. One main limitation of this investigation was that the mites were sampled from a total pool of mites from all the three apiaries and not from individual hives already identified to have DWV. This is because the number of mites in some hives was too low to allow for the collection of adequate mite samples for disease diagnosis. This is the first study investigating the presence of viruses in *Varroa* in Kenya and is significant because *Varroa* is a known vector of honeybee viruses (Bowen-Walker *et al.* 1999, Tentcheva *et al.* 2004, Shen *et al.* 2005b). These mites also have the ability changing the viral landscape of the viruses (Martin *et al.* 2012) theoretically leading to changes in the virulence level of the resultant viruses.

The prevalence of DWV was found to be neither correlated with hive weights nor the prevalence of *Varroa destructor* (Figure 15). In addition, the prevalence of this

pathogen did not vary with the type of hive used nor the apiary housing the bee hives. While this will be good news for farmers around the country, more data on the prevalence of DWV will be required for future studies where the prevalence of the pathogen is followed over a longer period of time.

The DNA isolation method employed for the investigation of *Nosema* in the honeybees of Kitui County was a combination of two techniques; CTAB and Phenol-Chloroform-Isoamyl alcohol (PCIA) and isolation techniques. The reason for this was that while the PCIA technique is suitable for breaking open the soft honeybee tissue for DNA isolation, it is not efficient for tough tissues such as the tough cellulose cover that is found on the surface of the non-germinated *Nosema* spore that lines the abdominal epithelium of infected honeybees. To break open tissues rich in cellulose, CTAB technique was optimal.

Real time PCR (qPCR) using the *Taq*Man assay was employed in the detection of *Nosema* DNA due to its higher sensitivity as compared to convectional PCR in disease detection especially when the expected quantities of pathogen are low (Chen *et al.* 2005, Hamiduzzaman *et al.* 2010, Traver and Fell 2011b). The results of this experiment showed the positive controls amplified while the negative controls together with the samples from Kitui showed no amplification (Plate 11). This means that the levels of *Nosema* in Kitui were either too low to be detected even with an assay as sensitive as qPCR or that the spores are absent. This conclusion would be in line with those reported by (Muli *et al.* 2014) which showed that the levels of *Nosema* in Kenya were very low and so far, only restricted to a few places in coastal

Kenya. The infective stage of the pathogen is the non-germinated spore which is ingested in contaminated pollen or honey (Higes *et al.* 2008b). This means that trade and movement in contaminated hive products from coast province to eastern province could lead to transportation of *Nosema* microspores from the coast to eastern province. *Nosema apis* was identified in some honeybee colonies collected in the coastal province of Kenya in 2010 (Muli *et al.* 2014).

Transmission of the parasite has been reported to occur horizontally by the worker bees to brood (Smith 2012) and to the queen (Higes *et al.* 2009) during feeding, and from infected drones to the rest of the colony *via* mating with the queen (Traver and Fell 2011a). This last route of transmission is a potential source of spread of *N. ceranae* from colony to colony because drones from different colonies congregate to mate with a virgin queen. *Nosema* can also be transmitted vertically from an infected queen to the eggs laid (Traver and Fell 2012). Kitui County is in part of the hybridization zone of honeybees forming a belt from Coastal Kenya-Eastern province-Central province highlands (Raina and Kimbu 2005). The fact that the coastal honeybees are infected with *Nosema* makes the honeybees of Eastern and Central province at risk of contacting the same pathogen due to the aforementioned routes of transmission between drones, queen, workers and brood.

4.3 ANALYSIS OF THE EFFECT OF HIVE TYPE ON COLONY GROWTH AND HEALTH

Honeybee health and growth is influenced by a variety of factors including floral resources (nutrition), pathogens, pests, and climatic conditions. Indeed, the health of an organism is not simply defined by the absence or presence of a pathogen, but by a complex of other factors as well.

The results from this work revealed that there was no difference in the prevalence of *Varroa* in the three types of hives in Kitui (Figure 13). These results differ with those reported by Ande *et al.* (2008) from a study conducted in Nigeria which concluded that KTB hives would perform better in Nigeria as compared to Langstroth hives due to their lower incidence of pests. This discrepancy could be explained that Ande and his team examined the prevalence of *Galleria melonella* (Greater wax moth), *Campanotus pennsylvanicas* (sugar ants), *Rana sp* (frog) and *Lactrodectus mactans* (Black Widow Spider). The prevalence of *V. destructor* was not examined. In addition, while the study tested the performance of traditional hives against modern hives, the performance of the traditional log hive was not examined (Ande *et al.* 2008).

Experiments comparing the number of *Varroa* in different apiaries also showed that there was no significant difference in the number of mites in beehives from different apiaries (Figure 14), in this site, SEKU Kitui. The reason for this could be that this assessment was done in one site (SEKU, Kitui) and that greater variation is expected at the site level. This was the case demonstrated in a recently done survey where the

high altitude areas such as the Aberdare Ranges and Mt. Elgon showed significantly higher numbers of mites than the lowland regions (Muli *et al.* 2014).

The seasonal analysis of the number of *Varroa* revealed that the number of mites were highest in August and October. The lowest record of mites was in January and March (Figure 16). This seasonal variation could be explained by the fact that the slight showers that were experienced in December enabled the bees to collect enough pollen and nectar to rear brood in the hives. Due to the fact that *Varroa* preferentially attach to honeybee brood as compared to adult worker bees (Ifantidis 1988), the results of mite count using adult bees tend to show lower numbers of the mite as compared to counts taken during non-brood rearing seasons. This is also supported by results showing the highest mite counts in August and October. August showed the highest mite count due to the fact that there were very few floral resources for the bees during this time. While February is the driest month of the year in Kitui, this month also saw the flowering of *Acacia sp,* a melliferous plant that is known to be a great source of pollen and nectar for African honeybees. This enabled the honeybee colonies to rear brood thus preferentially attracting the parasitic mite from the adult nurse bees to the brood. This will have implications for disease prevalence and transmission. Climate was seen to influence the prevalence of *Varroa* as inferred from the Sugar shake counts (Figures 16 and 17) where experiments conducted revealed that there were significant differences in the prevalence of *Varroa* sampled across different seasons. The highest prevalence was seen during the dry season which is a non-brood rearing season. The lowest prevalence was seen during the long

rains and the cold seasons which are peak brood rearing seasons due to abundance in forage material for the bees.

Finally, there was a strong positive correlation in the number of mites and colony weights (Figure 18), where as the weights of the honeybee colonies increase, the number of *Varroa* mites also increase. This result can be explained by the fact that honeybee reproduction and Varroa mite reproduction are linked. The mite reproduces in sealed honeybee brood. Therefore with increase in the number of brood, there is an increase in the number of the reproducing *Varroa*.

Clearly, the preference that *Varroa* has for brood (and more specifically drone brood) over adult bees is the main limitation of the Sugar roll technique of monitoring *Varroa* numbers and both researchers and apiculturists need to be aware of this. The best way to estimate the number of *Varroa* in a colony is to use a combination of sugar-shake technique and another technique that checks the number of mites in brood such as physically counting the number of mites in one hundred (100) brood cells at the pink eyed pupal stage.

Colony growth is an indicator of colony productivity and in this experiment two parameters were used in assessing colony growth; colony weights and number of frames occupied. These two parameters have previously been used by Muli and team (2014) to indicate colony productivity in different areas of Kenya.

The outcome of these analyses in Kitui show that Langstroth hives showed the highest average growth compared to both KTB and Log hives both when analysed

using hive weights data (Figure 22) and number of frames occupied (Figure 25). The reason for this could be that the Langstroth hive when supered has greater volume than both the Log and the KTB hives. This greater volume affords honeybees greater space within which to build combs for brood, pollen and honey storage.

Apiary B showed greater growth performance than both Apiaries A and C when assessed using both Colony weight (Figure 19) and Number of Frames Occupied (Figure 24). This could mean that the floral resources in Apiary B are better in quality and quantity compared to the resources in Apiary A and C. Therefore, the bees are better able to rear their young, build wax combs and have greater storage food reserves in the form of honey and pollen. Indeed, results from the comparative study conducted to assess the floral resources in the three apiaries show that honeybees in Apiary B had a greater diversity and amount of floral resources as compared to Apiary A and C (Figures 28 and 29). These resources go a long way in sustaining a colony through periods of dearth.

4.4 ANALYSIS OF THE KEY POLLEN SOURCES FOR HONEYBEES

Pollen processing using the acetolysis technique (Erdtman 1960) enabled for the removal of cellulose that covers the surface of the pollen grain. This removal exposed the surface of the pollen exine revealing shapes and patterns that are key in identification of pollen grains.

The results in Figure 26 indicate that the greatest amount of pollen was collected during in Season IV high rainfall, low temperature and high humidity (May) while the lowest amounts of pollen were recorded during Season II with low rainfall, high temperature and high humidity (February). Season IV falls within the months of March and April while Season II is in November and December. The reason for this could be that there are very few flowering plants in Kitui that bloom during the months of November and December, owing to the very high temperature. While February is the hottest month in Kitui, it is also the month of the year when Acacia, a highly melliferous plant, blooms.

The priorities of the bee hive changes with the change in climatic conditions in the different seasons of the year. During the drier seasons, there is low pollen and nectar flow and therefore low amounts of food for the hive. During these seasons, the queen reduces the amounts of eggs laid, drones are chased away from the hive and generally, there are fewer numbers of worker bees and thus smaller colony sizes. When the rainy season begins (October-November and March-April) flowers open up. In the hive, there is an urgency to replace the food resources consumed (more often than not to completion) by the honeybees that survived the dry period and also to revive the colony numbers. Therefore, the few foragers available collect pollen as the queen embarks on an egg-laying laying spree. As the eggs turn into larvae, the demand for pollen also increases and was larvae pupate and become adults, there is an urgency to feed the adult bees and therefore the greater emphasis in the colony shifts from pollen collection to nectar collection.

Apiary B had greater floral diversity than Apiary A and C with the latter having the least floral diversity with regard to plant families (Figures 27 and 28). This family level diversity is essential in two main ways. First, current research shows that plant diversity leading to forage/nutritional diversity is essential in boosting honeybee immunity (Di Pasquale *et al.* 2013). Secondly, this kind of diversity increases the availability of forage material over different climatic seasons, ensuring that the bees have forage all year round. Indeed, this was the case with Apiary B (Figure 28). Due to the presence of a huge diversity of plants including Acacia which flowers in February, the hottest month of the year, bees in this apiary had the least rate of absconding (Muli *et al* in prep) and the highest rate of growth in terms of increase in colony weights (Figure 19) and number of frames occupied (Figure 24). Majority of the honeys in Africa are said to be made from nectar from Acacia. In contrast, Apiary C had a high diversity of plant genera but low family diversity (Figures 28 and 29, respectively). This apiary had the lowest rate of growth and the highest absconding rates, up to 100% at a particular time (Muli *et al* in prep).

CHAPTER 5

5.0 CONCLUSION AND RECOMMENDATION

5.1 CONCLUSIONS

The honeybees of Kitui have been effectively identified as belonging to the sub-species *Apis mellifera scutellata*, with hybrids from the honeybees of the mountain *A. m. monticola* and those of the coast *A. m. litorea*. This mixed gene pool speaks to the high importance of the eastern belt of hybridisation as a source of high genetic diversity in the honeybees of Kenya, and hence, emphasises the urgent need for monitoring and conservation.

Deformed Wing Virus (DWV) is present in the honeybees of Kitui County but absent or in very low titres in *Varroa*. Israeli Acute Paralysis Virus (IAPV), Black Queen Cell Virus (BQCV) and *Nosema* are also absent in the honeybees of Kitui or very low titres that are undetectable by qPCR. Further monitoring is required to see how the presence of *Varroa* in this area will influence the occurrence and transmission of honeybee viruses in this area. This is the first documented study to investigating viruses in the mites in East Africa.

The honeybees of Kitui do not harbour *Nosema ceranae* as shown by the results of the qPCR assay. However, due to the high rate of hybridisation that takes place between the honeybees of Kitui County, Coast and Central provinces, close epidemiological monitoring should be carried out to make sure that should transmission of *Nosema* take place, notifications to the authorities such as OIE can be made in order to curb the spread of this pathogen to other regions.

This study also reports how the prevalence of *Varroa* varies with varying climatic conditions in the year, but not with apiary type or hive type. In addition, this work demonstrates how growth of honeybee colonies is affected by the type of hive (highest in the Langstroth hive), the apiary (Highest where there is greater family-level plant diversity) and season of the year (highest in the cold season after the long rains).

Finally, this work has revealed the fact that family-level plant biodiversity may be more important than genus level diversity in supplying resources for honeybee colonies to thrive.

Honeybee health (considered through prevalence of pathogens and pests) and growth (as as indicated by colony weights and number of frames occupied) is intricately connected to the ecology of an area (as reported by biotic factors such as pollen grains and abiotic factors such as the climate of an area and the type of hive housing honeybees).

5.2 OVERALL SIGNIFICANCE AND IMPLICATIONS

African bees have been described as resilient to the presence of honeybee pests such as *Varroa* and pathogens including viruses and microsporadia (Muli *et al.* 2014), although the reason for this resilience is still unknown. This is the first study investigating honeybee diseases in an African context where multiple hive types are utilized and pollen sources are polyfloral.

The study has demonstrated that honeybee health is not affected by the type of hive or the apiary. This is critical to farmers around the country as it gives them the freedom to choose the type of hive best for their bees, based on other factors such as productivity and growth, which have been shown to significantly differ with the type of hive and the apiary.

Finally, this work will strengthen the argument for extensive as opposed to intensive beekeeping. Our results show that family level floral diversity enables the bees to flourish better than floral monocultures or even genus level biodiversity.

Ultimately, the conclusions drawn from this work are expected to enable the beekeeper to practice profitable but sustainable beekeeping in an effort to conserve the pollinators of Africa.

5.3 RECOMMENDATIONS

Firstly, effort should be put in place to carry out studies similar to this one in other ecological zones where beekeeping is practiced as the prevalence of pests and diseases have been shown to significantly vary with change in ecological zones (Muli *et al.* 2014). In addition, it will be important to find out whether the types of beehive will a similar effect on honeybee growth or pathogens in a different ecological zone as they have in this dry land ecosystem. The recommendations drawn from these studies will go a long way in fortifying sustainable apicultural practices in Kenya.

Secondly, recent reports have documented the presence of honeybee pathogens in non-honeybee pollinators, a scenario that could be as a result of interspecific transmission of pathogens (Furst *et al.* 2014). Investigations into pollinator health and ecology should be extended to include other types of pollinators including carpenter bees and stingless bees.

Finally, beekeepers should be encouraged to continue practicing extensive beekeeping using beekeeping techniques that not only focus on increasing production of hive products but techniques that also ensure the sustainable wellbeing of the honeybee.

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