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The distribution, infestation levels and effects of honeybee parasites and pathogens on colony performance in two agro-ecological zones of Uganda

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List of abbreviations

ABPV	Acute bee paralysis virus
AEZ	Agro-ecological zone
AFB	American foulbrood
AmFV	*Apis mellifera* filamentous virus
Blast	Basic local alignment search tool
bp	Base pair
BQCV	Black queen cell virus
BSA	Basal solid agar
BSRV	Big Sioux River virus
CBPV	Chronic bee paralysis virus
cDNA	Complementary deoxyribonucleic acid
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	DeoxyriboNucleotide TriPhosphate
dsRNA	Double-stranded RNA
DWV	Deformed wing virus
EFB	European foulbrood
EFSA	European Food Safety Authority
EM	Electron microscopy
ERIC	Enterobacterial repetitive intergenic consensus
EU	European Union
FA	Formaldehyde
FC	Filament coils
FAO	Food and Agricultural Organisation
GA	Glutaraldehyde
GPS	Global positioning system

IAPV	Israeli acute paralysis virus
KBV	Kashmir bee virus
L-MEB	Laboratory for Molecular Entomology and Bee Pathology
LM	Light microscopy
LSV	Lake Sinai virus
MAAIF	Ministry of Agriculture, Animal Industry and Fisheries
MEGA	Molecular Evolutionary Genetics Analysis
MLPA	Multiplex Ligation-dependent Probe Amplification
mRNA	Messenger RNA
MYPGP	Mueller-Hinton broth, yeast extract, potassium hydrogen phosphate, glucose sodium pyruvate
NCBI	National Center for Biotechnology Information
NEMA	National Environmental Management Authority
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QENP	Queen Elizabeth national park
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
RT-qPCR	Reverse transcription quantitative (real time) PCR
SBPV	Slow bee paralysis virus
SBV	Sacbrood virus
SHB	Small hive beetle
SPSS	Statistical Package for the Social Sciences
SSU	Small subunit
TBE	Tris/Borate/EDTA
TEM	Transmission electron microscopy
UBOS	Uganda Bureau of Statistics
UEPB	Uganda Export Promotions Board

UNCST	Uganda National Council for Science and Technology
UrAc	Uranyl acetate
USA	United States of America
UV	Ultra violet
VDV-1	*Varroa destructor* virus 1
YGPSA	Yeast-glucose-starch agar

Summary

In Uganda, the honeybee, *Apis mellifera* is kept mainly for honey, other beehive products and for the provision of vital pollination services. Although the beekeeping sector is growing and providing a source of income, food and medicine to many rural households, the potential threat from honeybee parasites and pathogens poses challenges to the sector. Indeed, some of the parasites like *Varroa* mites which have devastated the beekeeping industry in Europe, Asia and North America have already been registered in Uganda. Although, such parasites and their impacts on European honeybee races are fairly known; their distribution, infestation levels and impacts on African honeybee races are poorly understood. In order to contribute to bridge this information gap, apiaries and feral colonies in the eastern and western highland agro-ecological zones of Uganda each with an altitudinal gradient and varying land uses were sampled during the dry and wet seasons between December 2014 and September 2015. The samples were screened for 11 common RNA viruses, *Paenibacillus larvae*, *Mellisococcus plutonius*, *Ascosphaera apis*, *Nosema* spp. and *Varroa* mites using standard techniques with the aim of establishing their infestation levels and their effects on colony performance in the two highland agro-ecological zones of Uganda. Five RNA viruses, *P. larvae*, three *Nosema* spp. and *Varroa* mites were detected. The infection rates of viruses varied: Deformed wing virus (51.9%), Black queen cell virus (20%), Acute bee paralysis virus (9.5%), Lake Sinai virus (2.5%) and Sacbrood virus (2.5%) in sampled colonies. Furthermore, one asymptomatic colony in a protected area of the western highlands was found positive for *P. larvae* ERIC I strain whose virulence was at least equally high as the reference strain on carniolan bees. A new microsporidian that is smaller than the known honeybee microsporidian parasites; *Nosema ceranae* and *Nosema apis* was detected in Ugandan honeybees. The new microsporidian has fewer polar filament coils (10 - 12), compared to 20 - 23 for *N. ceranae* and more than 30 often seen in *N. apis*. This new microsporidian was found at higher infestation rates compared to the other two known microsporidian parasites of honeybees. *Varroa* mite infestation levels in the eastern zone was significantly higher than that in the western during the dry season ($P = 0.02$). *Varroa* mites were spreading from lower to higher

elevations. Feral colonies were also infested with parasites and pathogens similar to those in managed colonies. Landscape factors like altitude and land use influenced honeybee parasite/pathogen distribution and infestation levels. Increase in viral diversity in Ugandan honeybee colonies and high *Nosema* spore loads reduced colony performance while *Varroa* mite infestation alone did not affect colony strength and productivity. The detection of these pathogens and parasites highlight the need for vigilance and development of a national honeybee health strategy to manage them in the country. Detailed studies on the new *Nosema* species and generally African honeybee health should be carried out in order to: 1) understand distribution, transmission, development, 2) identify associated effects on honeybees at individual and colony levels and 3) to reach a better understanding of the poorly known African honeybee pathogen complex especially in terms of the identities of pathogens present and survival mechanisms.

Samenvatting

In Uganda wordt de honingbij, *Apis mellifera*, hoofdzakelijk gezien als leverancier van verschillende natuurproducten zoals honing, bijenwas, konginnengelei en propolis, maar ze speelt tevens een belangrijke rol in de bestuiving van verschillende bloemen en planten. Hoewel de bijenteeltsector groeit en het een inkomstenbron, voeding en medicijnen verschaft aan vele gezinnen vormt de potentiële dreiging van parasieten en pathogenen een uitdaging voor de sector. Recent werd één van de belangrijkste parasieten, de varroamijt, in Uganda vastgesteld. Deze laatste wordt verantwoordelijk geacht voor de grote verliezen in de bijenteelt in Europa, Azië en Noord-Amerika. Hoewel, de gevolgen van deze parasiet op de Europese honingbij goed bestudeerd zijn, zijn de distributie, besmetting en hun effecten op de Afrikaanse honingbij weinig of niet onderzocht. In deze scriptie werden bijen bemonsterd van zowel beheerde als van wilde kolonies in twee agro-ecologische zones van Uganda elk met een hoogte gradiënt en wisselende landgebruik om deze problematiek verder uit te spitten. De bijen werden verzameld tijdens het droge en het natte seizoen tussen december 2014 en september 2015. De monsters werden onderzocht naar de aanwezigheid van 11 RNA-virussen, *Paenibacillus larvae*, *Mellisococcus plutonius*, *Ascosphaera apis*, Nosema spp. en de varroamijt met behulp van standaard technieken met als doel de prevalentie, besmettingsgraad en hun effecten op de kolonieprestaties te bestuderen. Vijf RNA virussen, *P. larvae*, drie Nosema spp. en de varroamijt werden gedetecteerd. De prevalentie van virussen in de onderzochte kolonies zijn: Deformed wing virus (51,9%), Black queen cel virus (20%), Acute bee paralysis virus (9,5%), Lake Sinai virus (2,5 %) en Sacbrood virus (2,5%). Bovendien werd een asymptomatische kolonie in een beschermd gebied van de westelijke hooglanden positief bevonden voor een *P. larvae* ERIC I stam. Zijn virulentie is te vergelijken met de referentiestam op carnioolse bijen. Een nieuwe parasiet behorende tot de microsporida familie werd geïdentificeerd. Deze nieuwe *Nosema* species is kleiner dan de veel voorkomende *Nosema ceranae* en *Nosema apis* en heeft minder polaire filament windingen (10 - 12) in vergelijking met *N. cerenae* en *N. apis* die respectievelijk 20 tot 23 en soms meer dan 30 windingen bezitten. Dit nieuw species werd met hogere prevalenties in de Ugandese

bijenpopulatie teruggevonden in vergelijking met de twee gekende species. In de Oostelijke zone was de graad van varroamijt besmetting significant hoger dan die in de Westelijke zone althans tijdens het droge seizoen (P = 0,02). Varroamijten verspreiden zich van lager naar hoger gelegen gebieden. Wilde en beheerde bijenkolonies waren vergelijkbaar besmet met parasieten en ziekteverwekkers. Omgevingsfactoren zoals hoogte en landgebruik beïnvloedden zowel de distributie als de infestatieniveas van de verschillende parasieten en pathogenen. De aanwezigheid van meerdere virusbesmettingen en hoge *Nosema* sporenlasten in de Ugandese bijenvolken verminderde de kolonieprestaties, terwijl het niveau van de Varroamijtbesmetting op zich geen invloed had op de koloniesterkte noch op de productiviteit. De detectie van deze pathogenen en parasieten onderstrepen de noodzaak tot waakzaamheid en de ontwikkeling van een nationaal programma die de gezondheid van de honingbij bewaakt. Meer doorgedreven onderzoek naar de nieuwe Nosema soorten en over het algemeen naar de gezondheid van de Afrikaanse honingbijen moet worden uitgevoerd om 1) hun verspreiding, overdracht en ontwikkeling te begrijpen 2) de bijbehorende effecten op individuele bijen en op kolonieniveau te identificeren en 3) om meer inzicht te krijgen in de totnogtoe slecht gekarakteriseerde Afrikaanse pathosfeer en naar de overlevingsmechanismen van de Afrikaanse honingbij.

CHAPTER 1

General introduction

1.1 Beekeeping: a source of sustainable livelihood

The beekeeping industry is an important source of food and employment for many rural households in developing countries (Bradbear, 2009). Beekeeping is also important in rural poverty alleviation, environmental conservation and diversification of national export bases; King *et al*., 2011)**.** Compared to other agricultural enterprises such as aquaculture, poultry and cattle farming, beekeeping is a relatively low-cost and low labor intensive enterprise that does not require a lot of land (Jacobs *et al*., 2006). This makes it viable for the people (e.g. women and youths) who are least likely to access production factors (Adjare, 1990; UEPB, 2005). In Uganda, women are actively involved in beekeeping and processing of beehive products: honey, beeswax (e.g. making candles), while the youth are involved in making beekeeping equipment and vending honey. Honey, pollen and bee brood are sources of carbohydrate and protein that rural people can obtain at minimal costs (UEPB, 2005). National and international pharmaceutical and cosmetic industries use bee products such as honey, propolis, royal jelly, bee venom and beeswax.

The most important service that honeybees give to mankind is pollination (e.g. Kevan & Phillips, 2001; Dietemann *et al*., 2009; Vaudo *et al*., 2012). Most crops (> 60% of the 1330 cultivated crop species) depend on pollination by bees (Zych & Jakubiec, 2006; Jacobs *et al*., 2006; Klein *et al*., 2007), indicating the vital role of these insects in our food security. In Uganda, the role of honeybees in pollination is gradually being appreciated. As such, honeybees are currently used for pollination of coffee, cotton, pulses, oil seeds, mangoes, oranges, peas, beans and spices (Munyuli, 2013).

Markets for Uganda's beehive products are continuing to grow both locally and internationally. For instance, the European Union (EU) licensed Uganda to export honey to its market in 2005, creating immense opportunities for the beekeepers and those involved in the honey export value chain (UEPB, 2005). As a result, the beekeeping sector in Uganda is growing and has the potential to provide more employment opportunities to several people. However, honeybees' susceptibility to environmental factors including attacks by numerous parasites and pathogens

like *Varroa* mites, viruses, bacteria and fungi pose significant threats not only to the health of honeybees and honeybee services, but to the production of beehive products. Therefore, there is need to maintain large and healthy populations of honeybees in Uganda for sustainable production and supply of honey, other beehive products and bee pollinated crops both to the local and international markets.

1.2 Honeybees

Currently, nine honeybee species in the genus *Apis* are recognized (*Apis florae* Fabricius, 1787; *Apis andreniformis* Smith, 1858; *Apis dorsata* Fabricius, 1793*; Apis cerana* Fabricius, 1793; *Apis mellifera* Linnaeus, 1758; *Apis laboriosa*; *Apis nuluensis*; *Apis koschevnikovi* and *Apis nigrocinta)* (Suwannapong *et al.,* 2011). Of the nine species*, A. mellifera* is the most important to the beekeeping sector. *A. mellifera* is endemic to Africa but has been introduced throughout the world (Gençer *et al*., 2004; Nedic *et al*., 2009). It has successfully adapted to the varied conditions across the globe resulting in over 25 races (Ruttner, 1988 and Sheppard *et al*., 1997).

In Africa, at least ten races of *A. mellifera* have been described (Franck *et al*., 2001; Figure 1.1). These include: *A.m. intermissa*; a North African honeybee race found north of the Sahara from Libya to Morocco (Chahbar *et al*., 2013). It is a very aggressive bee that swarms frequently. During droughts, over 80% of colonies may die but due to intensive swarming, colony numbers increase when conditions improve. Within a colony, *A.m. intermissa* rear numerous queens producing several queen cells. Another African honeybee race is *A.m. lamarckii* also called Egyptian bee which is found in North Eastern Africa primarily in Egypt and the Sudan along the Nile valley (Garnery *et al*., 2001). *A.m. lamarckii* has a reproduction strategy similar to *A.m. intermissa* in that they rear numerous queens with one colony recorded as rearing 368 queen cells and producing one small swarm with 30 queens. Another African honeybee race is *A.m. scutellata*, which is found in the savannahs of central and equatorial East Africa and most of South Africa (Franck *et al*., 2001). *A.m. scutellata* is small with a short tongue but highly aggressive and swarms frequently. They nest in a broad range of sites from cavities to open nests. In addition, *A.m. adansoni* is another African honeybee race found in West and East Africa and is yellow in colour

(Ruttner, 1982). They appear to be very similar to *scutellata* in their behaviours. Furthermore, *A.m. monticola* the mountain bee found at high altitudes (1500 - 3100 meters above sea level) in Kenya, Tanzania and Ethiopia is another African honeybee race (Prof. Frans Jacobs, personal communication). These bees are a dark and gentle race with longer hairs than other African races of bees. Another African honeybee race, *A.m. capensis* is found in South Africa and has a unique reproductive behaviour. Females other than the queen of *A.m. capensis* lay eggs which hatch into worker bees. Other races include: *A.m. jemenitica* in the northwest and eastern arid and semi-arid lowlands of Ethiopia (Al-Ghamdi *et al*., 2013), *A.m. bandasii* in the central moist highlands of Ethiopia and *A.m. woyi-gambell* in south western semi-arid to sub-humid lowland parts of Ethiopia (Amssalu *et al*., 2004). Although three races of honeybees (*A.m. scutellata*, *A.m. adansoni*, *A.m. monticola*) were believed to be present in Uganda, only two (*A.m. scutellata*, and *A.m. adansoni*) have been confirmed (Kasangaki, 2016).

Figure 1.1: Distribution of honeybee races in Africa according to Ruttner (1988).

1.3 Honeybee colony performance

The performance of honeybee colonies which includes their strength and productivity is an important factor in beekeeping. Colony strength is a critical factor for maximizing honey production in *A. mellifera* (Neupane *et al*., 2012) and is correlated to the honey yields (Jevtić *et al*., 2009). The strength of a honeybee colony is important in selecting colonies for pollination (Delaney and Tarpy, 2008; Abou-Shaara, 2014). The strength of a honeybee colony can be estimated by considering the number of adult worker bees in the hive, the brood pattern and the flight activities (Pokhrel *et al*., 2006; Vaudo *et al*., 2011; Ali, 2011; Delaplane *et al*., 2013). A new method of measuring colony strength has been developed which uses computer assisted digital image analysis (Delaplane *et al*., 2013). Colony productivity on the other hand is measured in terms of honey produced and pollen collected (Ali, 2011). The strength and productivity of honeybee colonies is influenced by factors including queen egg-laying capacity, availability of empty combs, climatic conditions and supply of both pollen and nectar (Ali, 2007). A good vegetation cover provides ample supply of forage for honeybees and drives brood production and increase of honey reserves (Amssalu, 2002). At higher altitudes, the temperature is cooler and this makes honeybees spend much of their time warming the brood and the hive hence limiting foraging activities (Hemp, 2005). Honeybee colony strength and productivity can be improved through proper bee management practices including; hive inspection, pest control and provision of water (Kumsa and Takele, 2014).

In Uganda, the honeybees (*A. mellifera*) are mostly kept for their products such as honey, propolis, beeswax and more recently honeybee venom. However, the role of honeybees in pollination is gradually being understood and appreciated. Therefore, honeybees are currently used by some farmers for pollination of fruits and vegetables in Uganda (Munyuli, 2013). As the beekeeping sector in Uganda develops, it is important to understand the performance of honeybee colonies and how this might be affected by factors that influence honeybee health for informed planning.

1.4 Factors influencing honeybee health

Honeybee health is potentially influenced by factors that include; honeybee nutrition, environmental temperatures, pesticides, parasites and pathogens. Honeybees require carbohydrates, amino acids, lipids, vitamins, minerals and water for their survival and reproduction (Di Pasquale *et al*., 2013). These food nutrients originate from different sources; carbohydrates are obtained mainly from nectar or honey while amino acids, lipids, vitamins and minerals are acquired mainly from pollen. These nutrients must be present in the right proportions to ensure honeybee survival and reproduction. Any deficiencies or excess of some nutrients may result in increased parasite infestation and or impact on honeybee health. For instance, protein supplementation in honeybee colonies causes increased *Nosema* parasite infections (Mendoza *et al*., 2012). Also, nutritional deficiencies provide conducive conditions for multiplication of *Nosema* spores (Invernizzi *et al*., 2011) which impact the honeybee health.

Similarly, weather conditions can impact on honeybee health and have been suspected to cause honeybee colony losses. A study in the United States showed that regions with relatively lower average temperatures had higher colony losses (VanEngelsdorp *et al*., 2008). However, direct impacts of temperature on honeybee survival are expected to be insignificant in Uganda where average annual temperature is about 26^{o}C.

Pesticides applied to agricultural crops and in beehives to control honeybee parasites have been suggested to compromise the immune systems of honeybees (Quarles, 2011) leading to increased pathogen infestation levels (Pettis *et al*., 2012; Dively *et al*., 2015). This can be attributed to some pesticides significantly affecting the expression of genes involved in several core physiological pathways in honeybee workers (Schmehl *et al*., 2014). However, pesticide use in most Ugandan farms is still low and might not currently be of great concern. Moreover, beekeepers in Uganda to date are not using pesticides to control *Varroa* mites.

Parasites and pathogens such as *Varroa* mites, viruses and fungal pathogens have been shown to contribute to colony losses in North America and Europe (VanEngelsdorp *et al*., 2008;

Neumann & Carreck, 2010). Infestation by honeybee parasites and pathogens and their synergistic interactions with other suspected drivers of colony losses are therefore a major threat to the apiculture sector worldwide.

Honeybee colonies in Uganda are potentially threatened by honeybee parasites and pathogens like *Varroa* mites (Kasangaki *et al*., 2016; Chemurot *et al*., 2016) and viruses such as the Black queen cell virus (BQCV) (Kajobe *et al*., 2009; 2011) that have been confirmed in the country. These parasites and pathogens may impact on honeybee health which will directly affect food security if not managed appropriately.

1.4.1 Honeybee pathogens and parasites

1.4.1.1 Viruses

Honeybees can be infected by many viruses (Chen and Siede, 2007). A total of 23 viruses have been identified and characterized in honeybees (Chen *et al*., 2004; Chen and Siede, 2007; Runckel *et al.*, 2011; McMenamin and Genersch, 2015; Gisder and Genersch, 2015). Most of these viruses are single-stranded positive RNA viruses of which six are the most common infections and hence form the focus of most honeybee virus research activities (Chen and Siede, 2007). These include: Deformed wing virus (DWV), Black queen-cell virus (BQCV), Sacbrood virus (SBV), Kashmir bee virus (KBV), Acute bee paralysis virus (ABPV) and Chronic bee paralysis virus (CBPV) (Chen and Siede, 2007; De Smet *et al*., 2012).

The DWV and SBV belong to the genus *Iflavirus*, family Iflaviridae (Chen and Siede, 2007). The DWV has been detected worldwide (Ellis and Munn, 2005) and is the most prevalent honeybee virus (Chen and Siede, 2007). Infection of honeybees by DWV results in well-defined symptoms which include shrunken, crumpled wings, decreased body size and discoloration in adult bees (Chen and Siede, 2007). This honeybee virus has been detected in all stages (eggs, larvae, pupae and adult bees) of honeybee development (Chen and Siede, 2007; de Miranda and Genersch, 2010). Infection of honeybees by DWV can result in suppression of the immunity system of honeybees (Di Prisco *et al*., 2016), reduction in the lifespan of bees (Chen and Siede, 2007) and

colony losses (de Miranda and Genersch, 2010). However, in the absence of *V. destructor*, DWV normally persists at low levels within the honeybee colony with no detrimental effects (Chen and Siede, 2007; de Miranda and Genersch, 2010). Transmission of DWV occurs both horizontally (fecal-oral, cannibal, *Varroa*-mite vectored) and vertically (parent-offspring) (de Miranda and Genersch, 2010).

Like DWV, SBV has been detected in all continents where *A. mellifera* colonies are present (Chen and Siede, 2007). SBV attacks both brood and adult stages of honeybees (Shen *et al*., 2005; Chen and Siede, 2007). Larvae of about 2 days old are most susceptible to SBV infections (Chen and Siede, 2007) which result in larval death (Shen *et al*., 2005). Infection of adult bees by SBV causes no obvious signs of the disease but infected adult bees may have a shortened lifespan (Chen and Siede, 2007). The initial spread of SBV within a colony occurs when nurse bees become infected while removing larvae killed by SBV. Viral particles accumulate in the hypopharyngeal glands of nurse bees who can then spread the virus throughout the colony by feeding larvae with infected glandular secretions and exchanging contaminated food with other adult bees including foragers within the colony. Young larvae become infected with SBV by ingesting food contaminated with the virus. SBV starts to replicate in the larvae and infected larvae turn pale-yellow after the brood is capped. As the disease progresses, the skin of the larva becomes leathery and the larva fails to pupate because it cannot digest the old cuticle. Sacbrood derives its name from the saclike appearance of diseased larvae (Chen and Siede, 2007).

The BQCV is an RNA virus within the genus *Cripavirus* (Family Dicistroviridae) (Mayo, 2002). It is one of the common viruses that affect honeybees (Chen and Siede, 2007). However, its implication in honeybee mortality remains poorly understood (Higes *et al*., 2007a). While the BQCV was reported as the major cause of queen larvae mortality in Australia (Somerville, 2010), the virus was detected in 86% of adult samples and 23% of pupae on a survey in healthy French bee colonies (Tentcheva *et al*., 2004), and recently in Austrian apiaries (Berényi *et al*., 2006). Diseased larvae have a pale yellow appearance and a tough sac-like skin, a symptom also observed in SBV infected larvae (Chen and Siede, 2007). The BQCV multiplies rapidly in pupal

stages of honeybees resulting in dark coloration and rapid death. The wall of the queen cell eventually becomes dark colored, a characteristic symptom of BQCV infection (Chen and Siede, 2007). BQCV disease outbreaks have been linked with the microsporidian *Nosema apis* infection in honeybee colonies (Higes *et al*., 2007a). It is thought to be transmitted to queen brood via glandular secretions by nurse bees during feeding (Chen and Siede, 2007). Since *N. apis* infects the midgut of adult honeybees (Fries, 1988), it increases the susceptibility of the alimentary canal to BQCV infections (Chen and Siede, 2007).

The ABPV, KBV and Israeli acute paralysis virus (IAPV) are closely related viruses from the Family Dicistroviridae (Mayo, 2002; Chen and Siede, 2007). Apart from having close genetic relationship, these viruses share a number of biological characteristics, such as the principal routes of transmission, the primary host life stage, and a low but widespread prevalence with a predominantly sub-clinical etiology that contrasts sharply with the extremely virulent pathology encountered at elevated titres, either artificially induced or encountered naturally (de Miranda *et al*., 2011). ABPV can be detected in brood and adult bees. And in the field, it is commonly found in apparently healthy colonies (Chen and Siede, 2007). ABPV spread in colonies occurs via infected salivary gland secretions from adult bees to young larvae or that is mixed with pollen. In addition, *Varroa* mites have been suggested as vectors of ABPV. This virus in association with *Varroa* mites has been linked to colony declines and collapse in Europe and America (Chen and Siede, 2007; de Miranda *et al*., 2011).

KBV infects all life stages (eggs, larvae, pupae, adults) of the honeybee causing mortality without clear specific symptoms (Chen and Siede, 2007). The KBV is considered the most virulent honeybee virus under laboratory conditions because it multiplies quickly once a few viral particles are introduced into the hemolymph causing bee death within three days (Chen and Siede, 2007). KBV does not cause infection when adult bees are fed with food mixed with viral particles suggesting that the virus invades the bees through the cuticle. *Varroa* mites have been shown to vector KBV (Shen *et al*., 2005; Chen and Siede, 2007) and high mite infestation is linked to high virulence of KBV in infected colonies (Chen and Siede, 2007).

CBPV is a double stranded RNA honeybee virus that has not officially been assigned to a genus. The CBPV attacks mainly adult bees causing paralysis; trembling of the body and wings, crawling on the ground due to flight inability, bloated abdomen and dislocated wings (Chen and Siede, 2007; Ribière *et al*., 2010). Another clear sign of CBPV infection is the presence of hairless, shiny and black appearing bees (Ribière *et al*., 2010) that are normally attacked and rejected from returning to the colonies at the entrance by guard bees (Chen and Siede, 2007). Although CBPV and ABPV cause similar symptoms such as trembling and inability to fly in infected bees, CBPV is less virulent (Chen and Siede, 2007). Spread of CBPV occurs via contaminated food and body contacts in overcrowded colonies (Ribière *et al*., 2010).

Although, only one honeybee virus (BQCV) had been confirmed in Uganda before my present study (Kajobe *et al*., 2011), there has been growing concern regarding the health status of local honeybee colonies especially with the discovery of the recently introduced *Varroa* mites which are vectors of different viruses. The detailed Ugandan honeybee viral pathosphere and distribution had not been clearly understood despite the documented impacts of some honeybee viruses in Europe and America.

1.4.1.2 Bacterial brood pathogens

There are two known serious brood bacterial pathogens of honeybees. These are *Paenibacillus larvae*: the causative agent for American foulbrood (AFB), and *Mellisococcus plutonius*: the causative agent for European foulbrood (EFB) (Genersch, 2010b). These bacterial pathogens have been reported worldwide (Ellis and Munn, 2005; Genersch, 2010a; Morrissey *et al*., 2014) and are threats to the health of honeybee colonies (Genersch, 2010b). For instance, *P. larvae* produces spores that can survive for long periods and are resistant to heat and chemical agents (Genersch, 2010a). As a result, AFB is listed in the Office International des Epizooties – the World Organization for Animal Health (OIE) Terrestrial Animal Health Code (2011) and member countries and territories are obliged to report its occurrence (OIE, 2011).

AFB transmission occurs mainly through the horizontal routes (Mill *et al*., 2014) via various bee behaviours (robbing and drifting), beekeeping practices and infected honey (Genersch, 2010),

but it is also known to be transmitted vertically as honeybees swarm (Fries *et al*., 2006). Although *Paenibacillus larvae* has a worldwide distribution (Ellis and Munn, 2005), few confirmations of the AFB disease have been made in Africa (Pirk *et al*., 2015).

Similarly, EFB is transmitted horizontally with adult worker bees acting as carriers of the bacterium both within the colony and between colonies and apiaries (Forsgren, 2010). Like AFB, EFB has a worldwide distribution (Forsgren, 2010; Budge *et al*., 2010) but no confirmations have been made in several African countries including Uganda. In Africa, EFB has been reported in Algeria, Libya, Morocco, Tunisia, Tanzania, Guinea-Bissau and Senegal (Hussein, 2001). Since African honeybee races abscond and swarm often (Pirk *et al*., 2015), pathogens such as *P. larvae* might be circulating between feral and managed colonies but at low infestation levels due to the predominant use of fixed comb beehives (Gupta, 2014). However, with the increasing promotion and use of frame beehives, the situation could change necessitating planning for the management of the parasites and pathogens circulating in local honeybee populations.

1.4.1.3 Fungal brood parasites

Ascosphaera apis is an important fungal brood pathogen of honeybees causing the chalkbrood disease (Jensen *et al*., 2009). *A. apis* only produces sexual spores and is heterothallic, thus spores are only produced when mycelia of the two opposite mating types come together and fruiting bodies are formed (Aronstein *et al*., 2007). Infection of honeybee larvae primarily occurs when they ingest food contaminated with spores (Jensen *et al.*, 2009). The spores germinate in the lumen of the gut because of the presence of suitable conditions such as high carbon dioxide concentration. Infected larvae are covered by a white, chalky mycelium, dying after their brood cells are sealed. Larvae infected by this fungus are mummified and show different colors (Jensen *et al*., 2013).

1.4.1.4 Microsporidia

The microsporidia are specialized parasitic fungi which invade the midgut epithelial cells of honeybees. Two species of Microsporidia (Family Nosematidae), *Nosema apis* (Zander, 1909) and

Nosema ceranae (Higes *et al*., 2006; Huang *et al*., 2007; Traver *et al*., 2012) infest the honeybee worldwide. *N. ceranae* was originally described as a parasite of *Apis cerana* (Fries *et al*., 1996) but was later reported as a pathogen of *A. mellifera* (Higes *et al*., 2006; Huang *et al*., 2007). Both microsporidian species are obligate intracellular parasites of the midgut of honeybees and have been reported in all continents (Ellis and Munn, 2005). *N. apis* and *N. ceranae* have similar morphologies under an optic microscope and SSU rRNA sequence identity is 92% (Fries *et al*., 1996). However, under the transmission electron microscope, spores of *N. ceranae* are smaller than those of *N. apis* (Huang *et al*., 2007). In addition, spores of *N. ceranae* have fewer (20 to 23) polar filament coils (Fries *et al*., 1996) than *N. apis* which usually contains over 30 polar filament coils (Huang *et al*., 2007).

Some recent reports suggest an apparent displacement of *N. apis* by *N. ceranae* (Paxton *et al*., 2007; Martín-hernández *et al.*, 2012). However, this replacement of *N. apis* by *N. ceranae* is not supported when the distribution and prevalence of both microsporidia in different climatic conditions is considered. *N. ceranae* infections appear to dominate in the warmer and temperate regions, whereas *N. apis* is more common in colder climates (Martin-Hernandez *et al.*, 2012). Both species seem to have different seasonal patterns (Runckel *et al*., 2011; Martin-Hernandez *et al*., 2012).

N. apis infection in honeybees causes Nosemosis type A which results in significant damage of the gut tissue, reducing winter survival in temperate climates, honey production and pollination effectiveness (Pacini *et al.*, 2016). On the other hand, infection of honeybees by *N. ceranae* causes Nosemosis type C which can lead to nutritional and energetic stress (Mayack and Naug, 2009), suppression of the host immune response (Antúnez *et al.*, 2009) increasing susceptibility to other pathogens and decreased host survival at the individual level (Higes *et al*., 2007b). The prevalence of *N. ceranae* and *N. apis* worldwide has raised concerns within the beekeeping industry especially with the recent declines in honeybee populations.

Transmission of *N. apis* is primarily through the oral-fecal route and the same route of transmission has been demonstrated for *N. ceranae* (Smith, 2012). Honeybees become infected with *Nosema* by ingesting food contaminated with the spores. Under laboratory conditions, when a bee ingests *N. apis* spores, germination and production of spores occurs within four days post infection (Fries, 1988). For *N. ceranae*, by day 3 post infection, spore infections in the epithelial cells of the midgut could be detected (Higes *et al*., 2007b). Sporulation of *N. apis* occurs only in the host honeybee's midgut epithelium, while *N. ceranae* has been reported to infect the honeybee alimentary canals, malpighian tubules, hypopharyngeal glands, salivary glands and fat body (Chen and Huang, 2010).

1.4.1.5 *Varroa* mites

Varroa mites are ecto-parasites of honeybees parasitizing on both brood and adult bees. Four species of *Varroa* mites are recognized and these include: *Varroa jacobsoni* (Oudemans, 1904), *Varroa underwoodi* (Delfinado-Baker & Aggarwal, 1987), *Varroa rindereri* (De Guzman & Delfinado-Baker, 1996) and *Varroa destructor* (Anderson & Trueman, 2000). These mites were first described in their natural host, the Asian honeybee, *Apis cerana* (Goodwin & Eaton, 2001) which co-evolved with the mite to develop a natural resistance (Navajas *et al*., 2010; Le Conte, Ellis, & Ritter, 2010). However, the mites switched host to the European honeybee, *A. mellifera* (Zhang, 2000) because of movement of honeybee colonies to the Far East with the aim of improving honey production (Le Conte *et al*., 2010). *V. destructor* has spread to all continents. However, the extent of spread and infestation levels in most parts of Africa remains to be explored.

Female *Varroa* mites enter honeybee brood cells containing the last instar of honeybee larvae to lay their eggs. Normally, the first egg which is usually unfertilized is laid approximately 70 hours after cell capping and it develops into a male mite. Afterwards, fertilized female eggs are laid in intervals of 30 hours. As a result, the male mite develops first and mates with the females within the brood cell. On average, a single female mite will lay five eggs in worker brood and six eggs in drone brood. The eggs of the mite hatch to produce nymph that develops through two nymphal

stages, the protonymph and deutonymph before molting into an adult (Rosenkranz *et al.*, 2010). The *Varroa* mite feeds on the hemolymph of the last instar larvae, pupae and adult bees resulting in serious weight loss and reduction of honeybee life span (Rosenkranz *et al*., 2010). Infestation of honeybees by *Varroa* also results in severe impact on honeybee health since the mites suppress honeybee immunity (Navajas *et al*., 2008; Navajas *et al*., 2010) and transmit honeybee viruses (Rosenkranz *et al.*, 2010).

Until 2009, *V. destructor* was only found in honeybee colonies in Kenya but not in Uganda (Fazier *et al*., 2010). However, by 2013, the mite was found to be widely distributed in Uganda (Kasangaki *et al*., 2015). This suggests that *Varroa* mites could have invaded Uganda more recently than neighboring Kenya. *Varroa* mites get into new environments through movement of infested honeybee colonies (swarming/migration). In addition, human activities such as honeybee colony inspections and colony division practices contribute to the dispersal of *Varroa* mites (Navajas *et al*., 2010). With the on-going rapid movements of people and beekeeping goods, the rate of dispersal of this mite and other honeybee parasites and pathogens may increase. Currently, limited information is available on *V. destructor* infestation levels and effects on honeybee colonies in Uganda. Investigations are therefore needed to establish *V. destructor* infestation levels and their impacts on honeybee colonies for informed decision making.

1.4.1.6 Other honeybee parasites and pathogens

***Tropilaelaps* mites**

The *Tropilaelaps* mites are ectoparasites of honeybee brood with a short phoretic phase on adult honeybees. Adults of this parasite cannot fly and require honeybee brood to survive. Different species of *Tropilaelaps* mites including: *Tropilaelaps clareae*, *T. koenigerum*, *T. thaii* and *T. mercedesae* infest honeybees (Anderson & Morgan, 2007). The native primary hosts of *Tropilaelaps* are the non-domesticated giant Asian honeybees, *Apis dorsata*, *A. breviligula* and *A. laboriosa*. Following its host shift to *A. mellifera*, *Tropilaelaps* has spread from mainland Asia to bordering areas and is currently prevalent in Indonesia and the Philippines to Afghanistan, Iran, New Guinea and South Korea. The infestation and feeding activities of the *Tropilaelaps* mites reduces the western honeybee longevity and emergence weight and promotes DWV infections

(Khongphinitbunjong *et al.*, 2016). As such, the *Tropilaelaps* mites are one of the most damaging pests of the honeybees (Anderson & Morgan, 2007; Dainat *et al*., 2009). Currently, the *Tropilaelaps* mites remain confined to Asia and bordering areas but are recognized as emerging threats to world beekeeping (Anderson & Roberts, 2013).

Acarapis woodi

This is an internal parasitic mite of the honeybee respiratory system. It feeds on the hemolymph of honeybees. Honeybee infestation by *A. woodi* causes obstructions of the trachea, lesions and hemolymph depletion (Sammataro *et al*., 2013). It is considered to be associated with poor winter survival in honeybees (McMullan & Brown, 2009). A recent study detected *A. woodi* in *A. cerana* collapsing colonies in Japan (Kojima *et al*., 2011). This mite has been reported almost worldwide (Ellis & Munn, 2005) and in most countries in Africa (Matheson, 1993). Since overwintering does not occur at the same scale in Africa as Europe, mite infestations may not result in the severe damage as seen in European countries (Pirk *et al*., 2015). However, very limited data are available on the presence, infestation levels and effects of these mites in African honeybee colonies.

Trypanosomatid parasites

Honeybees are also parasitized by trypanosomatids which belong to the class Kinetoplastea (phylum Euglenozoa) and order Trypanosomatida. Parasites in the class Kinetoplastea are characterized by a kinetoplast DNA, a network of condensed mitochondrial DNA (Adl *et al*., 2013). Trypanosomatids have one flagellum and can change their morphology during their life cycle (Field & Carrington, 2009). Some groups in the class Kinetoplastea complete their lifecycle in one host (monoxenous), others like the human parasites *Trypanosoma* spp. and *Leishmania* spp. require a second host (dixenous) (Lukeš *et al*., 2014).

Although honeybees are known to be parasitized by trypanosomatids, limited studies have focused on these parasites. For instance, since *Crithidia mellificae* (Family Trypanosomatidae) was described in 1967 (Langridge and Mcghee, 1967), it remained ignored until molecular markers were developed (Schmid-Hempel & Tognazzo, 2010) and cell culture techniques became

available (Runckel *et al*., 2011). A recent study showed that *Lotmaria passim* is the predominant trypanosomatid of honeybees and was previously considered to be *C. mellificae* suggesting that all previous reports of *C. mellificae* should be reconsidered (Schwarz *et al*., 2015). *C. mellificae* was shown to contribute to honeybee colony collapses (Ravoet *et al*., 2013) hence highlighting the relevance of investigating it. In Africa, *C. mellificae* has been detected in Algeria (Menail *et al*., 2016). However, it remains to be investigated in most parts of sub Saharan Africa.

Hive beetles

The small hive beetle (SHB), *Aethina tumida* is a honeybee pest which is native to sub-Saharan Africa, where it was previously considered a minor pest (Neumann & Elzen, 2004; Cuthbertson *et al*., 2013). However, recent field observations in Malawi (Dr. Wim Reybroeck personal communication) and Uganda (Dr. Patrice Kasangaki, personal communication) indicate destructive effects of SHB on African honeybees. The beetles are harmful pests of the European honeybee subspecies (Hood, 2000). They are bee brood scavengers of *A. mellifera*, *Bombus* spp. (bumble bee) and *Melliponini* (stingless bees) (EFSA, 2013). The SHB is a flying, free-living coleopteran (Family: Nitidulidae) that can survive and reproduce on ripe fruits, but not on vegetables, plants or flowers (EFSA, 2013). Adult SHB can detect airborne volatiles produced by *A. mellifera* and *Bombus* spp. and can be attracted to the odours of bees and beehive products. Mature larvae of the SHB leave the hive and burrow in the soil to pupate. The SHB larval stage is destructive to bee populations, whereas the adults have little impact (EFSA, 2013).

Since 1998, there has been considerable international attention on the SHB due to their invasion of honeybees in the USA (Elzen *et al*., 1999), Australia (Neumann & Elzen, 2004b; Spiewok *et al*., 2007) and Italy (Mutinelli *et al*., 2014). This is attributed to heavy infestations of SHB inducing absconding (Ellis *et al*., 2003; Spiewok *et al*., 2006) and reducing honey stores (Ellis *et al*., 2003). Damage to honeybee colonies stems from the feeding habits of both adult and larval SHB (Elzen *et al*., 2001; Neumann & Elzen, 2004). The SHB larvae burrow through combs, eat honey and pollen, kill bee brood and defecate in honey which subsequently ferments leading to reduced quality and quantity of beehive products (EFSA, 2013). In contrast, successful reproduction of the

beetle in African honeybee colonies is often restricted to weak host colonies due to behavioral resistance mechanisms (Elzen *et al*., 2001; Neumann & Hartel, 2004), or is associated with after absconding events (Cuthbertson *et al*., 2013). Moreover, absconding is common in African honeybee subspecies (Chemurot *et al*., 2016) and can be triggered by parasite infestations and severe small hive beetle infestations (Hood, 2004). Therefore absconding is an important escape mechanism by honeybees to avoid pest or parasite infestation.

1.4.2 Anthropogenic factors influencing bee health

Human activities such as agriculture have impacts that affect a wide range of ecosystem services, including pollination and biodiversity conservation (Dale & Polasky, 2007). Intensive agriculture and continued movements of humans, honeybees and beekeeping equipment directly affect honeybees. This can be through exposure of honeybees to pesticides used in agriculture which may have lethal (Kevan, 1975; Greig-Smith *et al*., 1994) or sub-lethal behavioral or physiological effects (Thompson, 2003; Quarles, 2011). Such effects on honeybees may lower their immunity making them more vulnerable to parasites like *Varroa* mites. On the other hand, movements of people, honeybees and beekeeping equipment can lead to direct transfer of honeybee pests and pathogens. These movements may lead to increased contact of honeybees with new pests and infectious agents. In Uganda, agriculture is growing and involves movements of people and equipment from one place to another. Such movements could have led to introduction of new honeybee parasites into the country.

1.4.3 Honeybee traits influencing honeybee health

The behavior of honeybees just like other animals is influenced by internal factors. These internal factors influencing honeybee behavior are the genetic material that is inherited from the queen and drones. For instance, the hygienic behavior of honeybees which involves inspection, uncapping and removal of diseased and dead bee brood from the colony is determined by two independent gene loci (Stanimirovic *et al*., 2002). This behavior is believed to have an important role in honeybee defense against parasitic and infectious pathogens (Bekesi & Szalai, 2003; Palacio *et al*., 2010). A recent study by Nicodemo *et al*. (2013) showed that honeybee lines that

are selected for high propolis production also have superior hygienic behavior and increased honey and pollen stores. Moreover, it has been shown that infestation of honeybee colonies by parasites like *Varroa* mites and their impacts vary with honeybee heritable traits and behavioral adaptations (Rosenkranz, 1999; Buchler *et al*., 2010; Calderón *et al*., 2010; Rinderer *et al*., 2010; Emsen *et al*., 2012).

Furthermore, the honeybee grooming behavior is an important trait which reduces honeybee parasite infestation levels. For example, *V. destructor* infestation levels have been shown to be lower in honeybees with high grooming levels (Rosenkranz *et al*., 2010; Calderón *et al*., 2010). Also, the brood effect associated with suppressed *Varroa* mite reproduction (Harbo & Harris, 2005) which can be related to the hygienic behavior, short brood post capping periods or honeybee genetics (Rosenkranz *et al*., 2010; Calderón *et al*., 2010) can control *Varroa* mites. Consequently, the health, productivity and strength of honeybee colonies with these traits have been reported not to be affected by parasites and pathogens (Muli *et al*., 2014; Strauss *et al*., 2015).

1.4.4 Landscape factors influencing honeybee health

Studies on parasites including ticks (Gern *et al*., 2008; Shchuchinova *et al*., 2015) and mosquitoes (Eisen *et al*., 2008; Lozano-Fuentes *et al*., 2012) among others suggest that landscape factors influence their abundance and distribution. For instance, a study in Switzerland showed that tick densities at high elevations were lowest compared to low altitudes (Gern *et al*., 2008). The lower tick densities at high elevations were attributed to low temperatures that affect tick development (Gern *et al*., 2008; Shchuchinova *et al*., 2015).

Similarly, elevation has been cited to influence *Varroa* mite infestation levels (Muli *et al*., 2014; Mumbi *et al*., 2014). Furthermore, vegetation around apiaries influences parasite infestation levels. For example, epizootiological studies on the microsporidian honeybee gut parasite, *N. ceranae* in honeybee colonies have shown high infestation levels under *Eucalyptus grandis* plantations due to nutritional deficits (Invernizzi *et al*., 2011). Although the potential influence of

landscape factors on some honeybee parasite and pathogen infestation levels are fairly known, the exact mechanisms through which these factors influence parasite infestation remains to be explored.

Uganda has an altitudinal gradient ranging from 620 meters (Albert Nile) to 5,111 meters (Mt. Rwenzori) (Kabi *et al*., 2014). It is endowed with varying climatic conditions and vegetation types that support different land uses. Based on vegetation type, elevation, climatic conditions and the agricultural activities, Uganda is divided into ten (10) production or agro-ecological zones (Kajobe *et al*., 2009). Despite the known varying altitudes and land uses in Uganda, no studies had investigated the potential influence of these factors on the distribution of honeybee parasites and pathogens in the country. Knowledge of these parameters could be useful in planning for honeybee parasite and pathogen management in the country to reduce any likely risks. The honeybee sampling and analysis for this study was conducted in two AEZs having altitudinal gradients and varying land uses in order to capture the variations within the country.

1.5 Conceptual framework

Interactions between landscape components have consequences on honeybee parasites and pathogens (Figure 1.2). In general, landscape factors like altitude and vegetation directly or indirectly affect environmental conditions such as temperature, humidity, wind speed and availability of honeybee forage. Also, landscape factors influence land use types with intensive farming activities taking place at elevations that allow easy mechanization. Consequently, areas that have high farming intensities are likely to have reduced honeybee forage diversity and tend to have high pesticide levels. Horizontal spread of honeybee parasites and pathogens is also assumed to increase with intensified human activity in farmlands. Poor honeybee nutrition arising from high farming intensity and the use of pesticides is likely to lower the immunity of honeybees making them more susceptible to infections by parasites and pathogens. High parasite/pathogen infestation levels in honeybees may have negative impacts that include lowering of colony strength and productivity which consequently lead to poor incomes from beekeeping.

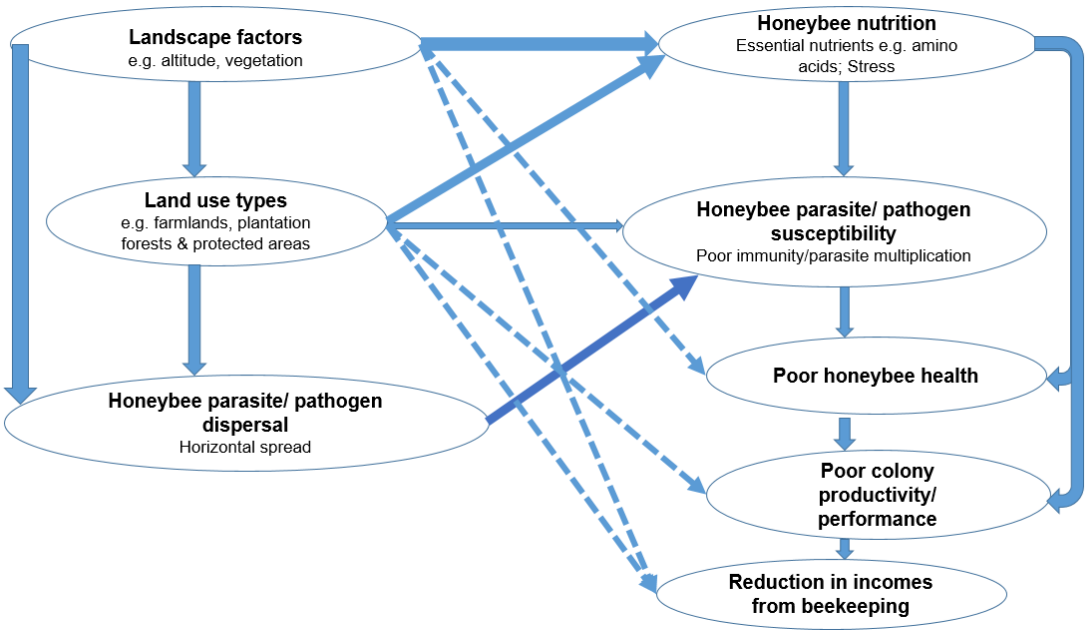


Figure 1.2: Interactions between landscape factor components and their consequences on honeybee parasites and pathogens.

1.6 Problem statement

Although the beekeeping industry in Uganda is growing and providing a source of food, medicine, income and employment to many rural households (UEPB, 2005), the potential threat from honeybee parasites and pathogens poses challenges to the sector. Some of the parasites which have devastated the beekeeping industry in Europe, Asia and North America have already been registered in Africa. For example, *Varroa* mites which cause varroosis have been confirmed in Uganda (Kasangaki *et al*., 2015; Chemurot *et al*., 2016) and Kenya (Fazier *et al*., 2009) among many *Varroa*-suspect countries. This mite is known to transmit ABPV, Kashmir bee virus (KBV) (Bakonyi *et al*., 2002) and DWV in honeybees (Shen *et al*., 2005) among others. However, before the current study, only one documented study in Uganda (Kajobe *et al*., 2010) investigated the presence of honeybee viral diseases. On the other hand, no studies had investigated the presence, prevalence, infestation levels and the effects of honeybee bacterial, protozoan and fungal diseases on honeybee performance in Uganda. The current study investigated the distribution and infestation levels of honeybee parasitic *Varroa*-mites, bacterial, fungal and viral pathogens and their effects on honeybee colony performance in the eastern and western

highlands AEZs of Uganda. It contributes to addressing honeybee health threats by assessing the potential influence of landscape and human factors on the distribution and infestation levels of honeybee parasites and pathogens in the two agro-ecological zones of Uganda.

1.7 Objectives

1.7.1 Overall objective

The general objective of this study was to investigate honeybee parasites and pathogens and their effects on colony performance in two highland agro-ecological zones (AEZs) of Uganda.

1.7.2 Specific objectives

1. To establish the infestation levels and distribution of honeybee parasitic *Varroa* mites, fungal, bacterial and viral pathogens.
2. To assess the potential influence of landscape factors on the infestation levels of honeybee parasites and pathogens.
3. To evaluate the effects of honeybee parasitic *Varroa* mites, bacterial, fungal and viral pathogens on honeybee colony performance.

1.8 Research questions

1. Which honeybee parasites and pathogens are present in honeybee colonies in the eastern and western highland agro-ecological zones of Uganda?
2. Are there any relationships between honeybee parasite/pathogen infestation level or distribution and landscape factors (altitude, vegetation, and human activities) in the eastern and western highlands AEZs of Uganda?
3. What are the effects of honeybee parasitic *Varroa* mites, fungal, bacterial and viral pathogens on honeybee colony performance in the eastern and western highlands AEZs of Uganda?

1.9 Justification of the study

This study was conducted in Uganda, East Africa. Uganda occupies a total area of 241,550.7 km^2 out of which 41,027.4 km^2 are open water bodies and wetlands while 200,523.2 km^2 is dry land (UBOS, 2014). The country has an altitudinal gradient ranging from 620 meters (Albert Nile) to 5,111 meters (Mt. Rwenzori). According to the 2014 national housing and population census, Uganda's population was 36.6 million persons and the majority (>80%) of this population depends on agriculture for their livelihoods (UBOS, 2014). This work was critical because the demand for honey and other beehive products in Uganda has been increasing. For instance Uganda was licensed to supply honey to the European Union in 2005 (UEPB, 2005) but is not meeting its quota (Mr. Dickson Biryomumaisho personal communication). Also, since majority of households in Uganda rely on agriculture which is dependent on bee pollination, threats to honeybee health directly impact on the livelihoods of the majority of the population. Furthermore, other livestock enterprises like poultry rely on feeds with crop origins such as sunflower seed cake. Therefore, Uganda must maintain a healthy honeybee population to meet the demand for beehive products and honeybee pollinated agriculture.

Effective honeybee disease management programs require adequate and accurate information on the prevalence and distribution of honeybee parasites and pathogens. Currently, there is no proper honeybee disease surveillance and management plan in Uganda partly due to lack of adequate information on the presence and distribution of honeybee parasites and pathogens. This study investigated the potential influence of landscape and human factors on the distribution of selected honeybee parasites and pathogens (*Varroa* mites, fungal, bacterial and viral pathogens due to the threats they pose elsewhere to beekeeping) in two highland AEZs of Uganda. In addition, this study assessed the effects of the selected honeybee parasites and pathogens on colony performance. It therefore provides baseline data on honeybee parasites and pathogens in the country. From this study, we understand current and potential threats to Ugandan honeybee health and honeybee dependent crop production. The findings provide an understanding of the magnitude of the honeybee health problem for creating awareness and developing a long term surveillance system for honeybee pests, parasites and pathogens.

Information from this study is vital and will contribute to the designing of honeybee disease surveillance and management plans to avert the likely honeybee health threats to the beekeeping industry and food security. Furthermore, the results of this study such as the detection of a new microsporidian highlight gaps that require more research for better understanding of several aspects including parasite development and epidemiology.

1.10 The research outline of this thesis

This study was designed to further our understanding of honeybee host-pathogen/parasite interactions under varying landscape factors in African honeybees. Specifically, 11 honeybee viruses in addition to *P. larvae*, *M. plutonius*, *A. apis*, *Nosema* spp. and *V. destructor* were investigated. These pathogens and parasites were selected because they are important threats to honeybee health worldwide. Moreover, some of these parasites and pathogens e.g. *V. destructor*, *Nosema* spp. and viruses like DWV and IAPV have been associated with colony losses in Europe and North America.

The output of this thesis is presented in four research chapters. In the first research chapter (Chapter 2), honeybee viral pathogens in Ugandan colonies were explored with the aims of: 1) understanding the landscape infection rates of honeybee viruses and 2) highlighting the possible extent to which the common RNA viruses are affecting honeybee colony performance. In Chapter 3, we employed pathogen culturing techniques to determine the presence of honeybee brood bacterial and fungal pathogens in Ugandan honeybee colonies. The aim here was to establish the presence and distribution of brood pathogens in honeybee populations in the two highland agro-ecological zones of Uganda and to determine if honeys that were locally retailed contained *P. larvae*, a lethal pathogen of honeybees. In Chapter 4, we explored the microsporidian parasite infestation in Ugandan honeybees with the aim of: 1) identifying the *Nosema* spp. present; 2) understanding the variation in infection levels among honeybee colonies located in areas of different land-use types and elevations and 3) determining effects of infections on honeybee colony performance. Finally, in chapter 5, we investigated the haplotype of *Varroa* mites and

potential factors that influence their infestation levels in the eastern and western highland AEZs of Uganda.

CHAPTER 2

Viral honeybee pathogens in Uganda

Personal contribution:	Designing the study
	Field data collection
	Laboratory analyses for honeybee viruses
	Data analyses
	Writing the manuscript

2.1 Abstract

Honeybee viruses pose significant threats to the health of the honeybee, *Apis mellifera*. In this study, the distribution of honeybee RNA viruses was investigated in two highland agro-ecological zones of Uganda each with an altitudinal gradient and varying land uses. The aim was to understand the landscape infection rates and the extent to which the common RNA viruses were affecting honeybee colony strength and productivity. Honeybee samples from colonies without any observable health problems were collected during the dry and wet seasons between December 2014 and September 2015. The samples were screened for common RNA viruses using PCR based techniques. Five honeybee viruses were detected in both the eastern and western highland agro-ecological zones of Uganda. These viruses include: Deformed wing virus (DWV) (51.9%), Black queen cell virus (BQCV) (20%), Acute bee paralysis virus (ABPV) (9.5%), Lake Sinai virus (LSV) (2.5%) and Sacbrood virus (SBV) (2.5%). Four of these viruses (DWV, BQCV, ABPV and SBV) were detected in feral colonies. Furthermore, multiple RNA viruses were prevalent in Ugandan honeybee colonies during the wet season. We show that the numbers of multiple honeybee virus infections were correlated to elevation, height of hive placement and distances to potential water sources, suggesting that environmental factors modulate honeybee viral infestation rates. Furthermore, *Varroa* infestation levels were positively correlated with the number of viral infections suggesting that *Varroa* was vectoring the viruses. However, surprisingly no honeybee viruses were detected in the sampled *Varroa* mites from virus-positive colonies. Increased viral diversity in Ugandan honeybee colonies reduced their performance. Based on these findings, honeybee health monitoring programs are urgently needed to keep track of the interactions between African honeybee races, viral pathogens and *Varroa* mite vectors.

Key words: *Apis mellifera*; Colony performance; Landscape factors; Pathogens; RNA viruses

2.2 Introduction

Honeybee viruses are of particular interest in beekeeping because they are suspected to be drivers of honeybee colony declines and have been reported globally (Manley *et al*., 2015). Until now, at least 23 viruses have been shown to infect honeybees, *Apis mellifera* (Chen *et al*., 2004; Chen & Siede, 2007; Runckel *et al*., 2011; McMenamin & Genersch, 2015; Gisder & Genersch, 2015). Most of these honeybee viruses are positive strand RNA viruses belonging to Picornavirales (Mayo, 2002).

The majority of honeybee viral infections are asymptomatic especially at low virus titer levels. However, at high virus titers associated with transmissions within the honeybee colony, symptoms such as deformities in wings for Deformed wing virus (DWV) (de Miranda & Genersch, 2010), swollen yellow larvae and/or dark brown larvae carcasses in the cells of worker bees for Sacbrood virus (SBV) (Shen *et al*., 2005), enlarged dark stained queen cell walls for the Black queen cell virus (BQCV) (Gajger, Bičak, & Belužić, 2014), hairless, dark and shiny bees for chronic bee paralysis virus (CBPV) can be observed (Chen & Siede, 2007; Ribière *et al*., 2010). Some viral infections also cause behavioral changes such as shivering, paralyses, disorientation, aggression or altered foraging preferences or changes in brood care (Ribière *et al*., 2010).

At least nine viruses have been reported in *A. mellifera* colonies from Africa (Pirk *et al*., 2015). These include: BQCV (Kajobe *et al*., 2011; Muli *et al*., 2014; Amakpe *et al*., 2015), DWV (Loucif-Ayad *et al*., 2013; Muli *et al*., 2014; Haddad *et al*., 2015; Menail *et al*., 2016) Acute bee paralysis virus (ABPV) (de Miranda *et al*., 2011; Muli *et al*., 2014; Amakpe *et al*., 2015), Lake Sinai virus (LSV) (Amakpe *et al*., 2015; Menail *et al*., 2016), *Varroa destructor* virus 1 (VDV-1), Israeli acute paralysis virus (IAPV) (Strauss *et al*., 2013), CBPV (Pirk *et al*., 2015), *Apis mellifera* filamentous virus (AmFV) (Pirk *et al*., 2015), and SBV (Mumoki *et al*., 2014; Pirk *et al*., 2015). However, the complete virus pathosphere in African honeybee colonies remains to be fully explored since relatively very few epizootiological studies have been conducted. Also, the intricate dynamics of multiple viral infections and their effects on African honeybee races has not been studied to date. Therefore, understanding the current distribution of potential virus threats to African honeybee health will be helpful in informed decision making.

A recent study in Uganda confirmed the presence of BQCV (Kajobe *et al*., 2011) raising concerns on the health of Ugandan honeybees. The combination of the recently introduced *Varroa* mites in the country (Kasangaki *et al*., 2015; Chemurot *et al*., 2016a) and the presence of BQCV (Kajobe *et al*., 2011) and *Paenibacillus larvae*, the causative agent for American foulbrood (Chemurot *et al*., 2016b), may negatively influence the honeybee health. In addition, *Varroa* mites are known to transmit honeybee viruses like DWV (Shen *et al*., 2005), IAPV and ABPV (Di Prisco *et al*., 2011). However, although *Varroa* mites had been detected in Uganda, these viruses had not been confirmed in the country until the present study. As such, honeybee viruses should be included in epizootiological studies in order to plan for better management of honeybee health.

This study investigated the presence and distribution of known honeybee viruses in Ugandan honeybee colonies. The colonies were sampled from two highland AEZs of Uganda each with an altitudinal gradient and varying land uses to; (i) understand the landscape infection rates of 11 disease causing common RNA viruses, (ii) study the possible correlation between virus infection rates and *Varroa* mite infestation levels, (iii) quantify and compare the infection rates of 11 RNA viruses in honeybee colonies located in farmlands and protected areas, and (iv) assess the extent to which 11 RNA virus infections are affecting honeybee colony performance. We screened for the most common RNA viruses that have been confirmed to affect honeybee health or linked to colony losses in North America and Europe; ABPV, KBV, IAPV, CBPV, BQCV, DWV, Aphid lethal paralysis virus strain Brookings (ALPV), SBV, Slow bee paralysis virus (SBPV), LSV and Big Sioux River virus (BSRV). We show that multiple RNA viruses were prevalent in some Ugandan honeybee colonies and present evidence for possible reduction in honeybee colony strength and productivity due to increased viral diversity.

2.3 Materials and methods

2.3.1 Study area

For proper agricultural planning, zoning of the land is critical in order to achieve increased productivity (Eledu *et al*., 2004). Agro-ecological zoning categorizes land areas based on factors that include: soil, altitude and climatic characteristics (FAO, 1996). Uganda is divided into ten (10)

agro-ecological zones (AEZs) based on vegetation type, elevation, climatic conditions and agricultural activities. Honeybee sampling for this study was conducted in two AEZs of Uganda namely; eastern and western highlands AEZs of Uganda (Figure 2.1). These study sites were purposively selected because they have beekeeping activities at varying altitudinal levels and in areas of varying land uses which allow comparative studies. The altitudinal and land use variations provide conditions for investigating the potential influence of landscape factors on the distribution of honeybee pathogens that can be used for designing honeybee disease management strategies.

The eastern highlands receive bimodal rainfall ranging from 900 to over 2100 mm per year. This zone has a minimum temperature of about 7.5^{o}C and the altitude within this AEZ varies from 1000 to 4000 m above sea level (Wasige, 2009; NEMA, 2009). The farming system here is intensive banana and coffee production. The districts that were sampled in this zone included Mbale and Kapchorwa (Figure 2.1) chosen on the basis of having beekeepers both in agricultural and non-agricultural areas. The climate in Mbale and Kapchorwa districts is classified as tropical with average temperatures of 23.0 °C and average rainfall of 1183 mm/year (https://en.climate-data.org/location/30666/; https://en.climate-data.org/location/780530/). In both districts, the driest month is January with lowest precipitation. Most precipitation falls in May.

The western highlands are a narrow zone along the western boundary of Uganda and include the Albertine rift valley famous for wildlife species diversity. Part of the western highlands is hot and dry with mean annual rainfall ranging from 875 - 1000 mm. In the higher altitude southern part (bordering the southern highlands), the mean annual rainfall exceeds 1875 mm. The mean altitude of the zone ranges from 600 - 4500 m above sea level (Kamanyire, 2000; Wasige, 2009;

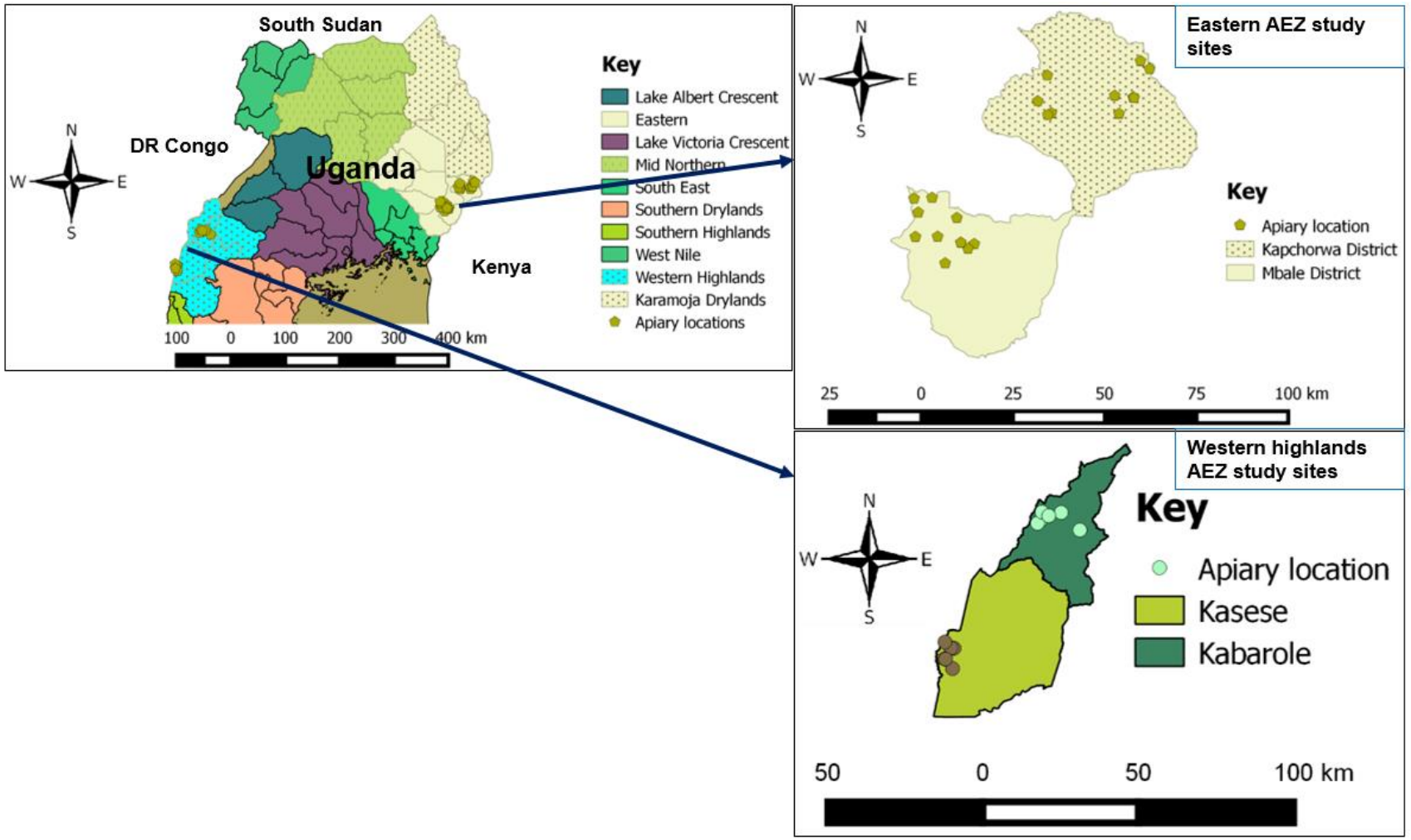


Figure 2.1: Location of study sites in the agro-ecological zones of Uganda.

NEMA, 2009). In this zone, the study districts included Kasese and Kabarole (Figure 2.1). The climate in Kasese and Kabarole districts is also considered tropical. The average temperature in Kasese is 23.1 °C while the average annual rainfall is 1475 mm/year (https://en.climate-data.org/location/505145/). For Kabarole district significant rainfall is received in most months and with a short dry season. The average temperature in Kabarole is 19.1 °C and the average rainfall is 1482 mm/year (https://en.climate-data.org/location/782724/). At the time of this study, beekeepers in Uganda were not treating for *Varroa* or any honeybee diseases and so far no *Varroa* related honeybee colony losses had been reported (Chemurot *et al*., 2016a).

2.3.2 Field sampling

For this study, two AEZs (eastern and western highlands) of Uganda were selected based on altitude and having beekeeping activities in and outside protected areas. Protected areas are public owned lands that currently receive government protection because of their recognized natural and ecological values. Within the AEZs, two districts in Mt. Elgon region and two districts around Queen Elizabeth National Park (QENP) selected based on altitude and beekeeping activities were sampled. In Mt Elgon region, Kapchorwa and Mbale districts were selected while around QENP, Kasese and Kabarole were selected. In each of the two AEZs, there were beekeepers practicing beekeeping in the protected area (National Park) following agreements with the park management while other beekeepers kept bees in farm lands and under *Eucalyptus* plantations. Here, a farmland refers to public or private land under agricultural crop cultivation while *Eucalyptus* plantations are areas with established *Eucalyptus* trees covering at least 0.5 hectares.

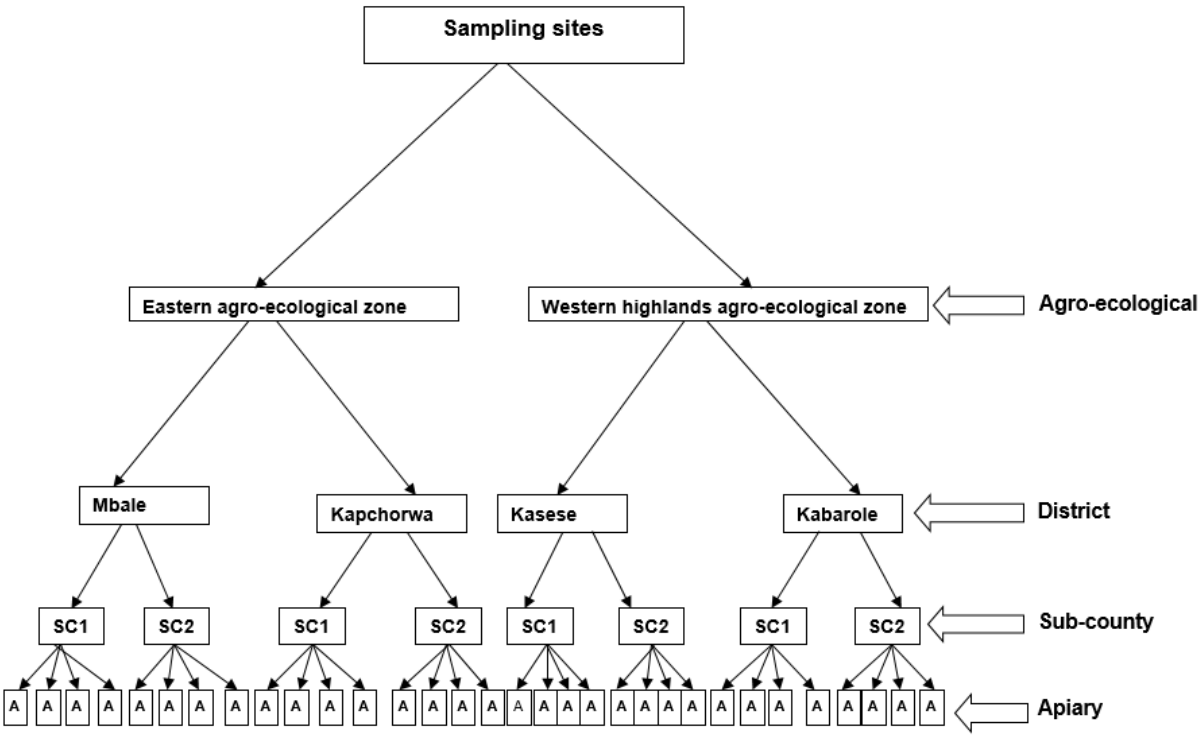
In each district, lists of beekeepers were obtained from the District Production offices and two sub-counties with the highest numbers of beekeepers were selected. Four apiaries in each sub-county were selected (Figure 2.2) on the basis of altitude (Figure 2.3) and land uses. In the present study, altitude in apiaries ranged from 920 – 2,400 m above sea level. Based on this, apiaries were stratified in each study district according to altitude into four strata; low (< 1,200 m), mid-

low (1,201 - 1,500 m), high (1,501 – 1,800 m) and very high (> 1,801 m) and at least one apiary was sampled in each strata.

In each apiary, honeybee colonies that were at least six months from the date of colonization were sampled to ensure that only established colonies were assessed. A total of 170 colonies from 32 apiaries and 7 feral colonies in the two AEZs were sampled during the dry season (December 2014 - February 2015). We decided to sample feral colonies because currently, beekeepers in Uganda majorly rely on feral colonies to populate their beehives and we were interested to know if feral colonies had similar parasites and pathogens with managed colonies. By the wet season, 38% and 45% of sampled managed colonies had absconded in the western highlands and eastern AEZs of Uganda respectively. During the second sampling (wet season: July - September 2015), 195 colonies were sampled. In cases where colonies sampled during the first sampling had absconded, new colonies within the same apiaries were sampled. Apiaries sampled were selected based on altitudinal gradients and land uses. The distance between one sampled apiary and the next was at least 3.5 km to minimize sampling honeybees foraging within the same area. The geographical coordinates and elevation at each apiary or feral colony nest site were taken using a global positioning system (GPS) receiver and observations made on human activities, apiary characteristics and landscape features such as the nature of slope and nearest potential honeybee water source. For this study, the nature of apiary slope refers to three categories of apiary slope gradient (steep, gentle and flat) while a potential water source is defined as the nearest stream/pond to the apiary. In apiaries sampled, there were between 3 to 64 honeybee colonies. Therefore, at each apiary, at least six honeybee colonies were randomly selected and sampled whenever it was possible. Beekeepers managed their apiaries in different ways. Based on this, apiaries were categorized into four: apiaries with no management; occasionally slashed; well slashed; slashed plus inspected regularly.

Furthermore, agriculture was being carried out at different levels in the study sites. Therefore, apiaries were categorized depending on the level of agricultural/farming intensity around them into four categories, namely: protected area; new farmland; old farmland with tree plantation;

and old farmlands without tree plantations. Here, new farmlands refer to areas that were opened recently (< 5 years) and still have remnants of natural vegetation. Old farmlands with tree plantations are areas that were opened for farming more than five years ago but have parts that were replanted with tree plantations. On the other hand, old farmlands without tree plantations are sites that were opened for farming more than five years but no efforts have been made to replant trees in them.



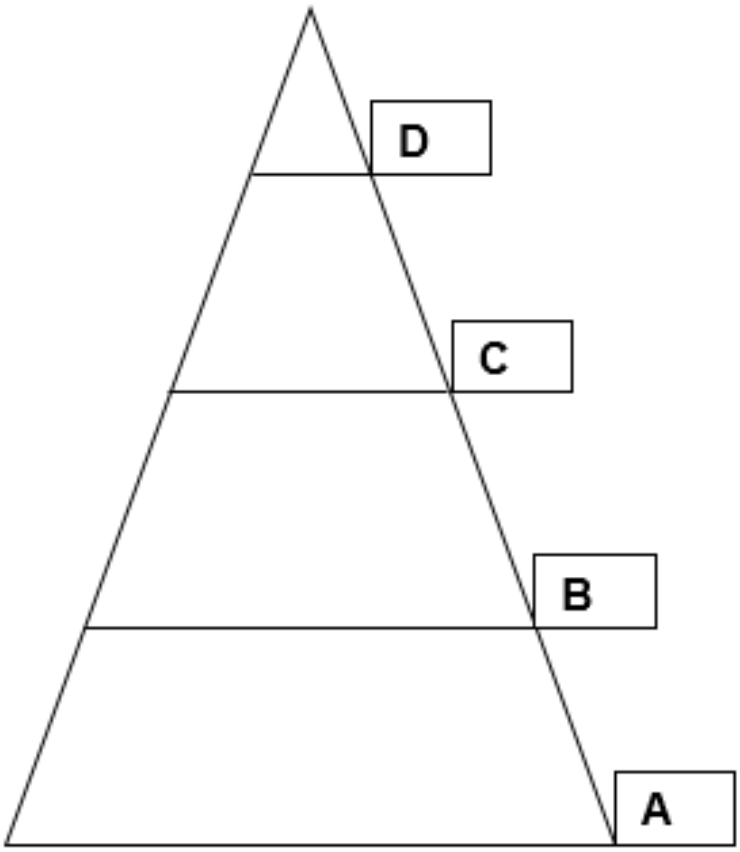
Where SC= sub county, A= apiary.

Figure 2.2: Illustration of sampling strategy

2.3.3 Evaluating mite infestation levels

Varroa mite infestation levels on adult honeybees were determined as described in Chemurot *et al*., (2016a). Approximately, 300 worker honeybees were collected from inside each honeybee colony into a 100 ml sample bottle. The bee samples were immobilized by spraying with 95% ethanol. In order to estimate the number of honeybees collected per sample, 10 bees were weighed using an electronic balance (sensitive to 0.1g) and used to estimate the weight per bee.

Then all the bees in the sample bottle were weighed and their number estimated. After that, the honeybees in each sample were poured into separate 600 ml plastic beakers and 95% ethanol poured to completely immerse them. The beakers were wrapped with parafilm and vigorously shaken for 5 - 10 min before the contents were separated using a sieve of 2 mm mesh size. After sieving, 30 honeybees were placed in 15 ml tubes while the rest were disposed. Mites that collected on the filter paper were picked using a pair of forceps, counted and placed in 1.5 ml vials. The mite infestation level here refers to the number of mites per 300 bees in a given honeybee colony. Samples of mites and honeybees were preserved in 95% ethanol and placed in a cold chain until analysis.



Where; A = < 1,200 m, B = 1,201 - 1,500 m, C = 1,501 – 1,800 m, D > 1,801 m.

Figure 2.3: Stratification of the study sites based on elevation.

2.3.4 Sample storage and transportation

Honeybee samples were kept in 95% ethanol in a cold chain for two months before being transported to the Laboratory for Molecular Entomology and Bee Pathology (L-MEB) in Ghent, Belgium for analyses.

2.3.5 Preparation of the honeybee sample homogenate

Ten bees per sample (previously preserved in 95% ethanol) were removed and washed in 10 ml phosphate buffered saline (PBS) for 48 hours in a cold room (4^{o}c). The bees were then transferred to 5 ml tubes and homogenized in 5 ml PBS by mechanical agitation in a TissueLyser at maximum speed for 5 rounds, each lasting 5 minutes in the presence of metal and zircornia beads. One (1) ml of the extract was pipetted into 1.5 ml Eppendorf tube, centrifuged at 1,500 rpm for 10 min and the supernatant pipetted into another tube and centrifuged at maximum speed (13,300 rpm) for 15 min. The final supernatant was pipetted into new Eppendorf tubes and kept in the freezer at -20^{o}c until used for RNA extraction.

2.3.6 RNA extraction

Total RNA was extracted from honeybee samples (n = 10 bees per colony) using the QIAamp® viral RNA kit following the manufacturer's protocol and descriptions by Ravoet *et al*. (2013) and Amakpe *et al*. (2015). Briefly, 560 µl of buffer AVL containing carrier RNA was pipetted into 1.5 ml Eppendorf tubes. Then, 140 µl of the homogenized honeybee sample was added and briefly vortexed and centrifuged. After adding 560 µl of absolute ethanol, the mixture was briefly vortexed and centrifuged. The mixture was then pipetted into QIAamp columns and centrifuged at 8,000 rpm for 1 min. The columns were washed with 500 µl of buffer AW1 and AW2 and centrifuged according to the manufacturer's protocol. Finally, the columns were transferred into 1.5 ml Eppendorf tubes and 50 µl of AVE elution buffer carefully added and left to stand at room temperature for 1 min. This was centrifuged at 8,000 rpm for 1 min and the eluted RNA was kept at -80^{o}C.

2.3.7 Detecting honeybee viruses

Honeybee samples were screened for the presence of 11 viruses using the Multiplex Ligation-dependent Probe Amplification (MLPA) technique following De Smet *et al*. (2012) and Ravoet *et al*. (2013). The MLPA technique can detect 8 honeybee viruses and one virus complex in one reaction and utilizes the reference gene β-actin (honeybee control gene) to validate negative results. This technique was selected because it works well even with highly degraded RNA since it requires only very short fragments of intact RNA (De Smet *et al.,* 2012). Moreover proper honeybee sample preservation was difficult to achieve during field work in Uganda to allow for other screening techniques that require intact RNA.

Each reaction was started with 1 µl RNA and 3.5 µl of the RT primer mix. The mixture was centrifuged before being placed in the thermocycler and programmed as follows: 1 min at 80^{o}C and 5 min at 45^{o}C. After denaturation of the sample, 0.68 µl of water, 0.68 µl SALSA enzyme dilution buffer and 0.15 µl reverse transcriptase were pipetted and mixed in each tube and incubated for 15 min at 37^{o}C and 2 min at 98^{o}C and stored at 25^{o}C. Then, the probe mix was added; 1.5 µl SALSA probe-mix and 1.5 µl MLPA buffer were pipetted in each tube and incubated for 1 min at 95^{o}C and 16 hours, 20 min at 60^{o}C. The hybridized probes were ligated by adding the ligase-65 mix solution consisting of 3 µl of ligase 65 buffer A, 3 µl of ligase 65 buffer B, 25 µl of water and 1 µl of ligase-65 for each sample. Before adding the ligation mix, the temperature of the reaction was reduced to 54^{o}C and finally the reaction was incubated for 15 min at 54^{o}C and 5 min at 98^{o}C. In the last step, the ligated probes were amplified using PCR. Here, 7.5 µl of water, 2 µl of SALSA primers and 0.5 µl SALSA polymerase were pipetted in each sample and mixed well. The PCR reaction had the following temperature cycles; 35 x [30 sec at 95^{o}C, 30 sec at 60^{o}C, 60 sec at 72^{o}C] 20 min at 72^{o}C. The amplified MLPA products were electrophoresed on 4% high resolution agarose gel, stained with 0.5µg/ml ethidium bromide and visualized using ultra violet (UV) light.

2.3.8 PCR to confirm viruses

All samples that were clearly positive or suspected to be positive under MLPA for ABPV, KBV, IAPV, CBPV, ALPV, SBV, SBPV and LSV were confirmed by reverse transcription (RT)-PCR. For

BQCV and DWV randomly selected samples from the two AEZs of Uganda that were positive under MLPA were analyzed by RT-PCR. The fragment of the genome of each of these 10 honeybee viruses (ABPV, KBV, IAPV, CBPV, BQCV, DWV, ALPV, SBV, SBPV and LSV) previously investigated using MLPA was amplified for virus confirmation using specific primers shown in Table 2.1. All reactions were carried out in 25 µl PCR solution containing 0.5 µM of each primer, 2.5 µl of 10x buffer, 0.25mM of dNTP, 2.5 mM $MgCl_2$, 0.2 U HotStarTaq Plus DNA Polymerase, and 1 µl of cDNA.

The cDNA was synthesized using random primers in the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit following Chemurot *et al*., (2016a). This process started with 5 µl RNA in a volume of 12 µl that was denatured for 5 min at 65^{o}C and paused at 25^{o}C. Then 4 µl of 5x reaction buffer, 1 µl of RiboLock RNase inhibitor (20 U/ µl), 2 µl of 10 mM dNTP mix and 1 µl of reverse transcriptase (200 U/ µl) were added to make a total volume of 20 µl. The solution was mixed gently and centrifuged before incubation for 5 min at 25^{o}C and 60 min at 42^{o}C. The reaction was finally terminated by heating to 70^{o}C for 5 min and the product used directly as a template for the different PCR. The PCR conditions for the different reactions are shown in Table 2.1. Aliquots of 10 µl of the amplified products were separated using 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide and visualized under UV light.

2.3.9 Establishing association between *Varroa* mites and honeybee viruses

In order to establish the association between honeybee viruses and *Varroa* mites, samples of 20 mites from virus positive colonies were screened for three viruses; DWV, BQCV and ABPV. First, RNA was extracted from individual whole mites using the QIAamp® viral RNA kit following the manufacturer's protocols and descriptions in Chemurot *et al*., (2016a). Briefly, 560 µl of AVL containing carrier RNA was pipetted into 1.5 ml Eppendorf tubes. Then, 140 µl of the *Varroa* sample (prepared by slicing each mite using a sterile blade and adding 200 µl PBS and centrifuging) was added and briefly vortexed and centrifuged. Then, 560 µl of absolute ethanol was added and the mixture vortexed and centrifuged. After this, 630 µl of the mixture was

pipetted into QIAamp columns in 2 ml tubes. Column washing and RNA elution was done as described in section 2.3.6. The RNA was kept at -80^{o}C until used for cDNA synthesis.

The cDNA was synthesized using random primers in a similar manner as it was done for the bees in section 2.3.8. To confirm the negative results, *V. destructor* actin primers described by Locke *et al*. (2012) were used in a reaction programed as follows: 95^{o}C for 5 min, 94^{o}C for 30 sec, 58^{o}C for 30 sec, 72^{o}C for 30 sec (35 cycles) and final extension 72^{o}C for 10 min. Ten µl of the amplified products were separated using 1.5% agarose gel stained with 0.5 µg/ml ethidium bromide and visualized under UV light.

2.3.10 Data analyses

To obtain information on landscape and management factors that influence honeybee parasite/pathogen infestation levels, data on environmental factors (elevation, distances to potential water sources, nature of apiary slope and season) and management factors (agro-ecological zones, farming intensity and height of hive placement) were recorded. The GPS was used to record elevation and to estimate distances to potential water sources from apiaries. Apiary slopes were categorized into three groups (steep, gentle and flat) and recorded in the two AEZs sampled. Farming intensity around each apiary was observed and categorized as follows: protected area; new farmland; old farmland with tree plantation; and old farmlands without tree plantations. In each apiary, the height (meters) from ground to the bottom of the beehive was measured using a tape measure for every sampled hive. During fieldwork, colony performance was estimated by making observations and or measurements on the strength and productivity of each sampled colony. The following indicators of honeybee colony performance were used: number of top-bars/frames with honey, brood, pollen, covered with bees and honey yield in kilograms that was harvested.

Data were analyzed using SPSS statistical program (version 16). To compare the number of honeybee viruses in honeybee colonies in different land use areas, Kruskal-Wallis tests were performed. When significant results were found, *post hoc* analysis with Mann-Whitney tests

were used to compare pairs of categories. Mann-Whitney test was also used to compare the number of honeybee viruses in honeybee colonies in the two AEZs of Uganda, two seasons (dry and wet) and in inspected and uninspected apiaries. Spearman's rank correlations were conducted to establish the relationships between the number of honeybee viruses (viral diversity) and the following: altitude, distances to the nearest water sources, height of beehive placement, *Varroa* mite infestation levels and colony performance. All tests were two tailed.

Regression models were built modeling the presence of honeybee viruses, *Nosema* and *Varroa* as a function of landscape and human factors that were thought to potentially impact on honeybee health. Because of the severe skewedness and the high amount of zero's in the outcome variables, a combination of ordinary and logistic regression were used as suggested by Fletcher *et al*. (2005). The goal was to build models indicating whether honeybee pathogens/parasites (viruses, *Nosema* and *Varroa*) were present or not, and another model indicating the viral diversity, number of *Nosema* spores and number of *Varroa* when they were present. From the original datasets, two datasets were created. One indicating whether pathogens/parasites were present or not, and the other showing the infestation levels when they were present. Dummies were created for the categorical variables. Forward stepwise logistic regression was used to model the presence of these pathogens and parasites. To model the viral diversity and infestation levels of *Nosema* and *Varroa* when these pathogens and parasites were present, forward stepwise linear regression was conducted. To fulfill all underlying assumptions of the models, square root transformations of the outcome variables were done in each case. A Bonferroni correction of all critical values was done to correct for type I errors.

2.4 Results

2.4.1 Infestation rates of honeybee viruses

Five honeybee viruses; DWV, ABPV, BQCV, SBV and LSV were detected in the investigated Ugandan honeybee colonies (Figure 2.4). Results showed that these five honeybee viruses were detected in both the eastern and western highlands AEZs of Uganda. The findings further showed that overall, 51.9% of the investigated samples were infected with DWV, 20% with BQCV, 9.5%

with ABPV, 2.5% with LSV and 2.5% with SBV. No samples tested positive for KBV, IAPV, CBPV, ALPV, SBPV and BSRV. The highest percentage of infections was found for DWV, which occurred in 29 out of 32 apiaries sampled. For BQCV, ABPV, SBV and LSV, infections were recorded in 23, 13, 7 and 7 out of the 32 apiaries sampled respectively. Moreover, in feral colonies, four honeybee viruses similar to those in managed colonies were detected. These were DWV (25%), ABPV (25%), BQCV (25%) and SBV (12.5%). Honeybees with deformed wings (Figure 2.5), a known symptom of DWV, were observed when brood was collected from the Lake Victoria AEZ of Uganda and incubated to allow young bees to emerge (personal observation).

2.4.2 Single and multiple viral infections

Table 2.2 shows the infection rates of single and multiple honeybee viruses in the eastern and western highlands AEZs of Uganda. Multiple honeybee viruses were more prevalent during the wet season compared to the dry season in managed colonies in both AEZs. Also, multiple viruses were detected in feral colonies during the dry season (Table 2.2). There were no significant differences in the number of honeybee viral infections (viral diversity) among honeybee colonies from the two AEZs (U = 9796, Z = -0.251, P = 0.802). However, the median number of viral infections in honeybee colonies located in areas of different land uses significantly varied (χ^2 (df = 2) = 6.671, P = 0.036). Apiaries located in *Eucalyptus* plantations had significantly higher median numbers of viruses (viral diversity) compared to protected areas (U = 35.0, Z = -2.215, P = 0.027) and farmlands (U = 617.0, Z = -2.075, P = 0.038). On the other hand, the median number of honeybee viral infections did not differ significantly between colonies located in farmlands and protected areas; U = 1855.0, Z = -1.510, P = 0.131).

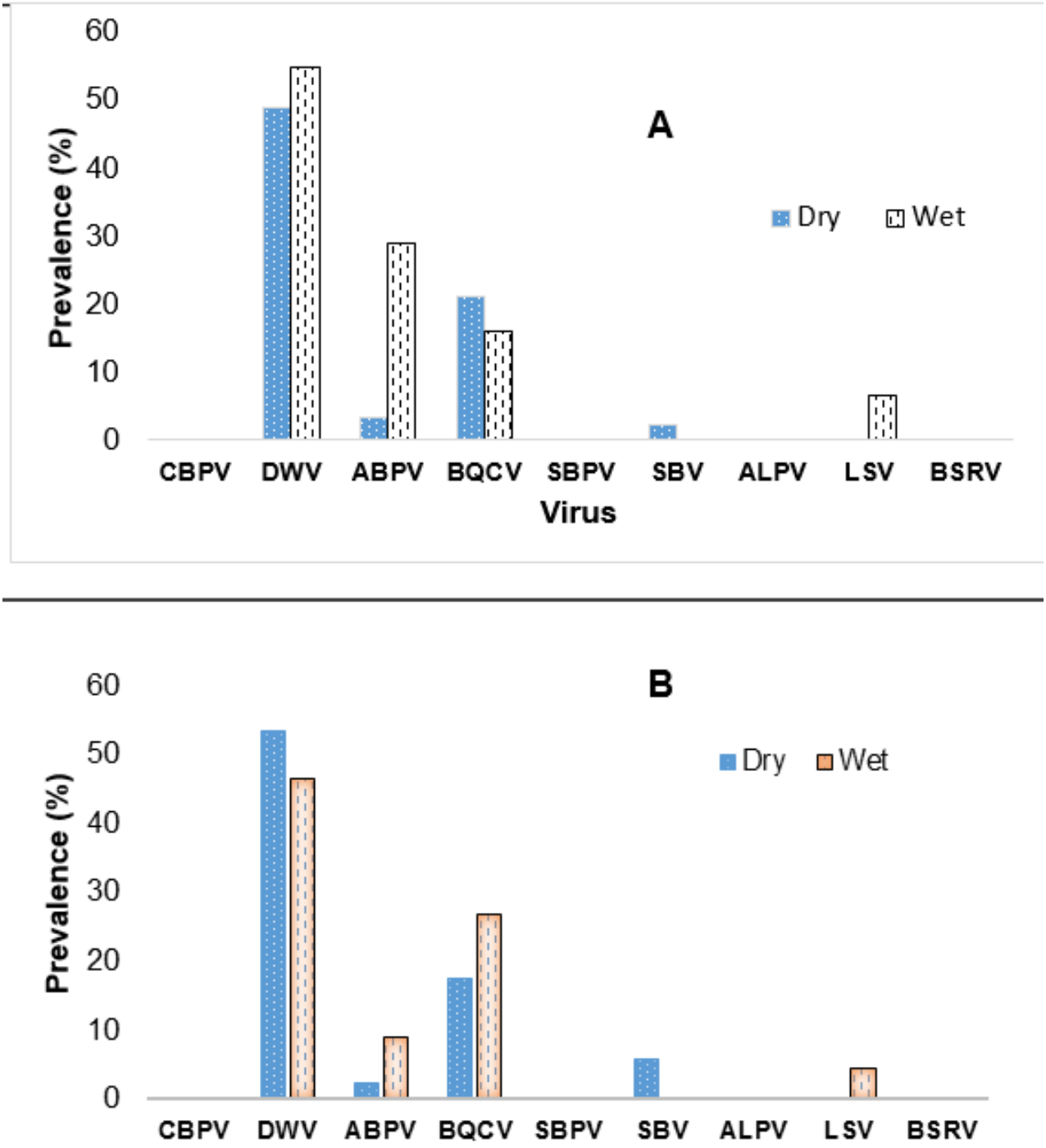


Figure 2.4: Infection rates of honeybee viruses in colonies: eastern (A) and western highland (B) agro-ecological zones of Uganda during the dry and wet seasons.

Table 2.1: Summary of the primers used for honeybee virus confirmations and cycling conditions.

Virus/gene	Primer	Primer sequence	Fragment length (bp)	Annealing temp	$MgCl_2$/25µl	Reference
ABPV	ABPV-F6548	TCATACCTGCCGATCAAG	197	48°C	1µl	de Miranda *et al*., 2011
	KIABPV-B6707	CTGAATAATACTGTGCGTATC				
KBV	KBV-F6639	CCATACCTGCTGATAACC	200			
	KIABPV-B6707	CTGAATAATACTGTGCGTATC				
IAPV	IAPV-F6627	CCATGCCTGGCGATTCAC	203			
	KIABPV-B6707	CTGAATAATACTGTGCGTATC				
CBPV	CBPV 1-1	TCAGACACCGAATCTGATTATG	570	55°C	1µl	Blanchard *et al*., 2008
	CBPV 1-2	ACTACTAGAAACTCGTCGCTTCG				
BQCV	BQCV-TOP-F	GGAGATGTATGCGCTTTATCGAG	316	63°C	1µl	Topley *et al*., 2005
	BQCV-TOP-R	CACCAACCGCATAATAGCGATT				
DWV	DWV-F1425	CGTCGGCCTATCAAAG	417	50°C	0.5µl	Forsgren *et al*., 2009
	DWV-B1806	CTTTTCTAATTCAACTTCACC				
ALPV	ALP-Br-F-2936	AACGTCGTATGCTACGATGAACTCG	464	60°C	1µl	Runckel *et al*., 2011
	ALP-Br-R-3400	GGGTTAAATTCAATTCCAGTACCACGG				
SBV	SBV-VP1b-F	GCACGTTTAATTGGGGATCA	693	51.5°C	1µl	Singh *et al*., 2010
	SBV-VP1b-R	CAGGTTGTCCCTTACCTCCA				
SBV	SB14 f	AAT GGT GCG GTG GAC TAT GG	597	55°C	1µl	Grabensteiner & Ritter, 2001
	SB15 r	TGA TAC AGA GCG GCT CGA CA				
SBPV	SBPV	GATTTGCGGAATCGTAATATTGTTTG	868	58°C	1µl	de Miranda *et al*., 2010
	SBPV	ACCAGTTAGTACACTCCTGGTAACTTCG				
LSV	LSVdeg-F	GCCWCGRYTGTTGGTYCCCCC	578	60°C	1µl	Ravoet *et al*., 2013
	LSVdeg-R	GAGGTGGCGGCGCSAGATAAAGT				
Vd-actin	Vd-actin-qF	CGACGGTCAGGTCATCAC	243	58°C	0.5 µl	Locke *et al*., 2012
	Vd-actin-qB	GTTGAGGGAGCCAAAGAGG				

Table 2.2: Seasonal infection levels (%) of uninfected, single and multiple honeybee viruses in honeybee colonies from the east and western highlands AEZs of Uganda.

Number of viruses	East		Western highlands		Feral colonies
	Dry (n=90)	Wet (n=62)	Dry (n=86)	Wet (n=45)	Dry (n=8)
0	42.2	29.0	34.9	44.4	37.5
1	43.3	43.5	51.2	24.4	37.5
2	13.3	17.7	14.0	26.7	12.5
3	1.1	9.7	0.0	4.4	12.5

2.4.3 Relationship between viral infections and *Varroa* infestation levels

Varroa mite infestation levels were generally low but varied from 1.52±0.30 to 3.79±0.54 mites per 300 bees in the two AEZs (Table 2.3). The number of viruses detected in honeybee colonies was positively correlated with *Varroa* mite infestation levels (rho = 0.212, n = 283, $P < 0.01$). However, no targeted viruses (DWV, BQCV and ABPV) were detected in the sampled mites from virus positive colonies.

Figure 2.5: Honeybees with deformed wings, a symptom for DWV.

Table 2.3: Mean *Varroa* mite infestation levels in the two AEZs of Uganda.

AEZ	Mean mites per 300 bees	
	Dry season	**Wet season**
East	3.79 ± 0.54	2.58 ± 0.78
Western highlands	2.25 ± 0.49	1.52 ± 0.30

2.4.4 Relationship between viral infections and environmental factors

Evaluation of the potential effects of environmental and human factors on honeybee viral diversity indicate that season and apiary location in relation to human activities influenced viral diversity in honeybee colonies (Table 2.4). The number of viral infections in honeybee colonies was significantly higher during the wet season compared to the dry season (U = 8305.5, Z = -2.045, $P < 0.05$). The number of viral infections in honeybee colonies was significantly negatively correlated with altitude of the apiary (rho = -0.162, n = 283, $P < 0.01$).

Table 2.4: Effects of environmental and human factors on viral diversity in honeybee colonies.

Factor		Viral diversity
Season	Dry	0.8±0.7 (176)*
	Wet	1.1±0.9 (109)*
Apiary location	Eucalyptus	1.4±0.5 (8)*
	Farmland	0.9±0.8 (257)*[a]
	Protected area	0.6±0.9 (18)*[a]
Nature of apiary slope	Flat	1.0±0.8 (98)
	Gentle	0.8±0.8 (132)
	Steep	0.7±0.8 (52)
Agro-ecological zone	Eastern	0.9±0.8 (152)
	Western highlands	0.8±0.8 (131)

Numbers indicate means and standard deviations, figures in brackets indicate the sample size (n), the * outside the bracket indicates there are significant differences in the mean viral diversity within the factor under consideration while the ∗a indicates no significant differences

Beehives that were sampled were placed at heights ranging from 0 – 4.5 m. The number of viral infections in honeybee colonies was significantly negatively correlated with the height of hive placement (rho = -0.152, n = 283, $P = 0.011$). Also, the number of viral infections in honeybee colonies was significantly positively correlated with the distances to potential water sources (rho = 0.140, n = 283, $P = 0.019$). Potential permanent water sources for honeybees were located between 10 – 2,000 m from the apiaries. The number of honeybee virus infections in colonies

under different apiary management practices did not differ significantly (χ^2 (df = 3) = 1.767, *P* = 0.622). Furthermore, the number of honeybee virus infections in colonies in apiaries with varying farming intensities did not differ significantly (χ^2 (df = 3) = 4.467, *P* = 0.215).

A binary logistic regression model developed to predict honeybee viral presence gave a Nagelkerke R^2 of 0.138 implying that the variables included in the model were able to explain 13.8% variance in the model. Although, the Chi Square 30.522 and df 6 was significant (*P* < 0.001), the findings indicates that the nature of apiary slope and apiary location among the explanatory variables (elevation, season, apiary location, farming intensity, apiary slope and apiary management) included in the model significantly predicted viral presence in the study sites. Change of apiary slope from flat to gentle and steep decreased chances of finding honeybee viruses by 0.513 times (Table 2.5). Generally, it is concluded that this binary logistic regression model does not have adequate integrity and hence not appropriate for predicting viral presence in honeybee colonies in the two AEZs of Uganda.

Table 2.5: Logistic regression model predicting presence of honeybee viruses in the study sites.

Factor	**B**	**S.E.**	**Exp(B)**	***P***
Elevation	0.001	0.001	1.000	0.746
Apiary location	*-1.387*	*0.497*	*0.250*	*0.005*
Nature of apiary slope	*-0.667*	*0.230*	*0.513*	*0.004*
Agro-ecological zone	0.213	0.282	1.237	0.452
Season	0.311	0.274	1.365	0.2572
Distance to potential water source	0.001	0.000	1.001	0.074
Constant	3.447	1.323	31.428	0.009

X2 = 30.522, df = 6, *P* < 0.001; Nagelkerke R2 = 0.138 (Italicized variable is statistically significant)

The linear regression model developed explains 8.8 % of the variance in viral diversity. Factors included in the model were: apiary location, nature of apiary slope, distance to potential water sources, altitude, season, agro-ecological zone and height of beehive placement. All together these factors significantly explained viral diversity (F(9,271) = 4.009, *P* < 0.01). However, only apiary slope, season and height of beehive placement significantly predicted viral diversity (Table 2.6).

2.4.5 Relationship between viral infections and colony performance

Comparison of honeybee colony performance indicates significant seasonal differences in the number of combs with honey, brood and pollen in each of the two AEZs of Uganda with the colonies showing better performance during the dry season in all cases except for pollen in the western highlands AEZ. The number of combs full with adult bees was only statistically different in the western highlands with the dry season colonies having more combs full with bees. Honey yield was generally very low in the colonies harvested during the study. Notably, average honey yield in the top-bar beehives was 3.4 ± 4.1 kg/hive (n = 20) in the eastern and 2.9 ± 3.8 kg/hive (n = 17) in the western highland AEZs of Uganda (Table 2.7). The number of combs with honey in the beehives was significantly negatively correlated with the number of multiple honeybee viral infections (Table 2.8). Other honeybee colony performance indicators were not significantly correlated with the number of multiple honeybee viral infections.

Table 2.6: Linear regression model predicting viral diversity infestation levels in the study sites.

Factor	Beta	T	*P*
(Constant)		1.44	0.151
Apiary location (Eucalyptus)	0.086	1.353	0.177
Apiary location (protected area)	0	0.007	0.994
Nature of apiary slope (flat)	*0.312*	*2.928*	*0.004*
Nature of apiary slope (gentle)	0.121	1.448	0.149
Elevation	0.136	1.434	0.153
Distance to potential water source	0.099	1.462	0.145
Season	*-0.198*	*-3.35*	*0.001*
Agro-ecological zone	0.033	0.475	0.635
Beehive placement height	*-0.236*	*-2.97*	*0.003*

Table 2.7: Seasonal variation in colony performance indicators in the two AEZs of Uganda.

AEZ	Season	Honey combs	Brood combs	Pollen combs	Combs with bees	Honey yield (kg)
Eastern	Dry	2.1±3.0 (77)*	1.7±1.6 (72)*	0.4±0.4 (58)*	12.2±6.5 (89)	3.4 ± 4.1 (20)
	Wet	1.6±3.2 (54)*	0.9±2.6 (37)*	0.03±0.1 (41)*	11.1±3.6 (49)	-
Western	Dry	2.9±2.3 (54)*	1.5±1.5 (46)*	0.4±0.3 (49)*	13.7±6.8 (86)*	2.9 ± 3.8 (17)
	Wet	0.5±0.7 (32)*	0.6±0.9 (15)*	0.8±2.9 (23)*	11.4±4.2 (45)*	-

Numbers indicate the means and standard deviations, figures in brackets indicate the sample size (n), while the * indicates there are significant seasonal differences in the particular AEZ.

Table 2.8: Correlation values of the number of honeybee viruses detected with honeybee colony performance indicators.

Colony productivity/strength indicator	Spearman's rho	N	*P*
Combs with honeybees	0.013	266	0.838
Combs with honey	*-0.191*	*215*	*0.005*
Combs with brood	-0.072	169	0.354
Combs with pollen	-0.036	170	0.641
Honey yield (kg)	0.108	37	0.525

2.5 Discussion

The huge loss of honeybee colonies in Europe and North America in recent years drew attention leading to the discovery of links of some honeybee viruses to colony losses. However, no major colony losses have been reported in Africa despite at least nine honeybee viruses being documented (Pirk *et al*., 2015). This paper represents the second molecular evidence of the presence of honeybee viruses in Ugandan apiaries after Kajobe *et al*. (2011). It documents four newly detected honeybee viruses in Uganda (DWV, ABPV, SBV and LSV) and shows presence of multiple viral infections in honeybee colonies. Compared to the infection rates detected in this study, the corresponding results from the previous study (Kajobe *et al*., 2011) show that the infection rate of BQCV was relatively higher. However, like in the previous study (Kajobe *et al*., 2011), the infection rates per AEZ reveals slightly higher BQCV infestation levels in the western highlands compared to the eastern AEZ.

At regional level (East Africa), only three honeybee viruses (BQCV, DWV and ABPV) had been reported before this study (Kajobe *et al*., 2009, 2011; Muli *et al*., 2014). The current study reports two additional honeybee viruses (SBV and LSV) but at low infection rates. Moreover, the sampling in this study was limited to two out of ten AEZs in Uganda. Therefore, there is need to sample other AEZs in the country and region. Also, further studies are necessary to understand the effects of these honeybee viruses on African honeybees at individual and colony levels especially since some of these honeybee viruses are associated with honeybee losses in Europe and North America.

Reports on honeybee viruses in African honeybees (e.g. Adjlane *et al*., 2015; Haddad *et al*., 2015), were based on reverse transcription-PCR (RT-PCR) on honeybee samples. This technique and the MLPA used in the current study are recommended for screening honeybee viral pathogens (de Miranda *et al*., 2013). However, in terms of sensitivity, the qPCR is the most sensitive technique since it can detect as low as 300 copies and is followed by RT-PCR. On the other hand, the MLPA can detect up to 1000 copies of the target in a sample but has the advantage of being able to detect infections in degraded RNA (De Smet *et al*., 2012) and hence is recommended if proper sample storage is difficult as was the case in this study.

The infestation rates of the DWV was highest among all honeybee viruses detected in the two AEZs of Uganda. DWV infestation rate in this study is higher than that reported in other African countries like Kenya (Muli *et al*., 2014), Algeria (Loucif-Ayad *et al*., 2013; Adjlane *et al*., 2015), Egypt, Libya, Morocco, Sudan and Tunisia (Haddad *et al*., 2015) but lower than that reported in European countries like Croatia (Gajger *et al*., 2014), France (Tentcheva *et al*., 2004), Belgium (Ravoet *et al*., 2013) and South American countries like Uruguay (Antúnez *et al*., 2006). One possible reason for the lower infection rates of honeybee viruses in the study areas could be because *Varroa* ecto-parasitic vector for honeybee viruses arrived relatively recently in Uganda (Fazier *et al*., 2010; Kasangaki *et al*., 2015; Chemurot *et al*., 2016a).

Like the study in Kenya (Muli *et al*., 2014), our data shows that *Varroa* infestation levels were positively correlated with the number of viral infections suggesting that *Varroa* is vectoring the viruses. However, unlike in Algeria, a North African country where honeybee viruses were detected in *Varroa* mites (Adjlane *et al*., 2015), in this study no honeybee viruses were detected in the sampled *Varroa* mites. Therefore the interaction between *Varroa* and honeybee viruses in the Ugandan population of *A. mellifera* obviously constitutes a unique case since honeybee viruses that were detected in honeybees were absent in mites.

One hypothesis for the unique interaction between honeybee viruses and *Varroa* is that *Varroa* mites were recently introduction in Uganda compared to other regions of the world. This

hypothesis is supported by the fact that *Varroa* mites were not detected in Ugandan honeybee colonies in 2009 (Fazier *et al*., 2010) but recently (Kasangaki *et al*., 2015; Chemurot *et al*., 2016a). Alternatively, since African honeybees have greater resistance to some honeybee viruses like DWV and BQCV (Hamiduzzaman *et al*., 2015), the variation could be due to honeybee genetic traits that reduce viral levels in honeybees to levels that considerably lower their chances of being transmited by the mites. However, a study by Di Prisco *et al*. (2016) showed that mutualistic symbiosis between *Varroa* infestation and DWV infections lead to reciprocal stimulation that escalates negative effects on the immunity and health of honeybees. As such, long-term honeybee health monitoring programs are needed to keep track of the interactions between African honeybee races, these viral pathogens and *Varroa* mite vectors.

Several honeybee viruses like KBV, IAPV, CBPV, ALPV, SBPV and BSRV were not detected in the study sites. Therefore, future honeybee health monitoring programs in Uganda should concentrate on the five viruses (BQCV, DWV, ABPV, SBV and LSV) which were detected if resources are limited. However, even if SBPV was not detected, its inclusion in future honeybee health monitoring programs should be considered since its infection rate is known to be naturally low (de Miranda *et al*., 2010).

Four honeybee viruses were detected in feral colonies in the two AEZs of Uganda although relatively fewer feral colonies were sampled. Beekeepers in Uganda currently rely on natural honeybee colonies to populate their beehives (Chemurot, 2011), therefore honeybee pathogens and parasites can easily spread from feral to managed colonies. On the other hand, since absconding and swarming are common among African honeybee races (Hansen & Brodsgaard, 1997), pathogens and parasites may also spread from managed to feral colonies. On this regard, feral colonies may act as reservoirs of honeybee parasites and pathogens which may have far reaching consequences on the health of managed honeybees if they succumb to these potential threats.

The numbers of multiple viral infections in honeybee colonies during the wet and dry season differed significantly. Also, the height of hive placement and the distances to potential water sources were negatively and positively correlated with the number of viral infections in honeybee colonies respectively. Furthermore, there was a correlation between elevation and number of viral infections in honeybee colonies. These results suggest that environmental factors (climate and landscape ecology) might be involved in mediating the transmission and the honeybee-virus interaction. Beehives placed higher have reduced contact with formerly diseased bees that die and are thrown out of the hive. At lower elevations, other honeybee virus reservoir hosts could be mediating honeybee viral transmission.

Transmission of honeybee viruses could be occurring at water sources and floral sources where honeybees from different colonies interact with each other in addition to interacting with other non-*Apis* hymenopteran species. A study by Singh *et al*. (2010) showed non—*Apis* hymenopteran species including *Bombus impatiens*, *B. vagans*, *B. ternarius*, *Xylocopa virginica*, *Ceratina dupla*, *Augochlora pura*, *Andrena* sp., *Vespula vulgaris*, *Polistes metricus*, *P. fuscatus* and *Bembix* sp. collected from flowering plants near apiaries were positive for several honeybee viruses including DWV, BQCV, SBV, KBV and IAPV and concluded that pollen is a route for inter-taxa virus transmission since viruses were detected in pollen. Based on the positive correlation between number of viruses and distances to potential water sources, we hypothesize that water sources might also act as virus transmission points. The effect of environmental factors on honeybee viral transmission needs to be explored in detail.

The productivity of honeybee colonies sampled in the two AEZs of Uganda was low but comparable to the findings from Adjumani district in the West Nile AEZ of Uganda (Chemurot, 2011). This low productivity per beehive may be attributed to several factors including; limited honeybee forage, lack of proper colony management to prevent swarming (e.g. no supplementary feeding), too many colonies in the apiaries (carrying capacity) and weakening influences such as pests, diseases and pesticides (Hussein, 2000). Importantly, in this study the number of viral infections was negatively correlated to the number of combs with honey

suggesting possible negative effects of viral infections on the general performance of Ugandan honeybee colonies. Other honeybee colony strength and productivity indicators were not significantly correlated with the number of viral infections implying that the extent of the effects of these viruses could still be low. This could be because there can be a time-lag before newly introduced pathogens cause considerable negative effects. A study in Hawai by Martin *et al*. (2012) showed that the *Varroa* mite arrival increased the prevalence of DWV, from ~10 to 100% within honeybee populations, which was accompanied by an increase in viral titer and a massive reduction in DWV diversity, leading to the predominance of a single DWV strain. Although African honeybees have greater resistance to some honeybee viruses like DWV and BQCV (Hamiduzzaman *et al*., 2015), the arrival of *Varroa* could result in the selection for DWV variants that are more virulent and might lead to honeybee health problems in future.

This study suggests that four new honeybee viral pathogens (DWV, ABPV, SBV and LSV) could have recently invaded honeybee populations in Uganda. The findings suggest that increased honeybee viral diversity decreases honeybee colony productivity in Uganda, at least in terms of number of combs with honey. Interestingly, numbers of honeybee virus infections were correlated to elevation, height of hive placement and distances to potential water sources, suggesting that environmental factors modulate honeybee viral infection rates. Based on these findings, beekeepers should be encouraged to provide water in their apiaries during the dry season in order to avoid long distances that might increase chances of viral pathogen transmission. In addition, beekeepers are advised to place their beehives at recommended heights (1m for top-bar, 1.5m for traditional (log) for ease of manipulation and to avoid possible pathogen infestation. Finally, the exact ways through which environmental and management factors could be involved in honeybee viral interactions should be investigated.

CHAPTER 3

Bacterial and fungal honeybee brood pathogens in Uganda

Personal contribution:
- Designing the study
- Field data collection and sample collection
- Pathogen screening
- Data analyses
- Writing the manuscript

Part of the results are published in SpringerPlus 2016 as:

First detection of *Paenibacillus larvae* the causative agent of American Foulbrood in a Ugandan honeybee colony

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3.1 Abstract

Honeybee brood is attacked by bacterial and fungal pathogens which are often lethal and can cause losses to beekeepers. Here, the presence of three honeybee brood pathogens: *Paenibacillus larvae*, *Mellisococcus plutonius* and *Ascosphaera apis* in the eastern and western highland agro-ecological zones of Uganda was investigated. This was done by collecting brood and honey samples from 67 honeybee colonies in two sampling occasions and cultivating them for these pathogens. Also, 8 honeys imported and locally retailed in Uganda were sampled and cultivated for *P. larvae*. One honeybee colony without clinical symptoms for American foulbrood in an apiary located in a protected area of the western highlands agro-ecological zone of Uganda was found positive for *P. larvae*. The strain of this *P. larvae* was genotyped and found to be ERIC I. In order to compare its virulence with *P. larvae* reference strains, *in vitro* infection experiments were conducted with carniolan honeybee larvae from the research laboratory at Ghent University, Belgium. The results showed that the virulence of the *P. larvae* strain found in Uganda was at least equally high as in the reference strain. The epidemiological implication of the presence of *P. larvae* in a protected area is discussed.

Key words: *Apis mellifera*; Brood pathogens; East Africa; Honeybee diseases; Prevalence

3.2 Introduction

Honeybee brood is attacked by a range of pathogens including: bacteria, viruses, protozoa, fungi and parasitic mites. However, the American foulbrood (AFB) and European foulbrood (EFB) caused by *Paenibacillus larvae* and *Mellisococcus plutonius* respectively are two of the most economically important brood bacterial diseases of honeybees (Forsgren, 2010). These bacterial pathogens pose a significant threat to the health of honeybee colonies and to the beekeeping industry because they cause considerable losses to beekeepers (Genersch, 2010b). Both AFB and EFB are widely distributed and potentially lethal to infected colonies (Forsgren, 2010).

Although AFB and EFB have a worldwide distribution (Forsgren, 2010; Budge *et al*., 2010; Genersch, 2010a; Morrissey *et al*., 2014), few confirmations of these diseases have been made in Africa. Therefore, there is still doubt as to whether they are present in extensive parts of Sub

Saharan Africa. AFB has been confirmed in Egypt (Masry *et al*., 2014), Guinea Bissau (Hansen *et al*., 2003) and South Africa (Human *et al*., 2011). In addition, spores of AFB have been detected in honey originating from Algeria (Adjlane *et al*., 2014), Libya, Morocco (Hussein, 2001) and Tunisia (Hussein, 2001; Fries & Raina, 2003; Hamdi *et al*., 2013). On the other hand, EFB has been reported in Algeria, Guinea-Bissau, Libya, Morocco, Senegal, Tanzania and Tunisia among African countries (Hussein, 2001; Ellis & Munn, 2005).

Honeybee larvae get infected by *P. larvae* when they are fed by nurse bees on feed contaminated with spores of *P. larvae*. Young larvae (< 36 h after hatching) are most susceptible to infection (Genersch, 2010b). Typical clinical symptoms of AFB are the brown, viscous larval remains forming a ropy thread when drawn out with a matchstick (de Graaf *et al*., 2006). The decaying brood desiccates into hard scales, tightly adhering to the walls of the cells, consisting of millions of bacterial spores which are the infectious stage of the pathogen (Genersch *et al*., 2006). AFB is spread both horizontally and vertically (Fries *et al*., 2006; Lindstrom *et al*., 2008). However, the most predominant route of spread is via the horizontal routes by both humans and bees. Horizontal transmission of AFB occurs when humans move contaminated honey or beekeeping equipment (Genersch, 2010b). In addition, drifting of adult bees between colonies and robbing behavior of foragers can lead to horizontal spread of AFB (Lindstrom *et al*., 2008).

For EFB infection to occur, bacterial cells of *M. plutonius* are ingested by larvae with contaminated food and multiply within the midgut of the honeybee larva (Forsgren, 2010). EFB affects mainly unsealed brood, killing honeybee larvae usually when they are 4 – 5 days old (Forsgren, 2010). Symptoms of EFB include: dead larvae displaced in its cell or twisted around the walls or stretched out lengthways; larval color changes from pearly white to yellow, then brown and finally, when they decompose, grayish black; sunken capping resembling the symptoms of AFB and a foul or sour smell (Forsgren, 2010).

Another important honeybee brood disease is chalk-brood caused by *Ascosphaera apis,* a fungus which also affects mainly the larvae of the honeybees. Infection of honeybee colonies by *A. apis*

can cause reduction in colony strength and productivity (Jensen *et al*., 2013). Chalk-brood disease has been reported in African countries including; Algeria, Egypt, Ethiopia, Nigeria, South Africa and Tunisia (Hussein, 2001; Ellis & Munn, 2005; Tesfay, 2014; Pirk *et al*., 2015). However, *A. apis* remains to be investigated in East African countries including Uganda, Kenya, Tanzania, Burundi and Rwanda.

Beekeeping is an important activity in many rural areas of Uganda where it is carried out mainly using traditional beehives and beekeeping practices (UEPB, 2005; Chemurot, 2011). Beekeepers in Uganda majorly target honey production (UEPB, 2005), although beekeeping provides several other benefits to people and the environment: production of propolis, beeswax, bee venom, pollen and pollination service (Jacobs *et al*., 2006; Genersch, 2010b). In doing so, honeybees contribute to food security, household incomes and biodiversity conservation. However, honeybees are threatened by numerous pathogens and parasites which can attack them. The most recent honeybee parasite reported in Uganda, is *Varroa destructor* (Chemurot *et al*., 2016a). In order to develop the beekeeping sector, it is essential to design effective honeybee pest and disease management plans. This requires accurate and adequate information on the distribution, infestation levels and impacts of honeybee pathogens in the country.

In this study, brood and honey samples were collected from honeybee colonies in the eastern and western highland AEZs of Uganda. Also, 8 honey samples from imported honeys that were locally retailed in Uganda were collected and cultivated for *P. larvae*, *M. plutonius* and *A. apis* to establish if they could be the source of these pathogens in the country. Here, data showing the presence of *P. larvae* in one honeybee colony without any clinical symptoms of AFB is presented and the epidemiological implication of the findings are discussed.

3.3 Materials and methods

3.3.1 Study area

This study was conducted in the eastern and western highland AEZs of Uganda. Details of the descriptions of these AEZ can be found in Chapter 2. In each AEZ, two districts were purposively selected and sampled. In each district, sub-counties known for beekeeping activities were chosen

in consultation with beekeeping extension workers. Then lists of beekeepers were obtained from the District Production Offices and the apiaries sampled were selected based on altitude and land uses. Six colonies were sampled per apiary whenever there were sufficient colonies.

A total of 67 honeybee colonies from 32 apiaries were sampled in two sampling occasions (dry and wet seasons) between December 2014 and September 2015 (Table 3.1). Only three honeybee colonies were sampled twice (during both seasons) because some colonies had either absconded or did not have brood at the time of second sampling. One brood comb from each honeybee colony was collected and frozen as soon as it was possible and later analyzed for *P. larvae*, *M. plutonius* and *A. apis* in the research laboratory at Ghent University, Belgium. During field work, observations on honeybee colony strength and productivity and clinical symptoms of AFB, EFB and chalkbrood were made and recorded. In addition, eight samples of honey imported and retailed in Uganda were collected directly from supermarkets in Mbale and Kabarole districts.

3.3.2 Culturing for *P. larvae*

In the laboratory, the culturing of *P. larvae* was performed according to routine protocols (de Graaf *et al*., 2006). Each brood sample was swabbed using cotton wool swabs (n = 10 cells; 5 on either side of the comb using two swabs) and the cotton wool washed in 5 ml Phosphate Buffered Saline (PBS). The sample was then heated in a water bath for 15 min at 80^{o}C and 150 µl pipetted onto MYPGP agar containing nalixidic (10 µg/ml) and pipemidic (20 µg/ml) acids. The agar was left to dry before being incubated at 37^{o}C for four days.

For honey, 5 g of the honey sample was measured in a 15 ml conical tube. Then, 5 ml of PBS was added into the honey sample and incubated for 15 minutes at 60^{0}C to dissolve the sugar in honey. The solution was vortexed for 10 seconds to mix it thoroughly before centrifuging at 3000 rpm for 30 minutes to separate debris from honey. The honey liquid was poured out and 150 µl of PBS added to the remaining debris. The mixture was vortexed before being incubated at 80^{0}C for 15 minutes. The culturing onto MYPGP agar was done in the same way as was for brood.

All agar plates were observed for bacterial growth. Bacterial colonies were observed for similarities with *P. larvae* reference strain (LMG 9820). Suspicious colonies were subjected to catalase tests and those which were catalase negative were gram stained and examined at 1000x magnification on a microscope. Colonies were confirmed as *P. larvae* by PCR (Dobbelaere *et al*., 2001).

3.3.3 Culturing for *M. plutonius*

M. plutonius was cultured on Basal solid agar (BSA) medium according to the protocol described in Forsgren *et al*. (2013). Briefly, each brood sample was swabbed using cotton wool swabs (n = 10 cells; 5 on either side of the comb using two swabs) and the cotton wool washed in 5 ml PBS. Then, 150 µl of the sample was pipetted in basal medium and the plates incubated for 7 days at 35°C anaerobically. All agar plates were observed for bacterial growth. Bacterial colonies were observed for similarities with *M. plutonius* reference strain. Suspicious colonies were gram stained and examined at 1000x magnification on a microscope.

3.3.4 Culturing for *A. apis*

In the laboratory, culturing for *A. apis* was done in yeast-glucose-starch agar (YGPSA) following Jensen *et al*. (2013). This was performed by pipetting 150 µl of the honeybee sample (also used for to culture for *M. plutonius*) into the YGPSA medium and incubating at 34^{o}C for 10 days. The plates were observed for fungal growth and colonies were matched with *A. apis* culture from a chalk-brood positive Belgian sample.

3.3.5 PCR for *P. larvae*

A colony of the suspected bacterial sample was suspended in 50 µl of distilled water and heated to 100°C for 10 min. The sample was then centrifuged at 13,300 rpm for 5 min and 1 µl of the supernatant was amplified in a 25 µl PCR mixture containing the following: 10x PCR buffer, 2.5 mM $MgCl_2$, 50 pmol of each primer (AFB-F: 5'-CTTGTGTTTCTTTCGGGAGACGCCA-3' and AFB-R: 5'-TCTTAGAGTGCCCACCTCTGCG-3') (Dobbelaere *et al*., 2001), 400 pmol of each deoxynucleoside triphosphate, and 1.25 U of *Taq* polymerase. The PCR conditions consisted of a 94°C (5 min) step; 30 cycles of 93°C (1 min), 55°C (1/2 min), and 72°C (1 min); and a final cycle of 72°C (10 min). As

a positive control, *P. larvae* LMG 9820 was used. The molecular weights of the PCR products were compared with those of the Generuler 1kb plus marker on a 1% agarose gel stained with ethidium bromide and visualized under UV light.

3.3.6 ERIC genotyping

ERIC genotyping was performed following the procedures described by Genersch *et al*. (2006). Briefly, the DNA sequences of the primers used for *P. larvae* DNA fingerprinting were 5'-ATGTAAGCTCCTGGGGATTCAC-3' (ERIC1R) and 5'-AAGTAAGTGACTGGGGTGAGCG -3' (ERIC2). The PCR were carried out in final volumes of 25 µl consisting of 1x reaction buffer (Qiagen) and final concentrations of 2.5 mM $MgCl_2$, 250 mM dNTPs, 10 mM primer and 0.3 U HotStarTaq polymerase (Qiagen). The reaction conditions were: an initial activation step (95^{o}C for 15 min); 35 cycles at 94^{o}C for 1 min, 53^{o}C for 1 min and 72^{o}C for 2.5 min, followed by a final extension step at 72^{o}C for 10 min. A 10 µl sample from the PCR was analyzed on a 0.8% agarose gel. A positive control for each ERIC genotype was used (LMG 9820, R 20833, LMG 16252 and LMG 16247).

3.3.7 Infection assay

The virulence test was conducted at the Laboratory of Molecular Entomology and Bee Pathology (L-MEB), Ghent University following the protocol described by de Graaf *et al*. (2013) using *Apis mellifera carnica* larvae. Briefly, plates each consisting of 24-wells were incubated at 34°C for 24 hours. A group of 30 larvae (in 3 wells) was treated with the Ugandan *P. larvae* isolate, another group of 30 larvae was treated with the *P. larvae* strain BRL 230010. Six (6) wells were left empty and filled with 1 ml of distilled water to avoid desiccation. Three hundred (300) µl of the spore-contaminated larval diet (20 spores of *P. larvae*/ µl feed) was added into each well of the treatment group. Three wells for the negative control group were left and fed on non-spore contaminated larval diet during the entire experiment. After 24 hours of infection, larvae were transferred to a pre-warmed, fresh normal larval diet (royal jelly, fructose and glucose) plate. The grafting tool was decontaminated between each group to avoid reinfection. Every treatment group received fresh larval diet every 24 hours and the plates were analyzed each day under a

stereo microscope to determine the health status of the larvae. Old feed was removed daily and replaced with pre-warmed fresh larval diet. After defecation (day 8), the larvae were transferred to pupation plates. The larvae were classified as dead when they stopped breathing (movement of tracheal openings stops) and lost body elasticity. The number of dead larvae was recorded every day. To determine whether *P. larvae* infection caused the death of a larva, dead larvae were plated out on MYPGP plates. Plates were incubated for three days at 37°C to allow the growth of vegetative bacteria. Positive AFB infection was confirmed by growth of *P. larvae*. Further confirmation was provided by performing *P. larvae*-specific PCR-analysis of colonies grown from larval remains.

Data analyses

Statistical analyses were conducted with the SPSS statistical program (version 16). The Kaplan-Meier method was used to compare the survival of honeybee larvae in the infection experiment (larvae fed on KAS-07 Ugandan *P. larvae* strain, ERIC I reference strain (BRL 230010) and uninfected (control).

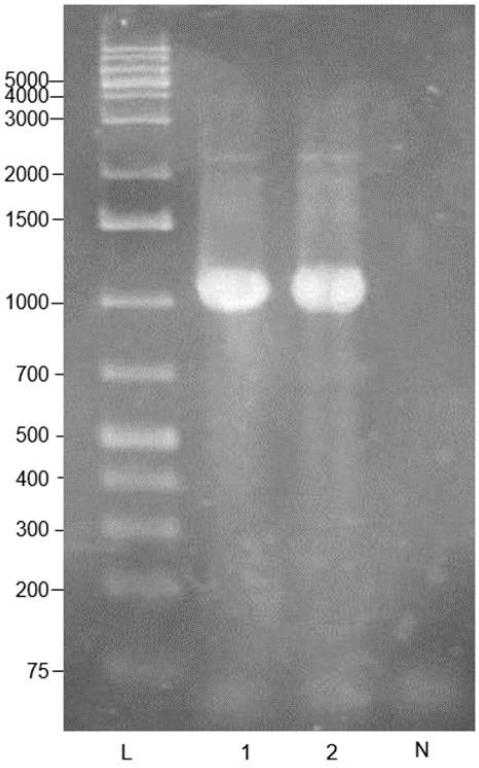
3.4 Results

M. plutonius and *A. apis* were not detected in all the honeybee brood samples. The presence of *P. larvae* in samples of honeybee brood and honey from the two agro-ecological zones of Uganda is shown in Table 3.1. A total of 59 brood samples from the two AEZs of Uganda were analyzed during the dry season. During the wet season, 11 brood and 13 honey samples were analyzed. No brood sample showed any clinical signs of AFB in the field. None of the honey samples were found to be contaminated with *P. larvae* spores. Of the 59 honeybee brood samples analyzed during the dry season, only one (sample KAS-07) (representing 1.7%) was confirmed positive for *P. larvae*. As expected, the PCR amplicon was around 1100-bp (1106-bp) determined based on the Generuler 1kb plus marker (Figure 3.1).

Table 3.1: Summary of the number of samples analyzed for *P. larvae* during the two seasons and results obtained.

S/N	Source of samples	Dry season		Wet season	
		Brood	Honey	Brood	Honey
1	Eastern AEZ	23 (0)	-	10 (0)	2 (0)
2	Western highland AEZ	36 (1)	-	1 (0)	3 (0)
3	Imported to Uganda	-	-	-	8 (0)

Figures indicate the number of samples analyzed; those between brackets indicate the *P. larvae* positive samples found.



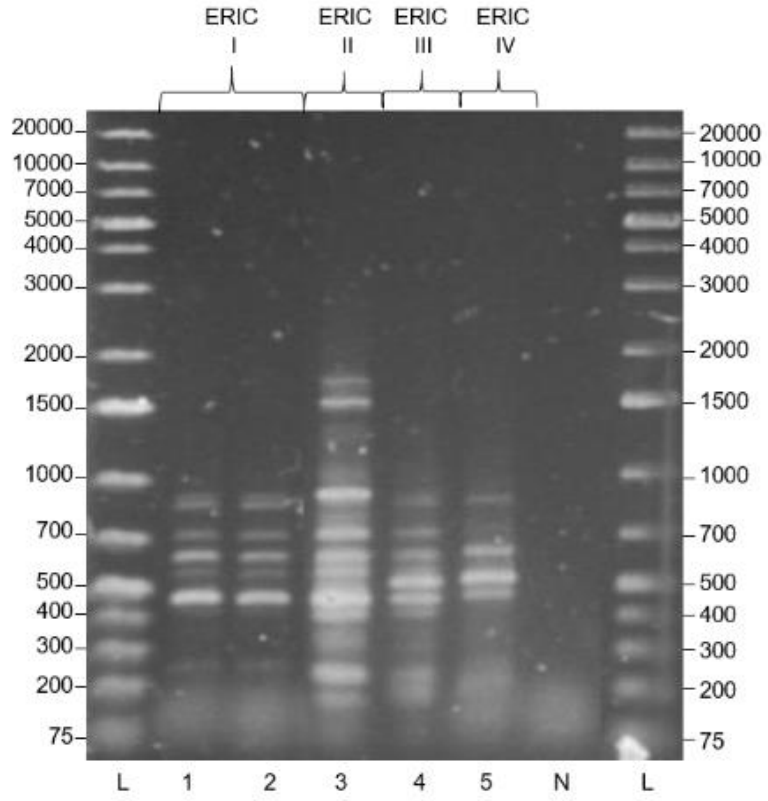
L= Generuler 1kb plus marker, 1= sample KAS-07, 2 = positive control (LMG 9820), N = negative control.

Figure 3.1: PCR product image.

ERIC PCR and virulence assay

The survival analysis using the Kaplan-Meier method on *A. mellifera carnica* larvae fed on *P. larvae* strain is shown in Figure 3.3. The results gave significant values (Log rank (Mantel-Cox: χ^2(df = 2) = 88.56, $P < 0.001$), Breslow (Generalized Wilcoxon: χ^2(df = 2) = 51.004, $P < 0.001$) and Tarone-Ware: χ^2(df = 2) = 67.790, $P < 0.001$) indicating that there were statistical differences in the survival times between treatments. Kaplan-Meier plots show that the uninfected (control)

bee larvae survived more than the bee larvae that were infected with both the reference *P. larvae* strain (BRL 230010) and the Ugandan (KAS-07) strain. The survival assay finding confirms that the strain obtained is at least equally virulent when compared to BRL 230010, which is an ERIC I genotype that was isolated from diseased colonies in the USA (Qin *et al*., 2006).



L= Generuler 1kb plus marker, 1= sample KAS-07 (found strain), 2 = LMG 9820, 3 = R 20833, 4 = LMG 16252, 5 = LMG 16247, N = negative control.

Figure 3.2: ERIC PCR product image.

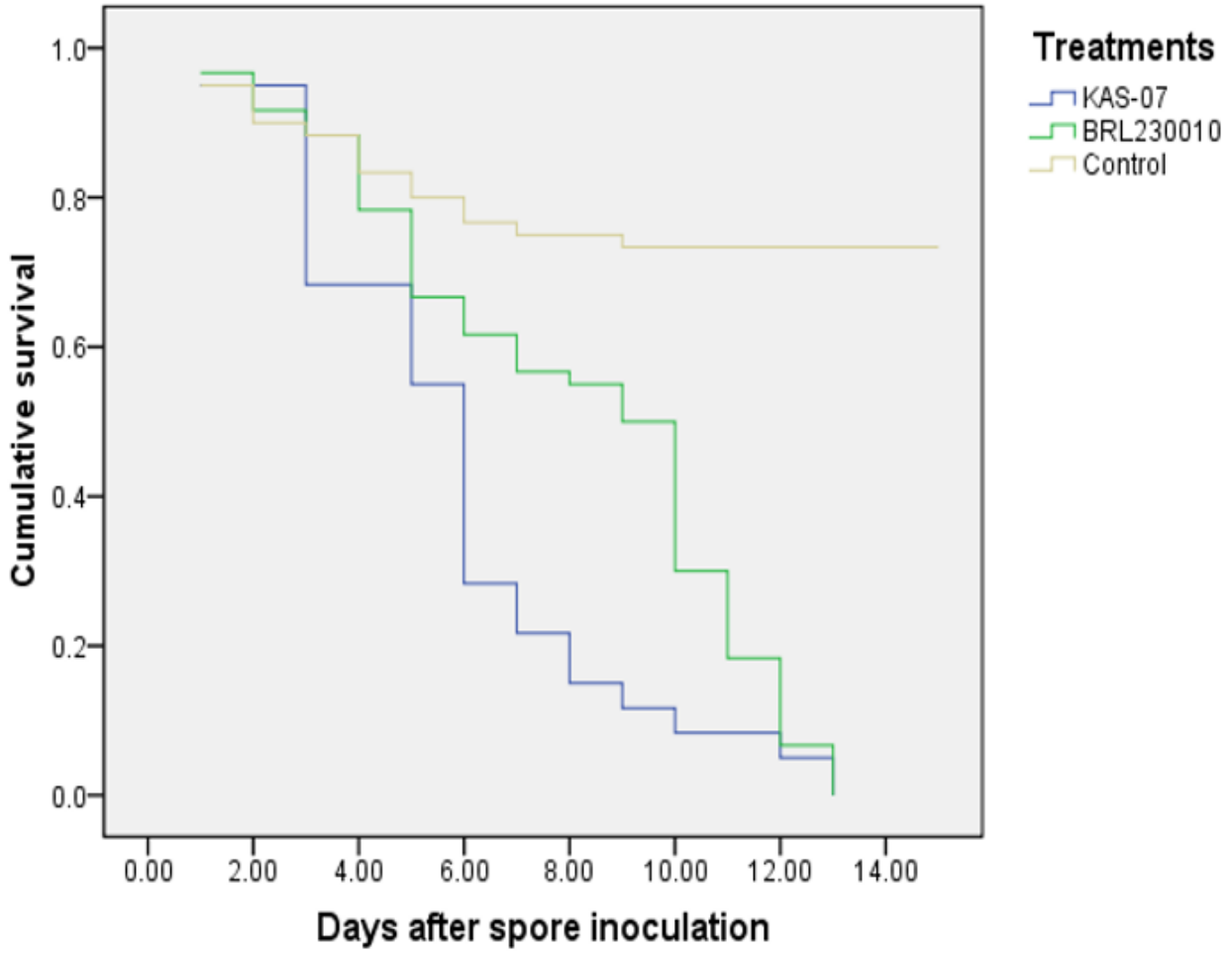


Figure 3.3: Kaplan-Meier survival plot of *A. mellifera* carnica larvae fed on KAS-07 Ugandan *P. larvae* strain, ERIC I reference strain (BRL 230010) and uninfected (control).

3.5 Discussion

Several reports on the presence of honeybee brood pathogens in *A. mellifera* populations in different African countries have been published (Hansen *et al*., 2003; Fries & Raina, 2003; Human *et al*., 2011; Masry *et al*., 2014; Adjlane *et al*., 2014). Most of these reports were based on honey analyses and not honeybee brood. Moreover, the sampling in most cases was limited to few honey samples, which did not permit the workers to detect the low pathogen levels typical of persistent inapparent infections. Here, the first survey of the infection rates of honeybee brood pathogens in seemingly healthy honeybee colonies from 32 apiaries, in two AEZs of Uganda is described. The apiaries sampled varied in altitude from 920 to 2400 m above sea level and included farmlands, *Eucalyptus* plantations and protected areas.

Of the three brood pathogens investigated by culture methods, *P. larvae* was the only pathogen detected in the colonies. In this study, 1.7% of the honeybee colonies sampled during the dry season were positive for *P. larvae*. Being the first finding of *P. larvae* in Uganda, it was necessary to perform ERIC genotyping and virulence tests to compare this strain with reference strains found in some western countries. Moreover, as clinical signs were not observed, it was difficult to conclude that this strain had any disease causing potential. Also, virulence tests of the *P. larvae* strain found were performed on *A. mellifera carnica* at the research laboratory of Ghent University, Belgium since there were neither facilities nor the required biosafety certificate to perform the experiments in Uganda. The results showed that the strain found was an ERIC I with at least equally high virulence when compared to BRL 230010 from the USA (Qin *et al*., 2006).

The infection rate (1.7%) of *P. larvae* in honeybee colonies and (3.03%) in apiaries recorded in this study is comparatively much lower than that reported in some Asian countries e.g. 37.3% in honeybee colonies in Pakistan (Anjum *et al*., 2015), 24.8% in honey samples from Taiwan (Chen *et al*., 2008) and some European countries e.g. 11% in Belgium (de Graaf *et al*., 2001), 66% in France (Mouret *et al*., 2013) and 5.3-9.8% in Latvian apiaries (Chauzat *et al*., 2014; Laurent *et al*., 2015). However, the infection rate of AFB recorded in the study apiaries is in the range of 1-5.7% in Estonia, 1.5-4.5% in Greece, 1.6-4.7% in Poland, 2% in Sweden, 2.6% in Slovakia, 2.2-2.7% in Italy (Chauzat *et al*., 2014; Laurent *et al*., 2015) and 1.6-3.2% in Spain (Garrido-Bailón *et al*., 2013). Long term epidemiological studies show that the prevalence levels of AFB vary over time. For example, in Uruguay, AFB prevalence levels fluctuated over 12 years after it was first reported (Antúnez *et al*., 2012) suggesting that the infection levels recorded in Ugandan apiaries could change over time.

In Africa, AFB has been confirmed in South Africa (Human *et al*., 2011), Guinea Bissau (Hussein, 2000; Hansen *et al*., 2003) and Egypt (Masry *et al*., 2014). *P. larvae* has also been detected in honey originating from Tunisia (Matheson, 1993; Hussein 2000; Fries & Raina, 2003; Hamdi *et al*., 2013), Algeria, Libya and Morocco (Hussein, 2000). Studies on *P. larvae* in Africa used culture techniques mostly on processed honey which normally gets contaminated when brood is heavily

infected. This reduces the chances of detecting the pathogen if the infestation level is low. Therefore, more sensitive sampling techniques such as collecting honey from combs within the brood areas should be used when investigating the presence of *P. larvae* in Africa. Despite *P. larvae* and other honeybee parasites like *Varroa destructor* being reported in Africa (Human *et al*., 2011; Muli *et al*., 2014; Strauss, *et al*., 2015; Chemurot *et al*., 2016a), no major colony losses have been reported yet. This could be associated with the higher levels of disease resistance in African honeybees (Human *et al*., 2011). However, such a trait may not persist if pathogens accumulate in hives especially with the promotion of frame beehives and honeybee breeding programs that do not consider disease resistance. Therefore, efforts should be made to prevent loss of the disease resistance in African honeybees.

Behavioral adaptations such as abscondment and swarming among African honeybee races may also explain their low levels of parasite infestation (Chemurot *et al*., 2016a). Two honeybee races, *Apis mellifera scutellata*, and *Apis mellifera adonsonii* have been confirmed in Uganda (Kasangaki, 2016). These honeybee races abscond from beehives more frequently when disturbed than other races of *A. mellifera* (Hansen and Brodsgaard, 1997). This behavioral trait could result in disinfection in honeybee colonies formally infected by *P. larvae* (Hansen and Brodsgaard, 1997).

The higher levels of hygienic behavior of African honeybees may also reduce the level of AFB infection (Fries and Raina, 2003; Human *et al*., 2011). In addition, the wax moth which is a very common pest in Africa and only affects weak colonies (Strauss *et al*., 2013) reduces AFB infestation levels by removing infected combs (NBU, 2014). The overall implication of this is that relatively very low AFB infection levels and extremely rare development of clinical symptoms are observed. During this study, wax moth larvae (Figure 3.4) were observed in 80% of the apiaries sampled during the wet season.

Figure 3.4: Wax-moth larva (red arrow) and the damage it causes on honeybee combs (yellow circle).

The *P. larvae* positive sample in this study was from a colony in a protected area suggesting that this pathogen could be present in feral honeybee colonies. Since beekeepers in Uganda rely on natural honeybee colonies to populate their beehives (Chemurot, 2011), *P. larvae* could spread from feral to managed colonies. On the other hand, absconding and swarming which are common among African honeybee races (Hansen and Brodsgaard, 1997) could also spread this pathogen from managed to feral colonies. However, the current predominant use of traditional and top-bar beehives (Chemurot, 2011) reduces chances of this pathogen accumulating in honeybee combs since beekeepers harvest the entire comb.

The results suggest that *P. larvae* is present in very few honeybee colonies in Uganda. This could be due to the beekeeping practices, absconding and swarming behavior of available honeybee races. Therefore, the prevailing beekeeping practices such as the use of traditional and top-bar beehives should not be drastically changed in order to keep this pathogen in balance and reduce potential production losses to beekeepers.

CHAPTER 4

Microsporidian parasite infestation in Ugandan honeybees

4.1 Abstract

The microsporidian parasites, *Nosema apis* and *Nosema ceranae* of honeybees are important threats to beekeeping in western countries. In order to provide baseline data and gain some insights into the infestation rates and seasonal patterns of microsporidian parasite infections in Ugandan honeybees, this study was conducted to: i) identify the *Nosema* spp. present, ii) understand the variation in spore infection levels among honeybee colonies located in areas of different land-use types and elevations and iii) determine how infection levels might affect honeybee colony strength and productivity. Microscopy and PCR for the detection and differentiation of *N. ceranae* and *N. apis* were used. The findings indicate that *N. apis* and *N. ceranae* were present in the sampled study sites. In addition, a newly detected microsporidian that is smaller than *N. ceranae* and has fewer polar filament coils is described. This new parasite was found at higher infestation rates compared to the other two known microsporidian parasites of honeybees. *Nosema* spore counts in colonies located in protected areas were significantly lower than those in farmlands ($P < 0.01$) and *Eucalyptus* plantations ($P < 0.01$). However, *Nosema* spore counts were not significantly different in colonies in *Eucalyptus* plantations and in farmlands ($P = 0.825$). The results also showed that there was a significant negative correlation between *Nosema* spore count and height of honeybee nest (beehive) from ground in both the eastern (rho = -0.333, n = 92, $P = 0.001$) and western highland agro-ecological zone (rho = -0.364, n = 86, $P = 0.001$) during the dry season. In the wet season, there was no correlation between spore count and height of beehives from ground (eastern: rho = 0.194, n =79, $P = 0.087$; western highland: rho = 0.142, n = 115, $P = 0.129$). Finally, the number of honeybee frames/top bars with honeybees and amount of honey which was harvested were negatively correlated with *Nosema* spore counts, suggesting that *Nosema* is negatively affecting honeybee colony performance in Uganda.

Key words: Honeybee; Colony productivity; Landscape factors; Microsporidia; *Nosema* species; Uganda

4.2 Introduction

Two species of Microsporidia are currently known to infect the honeybee, *Apis mellifera* worldwide. These are *Nosema apis* which was first reported over 100 years ago (Zander, 1909) and the relatively recent *Nosema ceranae* (Higes *et al*., 2006; Huang *et al*., 2007; Traver *et al*., 2012). Both *N. apis* and *N. ceranae* are obligate intracellular parasites of the midgut of honeybees. They have been reported in all continents where beekeeping with *A. mellifera* occurs (e.g. in Africa by Fries *et al*., 2003; Higes *et al*., 2009 and Muli *et al*., 2014; in Europe by Higes *et al*., 2006 and Paxton *et al*., 2007; in North America by Chen *et al*., 2008; in South America by Calderón *et al*., 2008; in Asia by Chen *et al*., 2009). The two species of *Nosema* cannot clearly be distinguished under the light microscope. However, under the electron microscope, *N. ceranae* has 20 - 23 polar filament coils while *N. apis* has more than 30 polar filament coils (Fries *et al*., 1996).

Infection of honeybees by *N. apis* and *N. ceranae* has negative impacts that vary with the geographical location and the *Nosema* species involved. Damage to colonies can include suppression of the honeybee immune system (Antúnez *et al*., 2009), shortening of worker bee lifespan (Mayack & Naug, 2009), decline in colony strength and productivity (Botías *et al*., 2013), queen supersedure (Alaux *et al*., 2011), increased winter losses and colony collapse (Higes *et al*., 2008). Such impacts demonstrate *Nosema* spp. as a threat to honeybees and hence the need to monitor its presence.

Beekeeping is an important economic activity in the rural areas of Uganda providing a supplementary source of income for rural households. However, limited information is available on honeybee pests and pathogens in the country that can be used to plan for their management. In order to provide baseline data and gain some insights into the infestation rates and seasonal patterns of microsporidian parasite infestation in Ugandan honeybees, this study was conducted to: i) identify the *Nosema* spp. present, ii) understand the variation in spore infection levels among honeybee colonies located in areas of different land-use types and elevations and iii) determine how this variation affects honeybee colony performance. Particularly, the interest was

to address this question uniquely through an altitudinal and land-use type stratification of study sites. Different detection methods for *Nosema* spp. including microscopy and a PCR for differentiation of *N. ceranae* and *N. apis* were used. Since several samples with visible microsporidia-like spores under the light microscope turned negative for the known *N. ceranae* and *N. apis*, Nosematidae family primers were used which gave positive results. Transmission electron microscopy (TEM) on the spores, amplification and sequencing of the entire 16S SSU rRNA were performed. The data confirmed the discovery of a new species of microsporidia that was found at high infestation rates in the study sites in Uganda. In addition, *N. apis* and *N. ceranae* were also present in the sampled study sites.

4.3 Materials and methods

4.3.1 Study area

This study was conducted in the eastern and western highlands AEZs of Uganda. Beekeepers in these two AEZs, practice beekeeping activities in agricultural and non-agricultural areas (protected areas) which allow evaluation of the potential influence of land use type on the health of honeybees. Details of the description of the study sites can be found in Chapter 2.

4.3.2 Field data and sample collection

Samples of worker honeybees were collected from the edges inside the beehives. A total of 175 colonies were sampled during the dry season and 195 during the wet season from the eastern and western highlands AEZs of Uganda. Honeybee samples were preserved in 95% ethanol and refrigerated as soon as possible until transported to Ghent University, Laboratory of Molecular Entomology and Bee Pathology for analyses. During fieldwork, the geographical coordinates and altitude of each apiary site were recorded using a GPS receiver. Also, height of beehives sampled from the ground, distance to the nearest water source, land uses and apiary management activities around the apiary were recorded. Detailed description of the field data and sample collection are provided in Chapter 2.

4.3.3 Colony strength and productivity

Because of the high level of defensiveness of African honeybees, the subjective methods of estimating colony strength were used as described by Delaplane *et al*. (2013). The adult bee population and amount of brood per colony were estimated in the two sampling moments. This was achieved by analyzing the top bars/frames covered by bees and quantifying the number of brood combs. Also the number of combs with pollen and honey were estimated whenever it was possible. The honey production in colonies that were harvested during the harvesting season (January-February 2015) were analyzed separately by weighing all the comb honey from each colony.

4.3.4 Detecting and counting *Nosema* spores

To determine the degree of infection of *Nosema* spp. a haemocytometer was used as described in the BEEBOOK (Fries *et al*., 2013). To detect and evaluate *Nosema* spp. infestation in the colonies, samples of bees (n = 10 bees per sample) were soaked in 10 ml PBS in 15 ml tubes and electronically agitated in a cold room (4^{o}C) for one day to wash off the ethanol. The PBS was changed the following day and the sample agitated in the cold room for another day to rehydrate the honeybees. Then, the honeybees were transferred into 5 ml self-standing tubes containing 5 ml PBS, about 0.25 ml zirconia beads and 5 metal beads. The honeybees were crushed in 5 rounds lasting 5 minutes each in the Bullet Blender Storm 5®/ VISUM IDPBW at maximum speed.

A volume of 20 µl of the sample was pipetted onto a haemocytometer (Bürker) and observations made at 400x magnification. When *Nosema* spores were noticed, counts were made in the counting chamber and estimates of the total number of spores per honeybee computed using the following formula modified from (OIE, 2013):

$$\text{Number of spores per bee} = \frac{\text{number of spores counted} \times 500}{\text{surface area counted (mm}^2) \times \text{depth of chamber (mm)} \times \text{dilution}}$$

For this study:

- Number of spores counted refers to the actual spore count made on the counting chamber.
- Surface area counted is the number of mini-squares where counting was done multiplied by the area of each mini-square (0.0025 mm^2).
- The depth of the chamber was 0.1 mm.
- Dilution in this case was 1 since no dilutions were made.
- The 500 was a constant (since 10 bees were suspended in 5 ml PBS, each bee was suspended in 500 µl PBS).

4.3.5 PCR to confirm species of *Nosema*

Total RNA was extracted from 10 bees per sample using QIAamp® viral RNA kit following the manufacturer's guidelines. Details of the steps followed in RNA extraction, storage and cDNA synthesis are shown in Chapter 2.

A singleplex PCR method was used to confirm the *Nosema* spp. present because it is currently the most sensitive method for discerning each species (Carletto *et al*., 2013). The PCR primers QNoUF2 (5' – GGA TTG TGC GGC TTA ATT TGA –3') and QNo.AR: (5' – CCT CAG ATC ATA TCC TCG CAG –3') were used to amplify a 77 bp fragment of *N. apis* 16s rRNA. On the other hand, for *N. ceranae*, the same forward primer QNoUF2 (5' – GGA TTG TGC GGC TTA ATT TGA –3') was used but the reverse primer was replaced with QNoCR (5' – ACC ACT ATT ATC ATT CTC AAA C – 3') targeting a 97 bp fragment of 16s rRNA. For each PCR reaction, 2.5 µl of 10X buffer, 1 µl of dNTP mix, 1.25 µl forward primer, 1.25 µl reverse primer, 0.25 µl of Taq polymerase and 13.75 µl of water were prepared (mixed thoroughly) and pipetted into PCR tubes. Then 5 µl of cDNA was added and the mixture centrifuged briefly before being placed in the PCR machine. The following temperature cycle for amplifying *Nosema* spp. DNA was used: 5 min at 94^{o}C, denaturation for 30 sec at 94^{o}C, annealing 30 sec at 60^{o}C, extension for 30 sec at 72^{o}C (35 cycles) and final extension for 3 min at 72^{o}C. The amplified PCR products were electrophoresed for 60 min at 100 volts

through 1.5% agarose TBE gel in standard TBE buffer, stained with ethidium bromide and visualized using UV illumination.

Some samples with visible spores under the light microscope gave negative PCR results for both *N. apis* and *N. ceranae*. Therefore, a set of Nosematidae family primers designed to amplify a 240 bp region of the 16S rRNA gene (Nos-F: 5' – TAT GCC GAC GAT GTG ATA TG -3' and Nos-R: 5' – CAC AGC ATC CAT TGA AAA CG -3') corresponding to nucleotides 644 to 883 (Higes *et al*., 2006; Fernández *et al*., 2012) were used. For each sample, the reaction consisted of 2.5 µl of 10X buffer, 1 µl of dNTP mix, 1.25 µl forward primer (NOS-F), 1.25 µl reverse primer (NOS-R), 0.25 µl of Taq polymerase, 13.75 µl of water and 5 µl of cDNA. The parameters of amplification were set as follows: 5 min at 95^{o}C, denaturation for 30 sec at 94^{o}C, annealing 30 sec at 52^{o}C, extension for 30 sec at 72^{o}C (35 cycles) and final extension for 10 min at 72^{o}C. Amplified PCR products were electrophoresed for 45 min at 120 volts through 2% agarose TBE gel in standard TBE buffer, stained with ethidium bromide, and visualized using UV illumination. Amplicons of three selected samples were sequenced. The sequences obtained were edited and alignments performed using Clustal-W and compared with those in the GenBank using BLAST.

4.3.6 Transmission electron microscopy (TEM) of spores

One sample of the honeybee homogenate with observable microsporidia-like spores under the light microscope but which was negative for both *N. apis* and *N. ceranae* was prepared for TEM. For primary fixation, 20 µl of the sample was placed in a fixative containing: 2% formaldehyde (FA) (EM-grade), 2.5% glutaraldehyde (GA) (EM-grade), 0.1M cacodylate buffer in a 1.5 ml tube. Fixation was gradually conducted by agitating the honeybee sample in the fixative for one hour before transferring it to another tube containing the buffer (three hours in total). Post fixation was done by putting the sample in reduced 1% OsO_4 and keeping it at 4^{o}C in an agitation machine for 1 h. The sample was then washed four times with ddH_2O at 4^{o}C in a rotating machine before being left for 2 h in 1% uracil acetate (in dark) for staining at 4^{o}C. Dehydration was done at 4^{o}C for 30 min each in 7%-15%-30%-50%-70% ethanol in a rotating machine. Thereafter, further dehydration at 4^{o}C for 30 min each in 95%-100%-100% dried ethanol in a rotating machine was

done. Infiltration was conducted at 4°C: 1/3 - 2/3 - 3 x 3/3 propylene oxide for one hour per step and at 4 °C: 1/3 – 2/3- 3 x 3/3 Spurr's resin for 8 h per step. Embedding was done in Spurr's resin and polymerization at 70°C. Light microscopy (LM) sectioning was done by making semi thin sections with an ultra-microtome (Leica EM UC6) at 0.5 mm and stained with 1% toluidine blue and 2% borax in distilled water. Electron microscopy (EM) sectioning was done by making ultrathin sections of gold interference colour made with an ultra-microtome (Leica EM UC6) and post-stained in a Leica EM AC20 for 40 min in uracil acetate at 20°C and for 10 min in lead citrate at 20°C.

4.3.7 Developing a diagnostic tool for the new *Nosema* species

A set of primers (Nos_ssu_18F: 5'-CACCAG GTT GAT TCT GCC-3' and Nos_ssu_1537r: 5'-TTA TGA TCC TGC TAA TGG TTC-3') designed to amplify the entire 16S rRNA gene of Nosematidae (Dong *et al*., 2010) were used on one sample (also used for TEM in section 4.3.6) with visible spores under microscopy but which gave negative results for *N. apis* and *N. ceranae*. The PCR program was set as follows: 2 min at 95°C, 30 sec at 94°C, 1 min at 50°C, 2 min at 68°C for 35 cycles and final extension 10 min at 68°C. The PCR product was purified using the GenJET Gel extraction kit #K0691 following the manufacturers' protocol with slight modifications (elution with 25 µl elution buffer instead of 50 µl). The purified PCR product was transformed into a plasmid using the TOPO® TA cloning® Kit for sequencing following the manufacturers protocol. The GenJet plasmid miniprep kit was used to extract the plasmids and sent for commercial sequencing. The sequences obtained were edited and alignments performed using Clustal-W and compared with those in the GenBank using BLAST. It was found to be 97% similar to *N. apis* (U97150.1).

We opted to work with the universal forward primer, QNoUF2 (5' – GGA TTG TGC GGC TTA ATT TGA –3') which is also used for *N. apis* and *N. ceranae*. A reverse primer was developed in the most variable region when compared with the known *Nosema* species. Two possible primers, Nos_new_R1 CCT CAA ATA GAA TCA TCG CCG G and Nos_new_R2 CAC TAG AAG TGT CAG TCC TAC were selected. The PCR program used was: 15 min at 95°C; 30 sec at 94°C; 30 sec at 60°C; 30 sec at 72°C: 35 cycles and final extension for 3 min at 72°C. When we conducted trial tests on the

developed primers on the new species, *N. apis* and *N. ceranae*, the universal forward primer and reverse (Nos_new_R2 CAC TAG AAG TGT CAG TCC TAC) were specific for the new microsporidian detected in Ugandan honeybees. The amplified PCR products were electrophoresed for 60 min at 100 volts through 1.5% agarose TBE gel in standard TBE buffer, stained with ethidium bromide, and visualized using UV illumination. All samples collected from Uganda, 21 samples from Greece and 96 Belgian samples were analysed using the developed primers to establish the presence of the new species.

4.3.8 Construction of the phylogenetic tree using the SSU rRNA gene sequences

The SSU rRNA gene sequences of 19 microsporidia were aligned and edited using the Clustal-W Program. Phylogenetic trees based on the resultant alignments were constructed using the Maximum Likelihood method of MEGA7 software (Kumar *et al*., 2016). In this method, initial trees were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated leaving a total of 1030 positions in the final dataset.

4.3.9 Infection experiment

Spores used in this infection experiment were obtained from a honeybee colony in Kasese district which had observable microsporidia-like spores under the light microscope but that were PCR negative for *N. apis* and *N. ceranae*. From this sample, the entire SSU rRNA was amplified, sequenced and TEM performed on the homogenate with the results clearly showing that it was a microsporidian (sub-sections 4.4.1 and 4.4.2). In order to confirm the viability of spores that were extracted from bees preserved in 95% ethanol (December 2014-March 2015) and kept in PBS at -20^{o}C (March 2015-July 2016), an infection experiment was conducted. This would also generate a new stock of spores for other infection experiments. Before the experiment, the spore suspension (1 ml) obtained from grinding 10 bees was filtered using sterile (Faltenfilter 615 ¼) filters. The filtrate was purified by centrifugation following Fries *et al*. (2013). Briefly, the filtrate

was centrifuged at 7,200 rpm for five minutes to produce a pellet of spores and the supernatant discarded. The pellet was re-suspended in distilled water by vortexing for five seconds and the procedure (centrifugation, supernatant discarding and pellet re-suspension) repeated 3 times.

The following steps were taken during the infection experiment:

1. Adult bees from colonies which were used in the infection experiment were tested for *Nosema* spp. by collecting 10 bees at the hive entrance, grinding with a mortar and pestle and adding distilled water and examining under a light microscope.
2. Sealed honeybee brood was collected from a *Nosema* negative colony and incubated at 34^{o}C to get newly emerged worker bees.
3. The young worker bees were starved for 2 hours and fed individually using a micropipette with 10 µl of a spore solution containing an estimated 2,500 spores and then kept for 3 hours without feeding.
4. Two 500 ml cages were stocked with 30 bees each (one control and the experiment).
5. The bees were fed on sucrose solution (50% weight/volume ratio) and Candipolline Gold (containing sugar, bee pollen sterilized with gamma rays, milk and egg proteins and vitamins) throughout the experiment.
6. The cages were monitored daily for any deaths and records taken.
7. On day 4 post infection, three honeybees were dissected and five body parts separated (head, thorax, mid-gut, deviscerated abdomen and rectum) and ground using a mortar and pestle. The ground parts were mixed with distilled water and examined under the light microscope for presence of spores. The hemocytometer was used to estimate the number of spores per bee.

4.3.10 Data analyses

The data were analyzed using SPSS statistical program. To compare the *Nosema* spore counts in honeybee colonies in the three different apiary locations, Kruskal-Wallis test was performed. When significant differences were found, *post hoc* analysis with Mann-Whitney tests were used to compare pairs of categories. Mann-Whitney test was also used to compare *Nosema* spore counts in the two AEZs of Uganda, two seasons (dry and wet) and in inspected and uninspected

apiaries. Spearman's rank correlations were conducted to establish the relationships between the *Nosema* spore counts and the following: altitude, height of bee nest/beehive from ground, distance of apiary to nearest water source, colony strength and productivity. All tests were two tailed.

4.4 Results

4.4.1 *Nosema* species present

The two *Nosema* spp. that infect honeybees were detected in the study sites. However, several samples with visible spores under the light microscope gave negative PCR results for both *N. apis* and *N. ceranae.* Further PCR analyses of these samples with Nosematidae family primers gave positive results. Amplicons (240 bp fragments) of the 3 samples that were sequenced were 95% identical to *N. apis* (FJ789798.1) with 9 base pair differences. To study the phylogenetic relationship between the new species and the known microsporidian species, we amplified the entire 16S SSU rRNA of the new species. The phylogenetic tree constructed with the obtained sequence and the sequences of other related Microsporidia in the GenBank clearly demonstrate that it is most closely related to *N. apis* (Figure 4.1).

4.4.2 Description of the new species

The transmission electron micrographs showed that the new *Nosema* spp. is smaller in size (length: 2.36 ± 0.14 µm and width: 1.78 ± 0.06 µm (n = 6) and has fewer polar filament coils (10 - 12) compared to both *N. apis* and *N. ceranae* (Figure 4.2 and 4.3; Table 4.1).

The SSU rRNA of the newly detected *Nosema* spp. consists of 1242 bp nucleotides and the GC content is 38.9%. It is between 93 and 97% identical with corresponding regions of the rRNA of other *Nosema* species (*N. apis*, *N. bombi*, *N. oulemae*, *N. thomsoni*, *N. Portugal*, *N. vespula*, *N. necatrix*, *N. ceranae*). The results also suggest that this isolate may be closely related to *N. apis*, with which it shares 97% identity of the entire SSU rRNA. From the phylogenetic tree (Figure 4.1) of the SSU rRNA, the newly detected microsporidian is an isolate of true *Nosema* and it is closely related to *N. apis* (U97150.1).

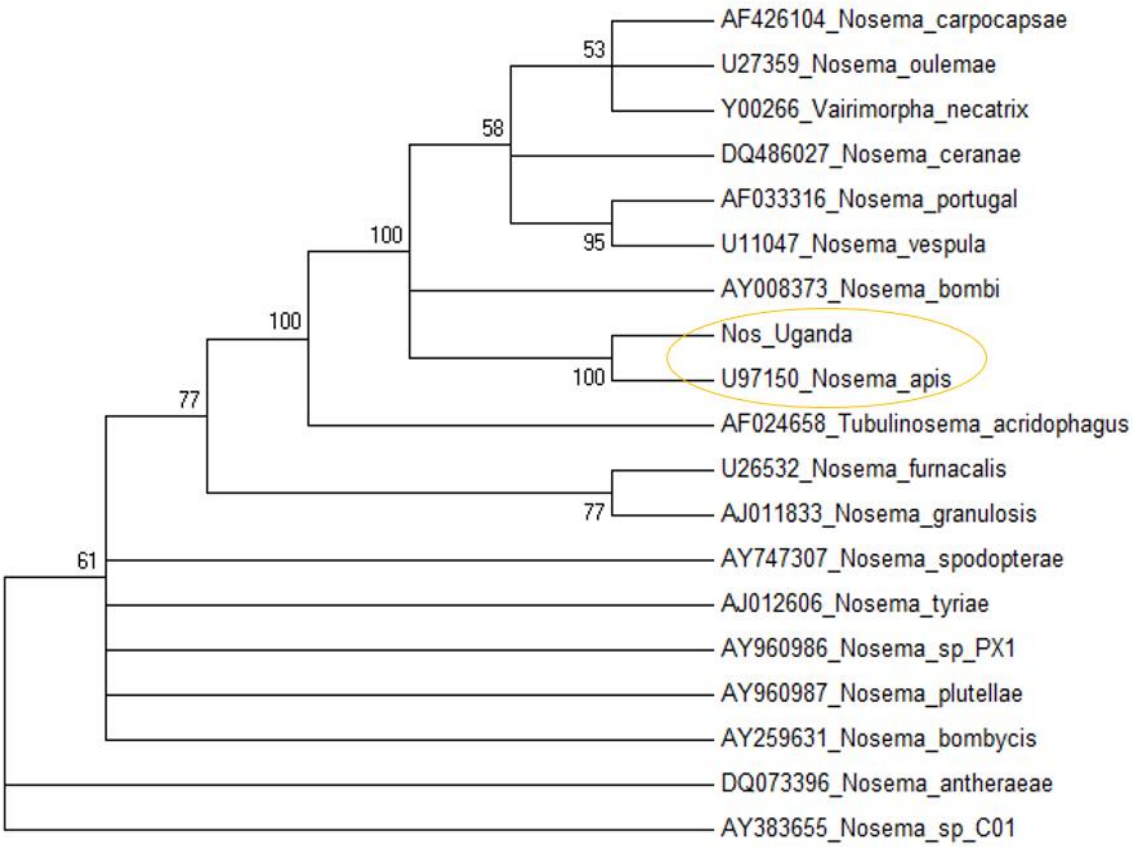
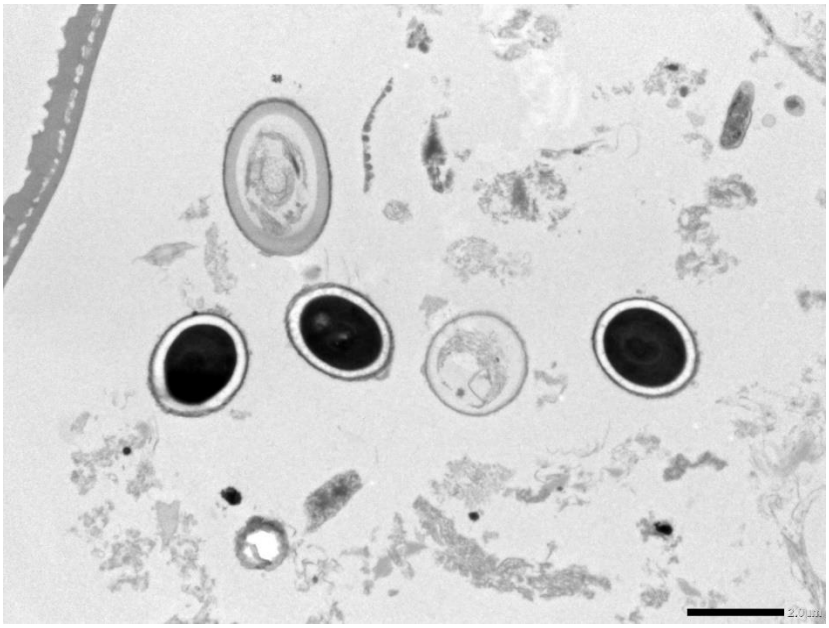


Figure 4.1: Phylogenetic tree constructed using the Maximum likelihood method showing the relationship of the newly detected *Nosema* species and other related microsporidia: full size 16s rRNA. The bootstrap values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the corresponding species name. The new *Nosema* spp (indicated in the tree as Nos_Uganda) and *N. apis* to which it is more closely related are encircled together in an orange oval.

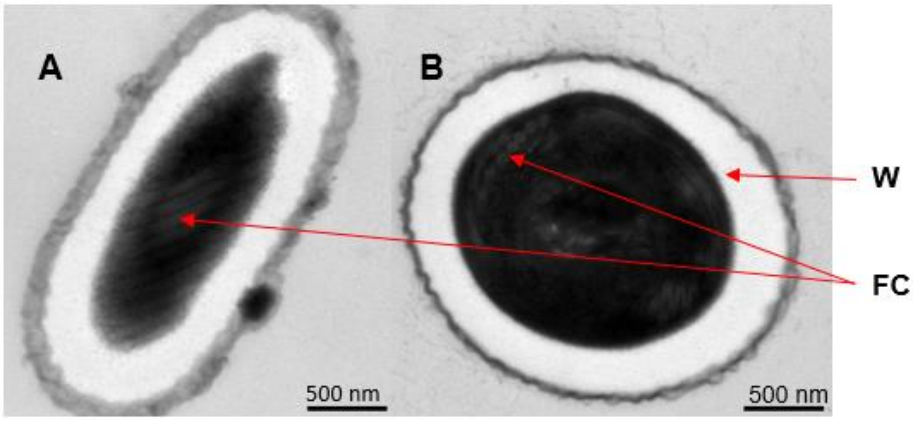
4.4.3 Development of a diagnostic tool

In order to study the infestation rate of the different *Nosema* species we developed a diagnostic tool. The laboratory tests on the tool showed that the universal forward primer (for *N. apis* and *N. ceranae*) QNoUF2 (5' – GGA TTG TGC GGC TTA ATT TGA –3') and reverse (Nos_new_R2 CAC TAG AAG TGT CAG TCC TAC) were specific for the new microsporidian detected in Ugandan honeybees.



(Length: 2.36 ± 0.14µm & width: 1.78 ± 0.06 µm (n = 6); Filament coils: 10 - 12) magnification 3000 x.

Figure 4.2: Electron micrograph of the new *Nosema* spp. spores.



(10 - 12) FC = polar filament coils, W = spore wall) Magnification 20,000 x.

Figure 4.3: Electron micrographs of the newly detected *Nosema* spp. spores with filament coils.

4.4.4 Seasonal infestation rates of *Nosema* spp.

There was varied infestation rates of *Nosema* species in colonies in the two AEZs of Uganda during the dry and wet seasons. The new *Nosema* species had higher infestation rates compared to *N. apis* and *N. ceranae*. Generally, the proportion of samples positive for the new *Nosema* spp. was higher in the dry than in the wet season (Figure 4.4).

Mean *Nosema* spore count in the eastern AEZ of Uganda was highest in the wet season (2800.63 ± 802.75 spores per bee) compared to the dry season (43.69 ± 24.21 spores per bee). In the western highlands AEZ of Uganda, the mean spore count was highest during the dry season (4247.61 ± 1578.65 spores per bee) compared to the wet season (2960.15 ± 1645.85 spores per bee). In both zones, the seasonal spore counts were statistically significant (eastern: Mann-Whitney U = 1269.5, Z = -7.478, $P < 0.01$; western highlands: Mann-Whitney U = 3389.0, Z = -3.942, $P < 0.01$).

Table 4.1: Comparison of the new *Nosema* spp. spores with *N. apis* and *N. ceranae*.

Characteristic	***N. apis***	***N. ceranae***	**New *Nosema* species**
Spore shape and size	✓ Oval or rod shaped 6.0 µm length and 3.0 µm width (Fries *et al*. 1996; Chen *et al*. 2009)	✓ Oval or rod shaped 4.4±0.41 µm length, 2.2±0.09 µm width (Fries *et al*. 1996; Chen *et al*. 2009)	✓ Oval or rod shaped 2.36±0.14µm length & 1.78±0.06 µm width: (n=6) **(Smaller)**
Nucleus	✓ Dikaryotic nuclei present in all developmental stages (Chen *et al*. 2010)	✓ Dikaryotic nuclei present in all developmental stages (Chen *et al*. 2010)	✓ Spores seem diplokaryotic
Polar filament	✓ Flexible polar filament in mature spores (Chen *et al*. 2010)	✓ Flexible polar filament in mature spores (Chen *et al*. 2010)	✓ Polar filament seen in spores
Spore Wall	✓ Mature spore with thickened wall (exospore and endospore) (Chen *et al*. 2010)	✓ Mature spore with thickened wall (exospore and endospore) (Chen *et al*. 2010)	✓ Spores with thickened wall
Polar filament coils	✓ More than 30 (Fries *et al*. 1989; Chen *et al*. 2009)	✓ 18-23 (Fries *et al*. 1996; Chen *et al*. 2009)	✓ 10-12 **(fewer)**

4.4.5 Analyses of samples from Greece and Belgium

In order to establish if the new *Nosema* species occurs also in Europe, honeybee samples collected from Belgium (Ravoet *et al*., 2013) and 10 regions of Greece (N. Marmaras, Komotini, Xanthi, Argos, Katerini, Paliouri, Volos, Kalampaka, Mytilene and Rethymno) (Hatjina *et al*., 2011) that were kept at L-MEB were analyzed for the newly detected *Nosema* spp. Interestingly, one

from Rethymno region of Greece out of twenty one samples from Greece tested positive for the newly detected species of *Nosema*. None of the 96 Belgian samples were positive for the new Microsporidia. The PCR amplicon generated on the Greece sample was sequenced and phylogenetic analysis conducted based on this sequence and sequences of related microsporidia in the GenBank. For the positive sample from Greece, the 240 bp fragment of the SSU rRNA was 100% identical to the newly detected *Nosema* sp. in Ugandan honeybees. It is also evident that the *Nosema* positive samples from Greece and Uganda cluster together in the phylogenetic tree (Figure 4.5).

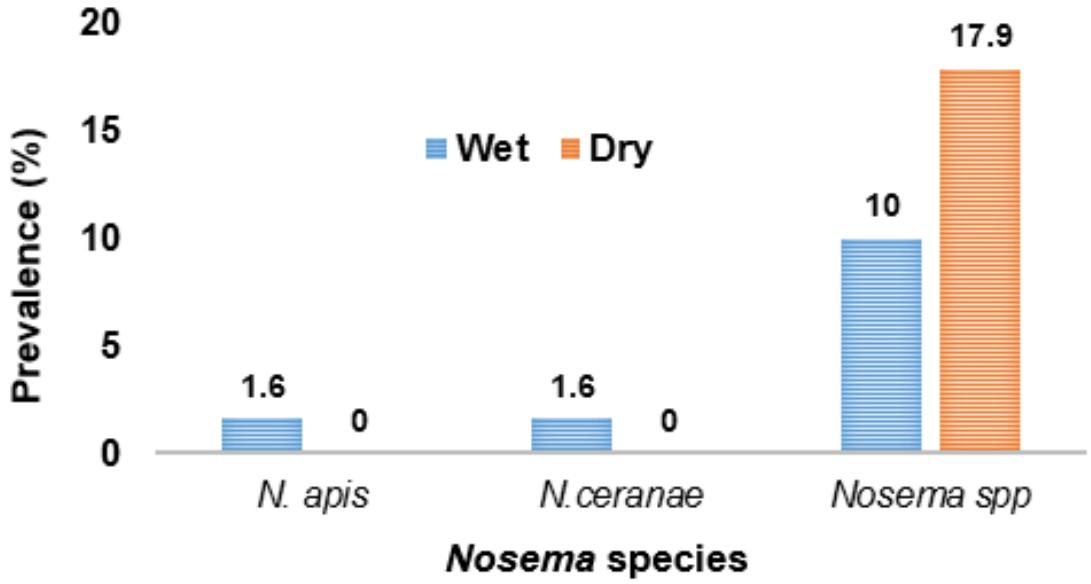


Figure 4.4: *Nosema* infestation rates in the study sites.

4.4.6 Potential factors influencing *Nosema* infestation levels

The results of the analyses show that the agro-ecological zone, season, apiary location in relation to human activities and nature of apiary slope influenced *Nosema* spore infestation levels (Table 4.2). The data indicates that *Nosema* spore count varied significantly across the three categories of apiary locations (χ^2(df = 2) = 18.662, $P < 0.01$). There were significantly lower *Nosema* spore counts in colonies located in protected areas compared to those in farmlands (U = 2930.5, Z = -4.276, $P < 0.01$) and *Eucalyptus* plantations (U = 59.5, Z = -3.027, $P < 0.01$). However, there were no significant differences in *Nosema* spore counts between colonies in *Eucalyptus* plantations and those in farmlands (U = 1422.0, Z = -0.222, $P = 0.825$).

The potential influence of elevation on *Nosema* indicates that spore count was correlated with altitude in some cases but not all. Specifically, there was a significant negative correlation between *Nosema* spore count with elevation in the eastern AEZ during the dry season (rho = -0.253, n = 91, *P* = 0.015). However, during the wet season, there was no significant correlation between *Nosema* spore count with altitude (rho = 0.118, n = 79, *P* = 0.302). In the western highland AEZ, the reverse trend was noticed with no correlation between *Nosema* spore count with elevation during the dry season (rho = -0.038, n = 86, *P* = 0.728) and a significant but positive correlation in the wet season (rho = 0.449, n = 115, *P* < 0.01).

Height of honeybee nest was negatively correlated to *Nosema* spore counts. Specifically there was a significant negative correlation between *Nosema* spore count with height of honeybee nest (beehive) from ground in both the eastern (rho = -0.333, n = 92, *P* = 0.001) and western highlands AEZ (rho = -0.364, n = 86, *P* = 0.001) during the dry season. In the wet season, there was no correlation between spore count with height of beehives from ground (eastern: rho = 0.194, n = 79, *P* = 0.087; western highlands: rho = 0.142, n = 115, *P* = 0.129).

The binary logistic regression model developed to predict microsporidian spore presence gave a Nagelkerke R^2 of 0.616 implying that the variables included in the model were able to explain 61.6% variance in the model. Moreover, since the Chi Square 136.541 and df 7 was significant (*P* < 0.001), the findings indicates that the explanatory variables (elevation, season, apiary location, farming intensity, apiary slope and apiary management) included in the model jointly influence the likelihood of having *Nosema* spores in colonies. Of great interest are elevation, apiary slope and farming intensity which significantly predicted spore presence (Table 4.3). Increase in elevation resulted in decreased chances of finding *Nosema* spores by 0.995 times while an increase in the farming intensity led to increased chances of finding the spores. Generally, it is concluded that this binary logistic regression model has some integrity and hence appropriate for predicting *Nosema* spore presence in the two AEZs of Uganda.

Table 4.2: Effects of environmental and human factors on Nosema spore infestation levels in honeybee colonies.

Factor		Spores/bee
Season	Wet	2074.7±10391.1 (177)*
	Dry	5097.6±18644.6 (111)*
Apiary location	Eucalyptus	634.7±1331.1 (8)*
	Farmland	3511.0±14884.6 (261)*
	Protected area	460.8±1473.7 (18)*
Nature of apiary slope	Flat	1086.3±1947.4 (100)*
	Gentle	2654.3±11496.3 (134)*
	Steep	8926.4±27194.6 (52)*
Agro-ecological zone	Eastern	1458.7±5297.5 (153)*
	Western highlands	5306.1±19950.8 (133)*

Numbers indicate means and standard deviations, figures in brackets indicate the sample size (n), the * outside the bracket indicates there are significant differences in the mean spore infestation level

The linear regression model developed explains 4.6 % of the variance in *Nosema* spore infestation levels. In the model, the factors included were: altitude, apiary location, nature of apiary slope, agro-ecological zone, season, distances to potential water sources and farming intensity. All together, these factors significantly explained *Nosema* spore infestation levels ($F(9,275) = 2.514$, $p < 0.01$). However, following the Bonferonni correction of the alpha, no individual factor independently could significantly predict spore infestation levels (Table 4.4).

Table 4.3: Logistic regression model predicting presence of *Nosema* spores in the study sites.

Factor	B	S.E.	Exp(B)	*P*
Elevation	*-0.005*	*0.001*	*0.995*	*0.000*
Apiary location	-0.731	0.966	0.481	0.449
Nature of apiary slope	*2.049*	*0.534*	*7.757*	*0.000*
Agro-ecological zone	0.060	0.617	1.062	0.923
Season	21.819	3199.837	2.992	0.995
Distance to potential water source	*-0.001*	*0.001*	*0.999*	*0.012*
Farming intensity	*0.856*	*0.386*	*2.353*	*0.027*
Constant	-16.323	3199.838	0.000	0.996

X2 = 136.541, df = 7, *P* < 0.001; Nagelkerke R2 = 0.616 (Italicized variable is statistically significant)

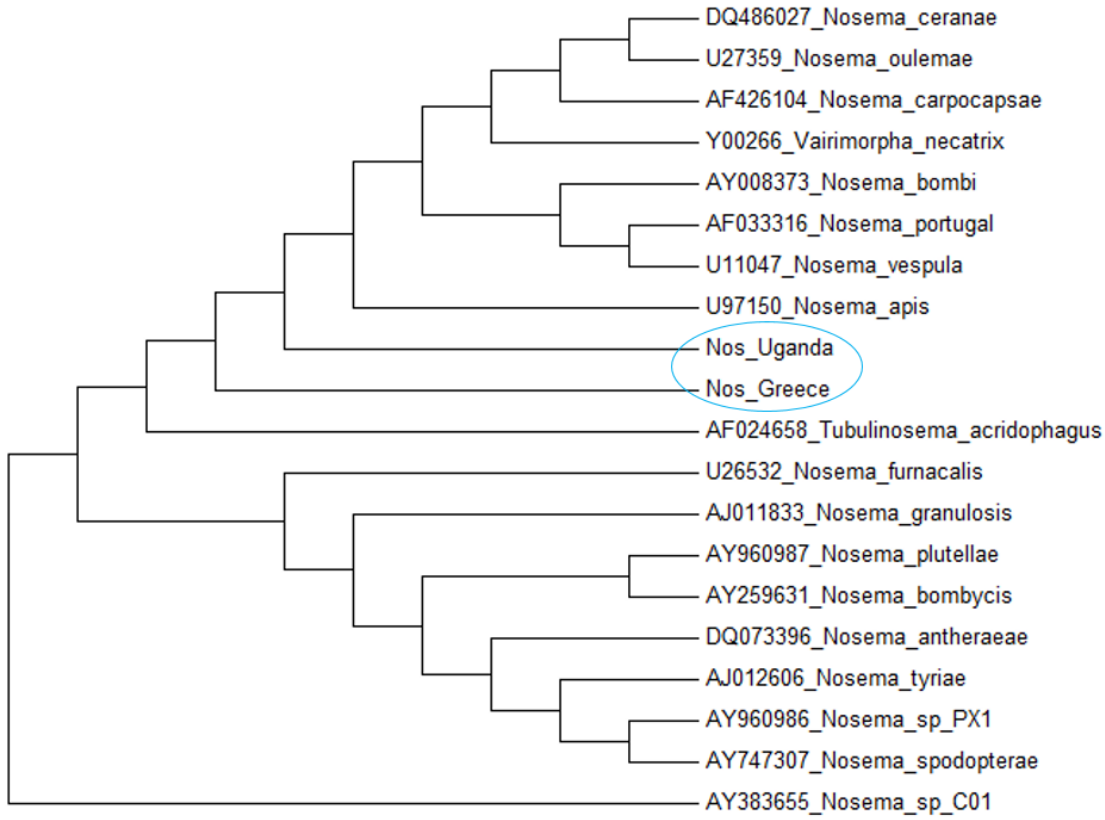


Figure 4.5: Maximum likelihood analysis of new *Nosema* spp. (sample from Uganda and Greece) with other related Microsporidia: 240 bp fragment of 16S rRNA. The GenBank accession number for each sequence is given adjacent to the corresponding species name. The new *Nosema* spp.: (indicated in the tree as Nos_Uganda and Nos_Greece) are encircled together in a blue oval.

Table 4.4: Linear regression model predicting *Nosema* spore infestation levels in the study sites.

Factor	**Beta**	**t**	***P***
(Constant)		2.035	0.043
Apiary location (Eucalyptus)	-0.029	-0.437	0.663
Apiary location (protected area)	-0.018	-0.296	0.767
Nature of apiary slope (flat)	-0.251	-2.444	0.015
Nature of apiary slope (gentle)	-0.146	-1.713	0.088
Elevation	-0.017	-0.199	0.842
Distance to potential water source	-0.01	-0.141	0.888
Season	-0.075	-1.263	0.208
Agro-ecological zone	-0.15	-2.192	0.029
Farming intensity (old farmland with trees)	0.17	2.433	0.016

4.4.7 Effects of *Nosema* on colony strength and productivity

The data show that the number of honeybee frames/top bars with honeybees and amount of honey which was harvested were negatively correlated with *Nosema* spore counts (Table 4.5), suggesting that *Nosema* is negatively affecting honeybee colony strength and productivity. The evidence of multiplication of this newly detected *Nosema* sp. on *A. mellifera* from Uganda is shown in Table 4.6. It can be observed that spore counts on the different body parts varied but the highest spore count was in the midgut.

Table 4.5: Spearman's correlation between *Nosema* spore count with honeybee colony strength and productivity indicators.

Factor	**Agro-ecological zone**	**Dry season**			**Wet season**		
		rho	**n**	***P***	**rho**	**n**	***P***
Combs with honey	Eastern	0.047	77	0.685	0.122	70	0.314
	Western highlands	-0.236	54	0.086	0.031	88	0.771
Combs with brood	Eastern	0.058	72	0.63	-0.032	45	0.834
	Western highlands	-0.082	46	0.588	0.145	41	0.365
Combs with pollen	Eastern	-0.128	58	0.338	-0.034	53	0.807
	Western highlands	-0.006	49	0.969	-0.139	49	0.341
Combs with bees	Eastern	0.055	89	0.61	-0.015	72	0.902
	Western highlands	*-0.411*	*86*	*<0.01***	0.067	107	0.493
Kg of honey harvested	*Both*	*-0.493*	*22*	*0.02***			

** and italics indicates a significant correlation

Table 4.6: *Nosema* spore count in honeybee body parts after 4 days post infection.

S/N	**Honeybee body part**	**Spores/bee**
1	Head	0
2	Thorax	5,000
3	Midgut	8,333
4	Devescerated abdomen	1,667
5	Rectum	3,333

(Spores fed per bee = 2,500 spores).

4.5 Discussion

This study is the first documentation of *Nosema* spp. in Ugandan honeybee colonies. It shows the discovery of a new Microsporidian in addition to confirming the presence of *N. apis* and *N. ceranae*. There are clear differences in the ultra-structural and molecular characteristics of the newly detected *Nosema* species compared to *N. apis* and *N. ceranae*, the other described honeybee Microsporidian parasites. Spores of the newly detected *Nosema* species are smaller (approximately 2.5 and 1.86 times shorter than *N. apis* and *N. ceranae* respectively) under the TEM. Also, the polar filament coils are fewer (10 – 12) in the new species compared to 20 - 23 for *N. ceranae* and more than 30 often seen in *N. apis* (Fries *et al*., 1996). Based on molecular and TEM data, this study confirms that the Microsporidia isolated from Ugandan honeybees are indeed a new *Nosema* species. Previous studies on honeybee microsporidia in Africa could have overlooked this new *Nosema* species because of the relatively small size of its spores compared to *N. apis* and *N. ceranae*. It seems also reasonable to believe that investigations only based on microscopic examinations might have misdiagnosed infections of the new species as *N. apis* or *N. ceranae* and thus should be revisited to ascertain the actual species involved. We could show that this newly detected *Nosema* species is present in Greece but absent in Belgian honeybee samples.

Nosema infestation rates of the new microsporidian were quite high compared to *N. apis* and *N. ceranae* although the spore counts were always relatively low in Ugandan honeybee colonies. This suggests that the new microsporidian is endemic in Ugandan honeybee colonies and that the local honeybees might be able to maintain the infestation levels low. The exact mechanisms how honeybees cope with this and other pathogens should be further investigated. Spore counts in honeybee colonies in apiaries located in *Eucalyptus* plantations and farmlands were significantly higher than those in protected areas. The high *Nosema* spore count in honeybees in farmlands and *Eucalyptus* plantations may be associated with nutritional deficiencies of honeybee forage around such apiaries and their surroundings. This is in agreement with findings in a study by Invernizzi *et al*. (2011) where infestation levels of *N. ceranae* were high when colonies were kept under *Eucalyptus grandis* plantations and were attributed to nutritional

deficits during periods of *Eucalyptus* flowering. The nutritional deficiencies provide adequate conditions for the multiplication of *Nosema* spores. Results presented here suggest that protected areas which are normally less degraded compared to farmlands and plantation forests offer a variety of forage options that meet the nutritional needs of the honeybees in a manner that does not favour *Nosema* parasite multiplication.

Although Mendoza *et al*. (2012) showed that protein supplementation in honeybee colonies causes increased *Nosema* infections without significant effects on bee health, no protein supplements were provided by beekeepers in the studied apiaries. Furthermore, laboratory and field experiments have shown that *Nosema* infections increase significantly in bees from pesticide-treated hives compared to bees from pesticide-untreated hives (Pettis *et al*., 2012). Moreover, in farmlands and *Eucalyptus* plantations, farmers are more likely to use pesticides to protect their crops from pests unlike in protected areas.

N. apis has been linked to BQCV outbreaks (Higes *et al*., 2007) because it infects the midgut of adult honeybees (Fries, 1988) and increases the susceptibility of the alimentary canal to BQCV infections (Chen and Siede, 2007). The detection of microsporidian parasites and BQCV which was reported much earlier (Kajobe *et al*., 2011) highlight the potential honeybee health threats in the country that require monitoring and effective management. In the meantime, since microsporidian parasitized bees fed with the polyfloral pollen live longer than bees fed with monofloral pollens (Di Pasquale *et al*., 2013), beekeepers and government should maintain semi-natural conditions in beekeeping zones to avoid reduction in diverse nutritional resources for the bees.

Nosema spore count was negatively correlated with honeybee colony productivity, suggesting that increase in the number of spores resulted in a decline in honey yield. This finding is in agreement with other reports (Antúnez *et al*., 2009; Mayack & Naug, 2009; Botías *et al*., 2013) on the impacts of *Nosema* spp. on honeybee colonies. Infections of honeybees by *Nosema* parasite shortens the lifespan of worker bees (Mayack & Naug, 2009) and lowers the strength

and productivity of colonies (Botías *et al*., 2013). In addition, *N. ceranae* infection significantly suppresses honeybee immune responses (Antúnez *et al*., 2009). This finding indicates that although the African honeybee has been famed for its high levels of tolerance to most pathogens, the pressures like natural habitat loss can make them susceptible.

The detection of the new microsporidian in Ugandan honeybees and in samples of bees from Greece emphasizes the need for control in movement of honeybee colonies to avoid the dispersion of this microsporidian through the exchange of beekeeping material between beekeepers. Finally, confirmation of the specific tissues parasitized and epidemiological studies on this new Microsporidian of honeybees are necessary to increase our knowledge of the factors involved in the transmission and survival of this microsporidian.

CHAPTER 5

Varroa mite infestation in Ugandan honeybee colonies

Personal contribution:
- Designing the study
- Field data collection
- Screening for *Varroa*
- *Varroa* haplotype identification
- Data analyses
- Writing the manuscript

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Factors influencing the prevalence and infestation levels of *Varroa destructor* in honeybee colonies in two highland agro-ecological zones of Uganda.
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5.1 Abstract

Varroa mites are ecto-parasites of honeybees and are a threat to beekeeping. In this study, the haplotype of *Varroa* mites was identified and potential factors that influence their infestation rates in the eastern and western highlands AEZs of Uganda evaluated. This was done by collecting samples of adult worker bees between December 2014 and September 2015 in two sampling moments. Samples of bees were screened for *Varroa* using the ethanol wash method and the mites were identified by molecular techniques. All DNA sequences obtained from sampled mite populations in the two AEZs were 100% identical to the Korean haplotype (AF106899). Mean mite infestation rates in the apiaries was 40% and 53% for the western and eastern zones respectively during the first sampling. Over the second sampling, mean mite infestation rates increased considerably in the western (59%) but not in the eastern (51%) zone. *Varroa* mite infestation levels in the eastern zone was significantly higher than that in the western highlands AEZ during the first sampling ($P = 0.02$). Factors that were associated with *Varroa* mite infestation levels include altitude, nature of apiary slope and apiary management practices during the first sampling. The results further showed that *Varroa* mites were spreading from lower to higher elevations. Feral colonies were also infested with *Varroa* mites at infestation levels not significantly different from those in managed colonies. Colony productivity and strength were not correlated to mite infestation levels. We recommend a long-term *Varroa* mite monitoring strategy in areas of varying landscape and land use factors for a clear understanding of possible changes in mite infestation levels among African honeybees for informed decision making.

Key words: *Apis mellifera*/ ecto-parasite infestation levels/ mite prevalence/ *Varroa destructor*

5.2 Introduction

Honeybee health is an important theme in apiculture because honeybees (*Apis mellifera*) play an important role in the pollination of both agricultural crops and wild plants (Ghazoul 2005; Melin *et al*., 2014). By doing so, honeybees contribute to food security and biodiversity conservation. Honeybees provide a direct source of income to beekeepers through sale of hive products and beekeeping is potentially a poverty alleviation tool in many developing countries (Jacobs *et al*.,

2006). Due to their importance, honeybee colony losses in Europe and North America drew attention (Martin, 2001; Moritz *et al*., 2010; Paxton, 2010; Le Conte *et al*., 2010; Cornman *et al*., 2012; van Dooremalen *et al*., 2013) leading to the discovery among others of the influence of parasites like *Varroa destructor* (Anderson & Trueman, 2000) in colony collapses (Le Conte *et al*., 2010; Dainat *et al*., 2012).

V. destructor is an ecto-parasite of honeybees that is a big threat to the beekeeping industry. *V. destructor* originated from Asia where its natural host is *Apis cerana* (Rosenkranz *et al*., 2010) and has since crossed host to *A. mellifera* and spread all over the world (Fazier *et al*., 2010; Dietemann *et al*., 2013; Strauss *et al*., 2013; Muli *et al*., 2014). In Europe and North America, *V. destructor* is a contributing factor for colony collapses (Shen *et al*., 2005; Dainat *et al*., 2012). This is attributed to the mites weakening the honeybee colonies by sucking the hemolymph (Martin, 2001; Martin & Medina, 2004; Rosenkranz *et al*., 2010) and spreading viruses (Bowen-Walker *et al*., 1999; Shen *et al*., 2005).

In 2009, samples of honeybees collected from Kenya and Tanzania were positive for *V. destructor* (Fazier *et al*., 2010) but not from Uganda. In 2011, *Varroa* mites were confirmed in Uganda and have been reported countrywide (Kasangaki *et al*., 2015) but it is unknown exactly when *Varroa* mites arrived in Uganda. In order to plan for better management of *Varroa* mites, it is essential to identify the mite haplotypes present as they may differ in virulence and gather information on their epizootiology. Currently, little information is available on *V. destructor* infestation rates in Uganda.

Infestation of honeybee colonies by *Varroa* mites and their impacts vary with honeybee heritable traits and behavioural adaptations (Rosenkranz, 1999; Buchler *et al*., 2010; Calderón *et al*., 2010; Rinderer *et al*., 2010; Emsen *et al*., 2012). Also, *Varroa* mite infestation levels are related to landscape factors such as elevation (Muli *et al*., 2014; Mumbi *et al*., 2014). Given the varying altitude, land uses and the common practice in Uganda of relying on natural swarms to populate hives, it is important to investigate the association between mite infestation levels and landscape

factors and management. Therefore, this study was conducted in two AEZs of Uganda that are spatially separated by about 546 km but are comparable in rainfall amounts, altitude and land uses. The aim was to determine if *V. destructor* infestation levels differed between the two AEZs and to identify factors that may be associated with the levels of infestation.

5.3 Materials and methods

5.3.1 Study area

This study was conducted in two AEZs namely; the eastern and western highlands of Uganda described in Chapter 2.

5.3.2 Data collection

A total of 170 honeybee colonies from 32 apiaries and 7 feral honeybee colonies in the two AEZs were sampled during the dry season. During the second sampling (wet season), 195 honeybee colonies were sampled. The details on data collection can be found in Chapter 2.

5.3.3 Estimating colony strength and productivity

For each selected honeybee colony, colony strength was estimated by counting top-bars or frames covered by honeybees and combs with brood. Counts of frames/top-bars were to the level of quarter full. Colony productivity was estimated by counting top-bars or frames with honey and pollen. In some honeybee colonies, comb honey was harvested and weighed. Details of the methods can be found in Chapter 2.

5.3.4 Evaluating mite infestation levels

Mite infestation levels on adult honeybees were determined using the ethanol-wash method described in Chapter 2.

5.3.4 *Varroa* haplotype identification

Varroa mite haplotyping was performed using RNA samples that were available for other purposes (i.e. virus analyses; data shown in Chapter 2), which was technically feasible because

mtDNA of the *Varroa* mite is entirely devoid of introns (Evans & Lopez, 2002). RNA was extracted from whole mite using the QIAamp® viral RNA kit following the manufacturer's protocols and used to synthesize cDNA using random primers in the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit as described in Chapter 2.

A 458 DNA base-pair fragment of *Varroa* mtDNA COXI corresponding to sites Co1F.F and Co1N.R (Evans & Lopez, 2002) was amplified for mite haplotype identification (Anderson & Trueman, 2000) using COXI/COXIR primers (COXI: 5'-GG(A/G) GG(A/T) GA(C/T) CC(A/T) AAT (C/T) T(A/T) TAT CAAC -3' and COXI-R: 5'-CCT GT(A/T) A(A/T)A ATA GCA AAT AC -3') (Navajas *et al*., 2010). Reactions were carried out in 25 µl of PCR solution containing 0.5 µM of each primer, 2.5 µl of 10X buffer, 0.25mM of dNTP, 2.5 mM $MgCl_2$, 0.2 U HotStarTaq Plus DNA Polymerase, and 5 µl of cDNA. The following method for amplifying *Varroa* spp. DNA described by Dietemann *et al*. (2013) was used: 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 51 °C and 1 min at 72 °C and final extension 10 min at 72 °C. Amplicons of 9 randomly selected samples from the two agro-ecological zones were sequenced. The sequences obtained were edited and alignments performed using Clustal-W. Thereafter, sequences were compared with those in the GenBank using BLAST.

5.3.5 Data analyses

The data were analyzed using SPSS statistical program. To compare the infestation levels of *Varroa* in honeybee colonies in different nest types and in the three different apiary slope categories, Kruskal-Wallis tests were performed. The same test was used to compare mite infestation levels in apiaries with different human activities. When significant results were found, *post hoc* analysis with Mann-Whitney tests were used to compare pairs of categories. Mann-Whitney test was also used to compare the infestation levels of mites in the two AEZs, two seasons (dry and wet) and in inspected and uninspected apiaries. Spearman's rank correlations were conducted to establish the relationships between the infestation levels of mites and the following: altitude, height of bee nest/beehive from ground, distance of apiary to nearest water source, colony strength and productivity. All tests were two tailed. Regression models were built

modeling the presence of *Varroa* as a function of landscape and human factors as described in chapter 2, sub-section 2.3.10.

5.4 Results

5.4.1 Seasonal infestation rates of *Varroa* mites

Varroa mites (Figure 5.1) were present in both the eastern and western highlands AEZs of Uganda during the dry and wet seasons. Mean infestation rates in apiaries during the dry season were 40% in the western and 53% in the eastern AEZs respectively. During the wet season, the mean mite infestation rates in apiaries increased considerably in the western highlands AEZ (59%) but not in the eastern AEZ (51%). These infestation rates did not differ significantly between seasons (U = 15694.5, Z = -1.743, n = 372, *P* = 0.08). However, *Varroa* mite infestation rates varied significantly along the altitudinal gradient during the first sampling (dry season) in both the eastern AEZ (U = 754.5, Z = -2.211, n = 91, *P* = 0.03) and western highlands AEZ (U = 650.5, Z = -2.067, n = 86, *P* = 0.04). During the second sampling (wet season), there were no significant differences in the infestation rates of mites along the altitudinal gradients in both zones. *Varroa* mite infestation levels were low during both the dry (mean infestation levels: eastern 3.79 ± 0.54 mites/300 bees, western highlands 2.25 ± 0.49 mites/300 bees) and wet seasons (mean infestation levels: eastern 2.50 ± 0.63 mites/300 bees, western highlands 2.02 ± 0.29 mites/300 bees). However, there were significant differences in mean mite infestation levels in colonies in the two zones during the dry season. Specifically, colonies in the eastern AEZ had a higher infestation level of mites compared to those in the western highlands AEZ during the first sampling (dry season) (U = 3195, Z = -2.292, n = 177, *P* = 0.02). During the wet season, mite infestation levels did not differ significantly in the two AEZs (U = 4522, Z = -0.162, n = 195, *P* = 0.87).

5.4.2 *Varroa* haplotype

For all the 9 analyzed mite samples from the two AEZs of Uganda, the COI sequence between primer sites Co1F.F and Co1N.R (Evans & Lopez 2002) were 100% identical to the South Korean haplotype of *Varroa destructor* (GenBank entry AF106899).

Figure 5.1: Adult *Varroa* mites on a bee (A) and magnified (B).

5.4.3 Potential influence of landscape and apiary management factors on *Varroa* mite infestation levels

Analyses of the potential effects of environmental and human factors on *Varroa* mite infestation in honeybee colonies shows that the nature of apiary slope influenced mite infestation levels (Table 5.1). The mean ranks of mite infestation levels were significantly different among apiaries in the different apiary slopes (χ^2(df = 2) = 18.074, P < 0.01) with the steep slopes having significantly lower mean ranks compared to the flat and gentle slopes during the first sampling (dry season) (flat vs. steep; U = 2882.5, Z = -4.168, P < 0.01: gentle vs. steep; U = 3267.5, Z = -3.739, P < 0.01; flat vs. gentle; U = 11718.5, Z = -0.772, P = 0.44). In the second sampling (wet season), no significant differences were observed in mite infestation levels across the different apiary slopes.

Varroa mite infestation level was negatively correlated with altitude during the first sampling (dry season) in both AEZs (eastern: rho = -0.213, n = 91, P = 0.04 and western: rho = -0.284, n = 86, P < 0.01). However, by the second sampling (wet season), there was no significant correlation between mite infestation levels and altitude in both AEZs (eastern: rho = 0.117, n = 79, P = 0.31 and western: rho = -0.041, n = 116, P = 0.66).

Table 5.1: Effects of environmental and human factors on Varroa mite infestation in honeybee colonies.

Factor		***Varroa* mites/300 bees**
Season	Wet	3.0±4.9(177)
	Dry	2.1±4.8 (110)
Apiary location	Eucalyptus	4.2±4.6 (8)
	Farmland	2.6±4.9 (261)
	Protected area	2.9±4.8 (18)
Nature of apiary slope	Flat	3.2±5.1 (100)*[a]
	Gentle	2.9±5.3 (134)*[a]
	Steep	0.9±2.1 (52)*
Agro-ecological zone	Eastern	3.3±5.6 (153)
	Western highlands	2.0±3.9 (133)

Numbers indicate means and standard deviations, figures in brackets indicate the sample size (n), the * outside the bracket indicates there are significant differences in the mean *Varroa* mite infestation level within the factor under consideration while the ∗a indicates no significant differences

Honeybee colonies were sampled from different habitat categories: protected areas, newly opened farmlands, old farmlands with small plantation forests and old farmlands without tree plantations. The infestation levels of *Varroa* mites did not vary with the location of the apiary (χ^2(df = 3) = 7.488, P = 0.06). Furthermore, the infestation levels of mites in honeybee colonies was not correlated to distance to nearest water source and height of beehive from ground (Spearman's rho = -0.050, n = 177, P = 0.51 and Spearman's rho = -0.023, n = 177, P = 0.74 respectively).

Beekeepers in the two AEZs of Uganda used five different beehive types (Traditional, Johnson's, Kenyan top-bar, Tanzanian top-bar and Langstroth beehive types). Seven feral honeybee colonies were also identified by beekeepers and these were sampled. A comparison of *Varroa* mite infestation levels in the different nest types shows that honeybee nest type did not have any influence (χ^2(df = 5) = 9.733, P = 0.08). Honeybee colonies in hives that were inspected had significantly higher mean ranks of mite infestation levels compared to those that were not inspected during the first sampling (U = 2881, Z = -2.871, n = 177, P < 0.01). However, during the second sampling, there were no significant differences in mean ranks of mite infestation levels between inspected and uninspected colonies (U = 24015.5, Z = -1.936, n = 195, P = 0.05).

A binary logistic regression model developed to predict mite presence gave a Nagelkerke R^2 of 0.079 implying that the variables included in the model were able to explain 7.9% variance in the model. This was considered low; moreover the Chi Square 9.439, df 8 was not significant (P = 0.31) indicating that all explanatory variables included in the model jointly did not influence the likelihood of having *Varroa* mites in colonies. However, the nature of apiary slope significantly predicted mite presence (Table 5.2). Change of apiary slope from flat to gentle and steep decreased chances of finding *Varroa* mites by 0.497 times. Generally, it is concluded that this binary logistic regression model does not have adequate integrity and hence not appropriate for predicting *Varroa* mite presence in the two AEZs of Uganda.

On the other hand, the linear regression model developed explains 5.3 % of the variance in mite infestation levels. The following factors were included in the model: altitude, season, farming intensity and nature of apiary slope. All together these factors significantly explained mite infestation levels ($F(7,364) = 3.957$, $p < 0.01$). However, only apiary slope significantly predicted mite infestation levels (Table 5.3).

5.4.4 Relationship between colony strength, colony productivity and *Varroa* mite infestation levels.

The productivity and strength of honeybee colonies was not correlated to *Varroa* mite infestation levels (Table 5.4). Specifically, number of frames/bars with bees, honey, brood, pollen combs and honey yield in kilograms from hives harvested were not correlated with *Varroa* mite infestation levels.

Table 5.2: Logistic regression model predicting presence of *Varroa* mites in the study sites.

Variable	**B**	**S.E.**	**Exp (B)**	***P***
Agro-ecological zone	-0.132	0.234	0.876	0.57
Elevation	0.000	0.000	1.000	0.85
Farming intensity	0.254	0.126	1.289	0.04
Season	0.379	0.218	1.461	0.08
Apiary slope	*-0.699*	*0.203*	*0.497*	*<0.01****
Apiary management	-0.116	0.217	0.890	0.59
Constant	0.080	0.854	1.084	0.93

X2 = 9.439, df = 8, *P* = 0.31 (Hosmer and Lemeshow Test); Nagelkerke R2 = 0.079 (Italicized variable is statistically significant)

Table 5.3: Linear regression model predicting infestation levels of *Varroa* mites in the study sites.

Factor	**Beta**	**t**	***P***
(Constant)		2.327	0.02
Elevation (altitude)	-0.057	-0.820	0.41
Season (dry)	-0.052	-1.027	0.31
Farming intensity (protected area)	0.034	0.635	0.53
Farming intensity (new farmland)	0.136	2.533	0.01
Farming intensity (old farmland with trees	-0.050	-0.917	0.36
Apiary slope (flat)	*-0.281*	*-3.105*	*<0.01****
Apiary slope (gentle)	*-0.256*	*-3.443*	*<0.01****

Italicized variable is statistically significant

Table 5.4: Correlation values of *Varroa* mite infestation levels with honeybee colony productivity and strength indicators.

Colony productivity/strength indicator	**Spearman's rho**	**n**	***P***
Combs with honeybees	0.062	230	0.35
Combs with honey	0.014	194	0.85
Combs with brood	0.021	141	0.80
Combs with pollen	0.024	149	0.77
Honey yield (kg)	0.366	27	0.06

5.5 Discussion

The randomly selected *Varroa* mite samples were similar to the Korean haplotype. This haplotype has also been reported in other African countries such as Kenya (Fazier *et al*., 2010), Nigeria (Akinwande *et al*., 2012), and Madagascar (Rasolofoarivao *et al*., 2013). This haplotype infests all European honeybee races worldwide and African and Africanized honeybee races (Solignac *et al*., 2005) and is considered a more virulent haplotype compared to the Japanese haplotype (Anderson & Trueman, 2000). Although no honeybee colony losses due to the *Varroa* mites have been reported in Sub Saharan Africa, their presence is a potential threat to the beekeeping sector and should be monitored.

Varroa mite infestation rates recorded in this study are comparatively lower than those reported in Kenya (Muli *et al*., 2014), Nigeria (Akinwande *et al*., 2012), South Africa (Strauss *et al*., 2014) and European countries (Nguyen *et al*., 2011; Meixner *et al*., 2014). This could be attributed to the mites being relatively new in Uganda. Moreover, the data presented here support the idea that mite populations have not yet entirely settled and that a process of spreading is still ongoing from the east to the west. The data shows that mean *Varroa* mite infestation levels in colonies in the eastern AEZ was significantly higher than that in the western highlands AEZ during the first sampling. Mite infestation rates increased considerably in the western highlands AEZ from 40 to 59% between the two sampling moments. This is also supported by historical data: in 2009, *Varroa* was found in Kenya and not in western Uganda (Fazier *et al*., 2010) whereas in 2011, they were found throughout Uganda. In 2009, samples were not collected from eastern Uganda but samples from western Kenya tested positive suggesting that the eastern AEZ of Uganda had *Varroa* mite infestation before the western highlands AEZ.

Mite infestation levels reduced with increase in altitude during the first sampling. The nature of apiary slope was also correlated with mite infestation levels. These findings conform with reports by Mumbi *et al*. (2014) but contradict Muli *et al*. (2014) who reported a positive correlation between *Varroa* mite infestation levels and elevation in Kenya. Although the significance of altitude on *Varroa* mite infestation levels in honeybee colonies is not clear, at lower elevation,

temperatures are relatively high compared to higher elevations. Such higher temperatures could favor mite reproduction and spread (Rosenkranz *et al*., 2010) because honeybees in such conditions are likely to produce brood throughout the year. Altitudinal studies on *Ixodes ricinus* ticks in Switzerland revealed that tick density decreased with increasing altitude and was attributed to high moisture which negatively affects tick survival (Gern *et al*., 2008). But, since the life cycle of *Varroa* mites is closely linked to their hosts and they lack free living stages (Moretto & Leonidas, 2003; Rosenkranz *et al*., 2010), the effects of environmental factors like temperature and humidity act indirectly via the host. Such effects may include influencing the amount of brood and the hygienic behavior (Rosenkranz *et al*., 2010). By the second sampling, the infestation levels of *Varroa* mites at different altitudinal levels became similar suggesting, mites first infested honeybee colonies at lower altitude areas and were spreading to higher altitudes. However, since the mites are still spreading, follow-up studies are needed to monitor any changes in mite infestation levels that may occur along altitudinal gradients.

There were no significant seasonal differences in mite infestation levels. These results are similar to findings in Nigeria (Akinwande *et al*., 2012) and in Mexico (Ruíz-Flores *et al*., 2012) and can be attributed to tropical climate in the two AEZs of Uganda. In the tropics where temperatures are high throughout the year, honeybee brood is available throughout and hence mite *Varroa* mite populations are likely to be relatively constant.

Honeybee colonies that were inspected had significantly higher *Varroa* mite infestation levels compared to uninspected colonies during the dry season. However, during the wet season, there were no significant differences in mite infestation levels between these colonies. The end of the dry season (major honey harvesting period) is usually characterized by honeybee colony propagation through swarming and abscondments are also high when bees are attacked by pests. In both cases, honeybee brood cycle is broken and *Varroa* load gets reset to low levels in the next season, irrespective of the difference observed earlier. This seems to be a successful mechanism by which honeybees in Africa get *Varroa* infestations under control. The same mechanism minimizes the potential impact of land use type on *Varroa* load as seen in this study. In addition,

***Varroa* mite infestation in Ugandan honeybee colonies**

African honeybees have higher levels of resistance to *Varroa* mites (Rosenkranz, 1999; Calderón *et al*., 2010; Pirk *et al*., 2015) associated with their hygienic behavior (Fazier *et al*., 2010), grooming and short brood post capping periods (Rosenkranz *et al*., 2010; Calderón *et al*., 2010) which also reduce mite infestation levels. Consequently, *Varroa* does not affect African honeybee colony productivity and strength as confirmed in this study and reports by Muli *et al*. (2014) and Strauss *et al*. (2015). Although, no honeybee colony losses have been reported in Uganda, there are honeybee pests and diseases like the BQCV (Kajobe *et al*., 2009; 2011) that may work alongside *Varroa* mites to negatively impact on honeybee health.

In this study, no impact of *Varroa* mites on honeybee colony strength and productivity was observed just like other studies in Africa (Dietemann *et al*., 2009; Muli *et al*., 2014; Strauss *et al*., 2015). Therefore, interventions in the management of *Varroa* mites in Africa should consider allowing the process of natural selection in honeybees. This is because lessons from Europe and America indicate that beekeeping has become more labor intensive and expensive due to activities related to *Varroa* mite management (Beetsma, 1994). The arrival of *Varroa* mites in these continents over 30 years ago and the approach taken to develop and use chemicals did not completely solve the problem. Mites have been developing resistance against chemicals and beekeeping costs have gone up due to *Varroa* mite control activities. Since African honeybees can survive without the need for treatment, it is important to allow natural selection to act against genotypes of honeybees that are susceptible to *Varroa* mites leaving resistant ones. An important honeybee pest to learn lessons from is the small-hive beetle which is commonly considered a minor pest of African honeybees (Spiewok *et al*., 2007). It is likely that African honeybees adapted to cope with small-hive beetles over time. Natural selection against honeybees not tolerant to the small-hive beetles worked over time leading to the emergence of predominantly tolerant honeybees currently available in Africa. Therefore, considerations on *Varroa* mite management that allow natural selection should be encouraged and a long-term *Varroa* mite monitoring strategy for a clear understanding of African honeybee interactions with this new ecto-parasite is recommended.

CHAPTER 6

General discussion, conclusions and recommendations

6.1 General discussion

6.1.1 Honeybee parasites

Several honeybee parasites and pathogens have been detected recently in some African countries. These include nine honeybee viruses (Pirk *et al*., 2015), *N. apis* and *N. ceranae* (Ellis & Munn, 2005*;* Pirk *et al*., 2015)*, V. destructor* (Fazier *et al*., 2010; Muli *et al*., 2014; Mumbi *et al*., 2014; Kasangaki *et al*., 2015; Chemurot *et al*., 2016a) and *P. larvae* (Ellis & Munn, 2005; Pirk *et al*., 2015; Chemurot *et al*., 2016b) among others. However, some of the parasites and pathogens have only been reported in a few countries partly due to limited studies. This study confirmed the presence of honeybee pathogens and parasites in Uganda that are possible threats to the beekeeping industry if not managed appropriately. Specifically, five honeybee viruses, *P. larvae*, three *Nosema* spp. and *V. destructor* were detected in the two AEZs of Uganda.

Importantly, *Varroa* mites, *Nosema* spp. and *P. larvae* were detected at low infestation levels in the two AEZs of Uganda. Also for *P. larvae*, there were no clinical signs in the positive colony. Different factors contribute to the observed low parasite/pathogen infestation levels in African honeybees. First, behavioral traits such as abscondment and swarming among African honeybee races are key factors that explain their low levels of parasite infestation (Hansen and Brodsgaard, 1997; Chemurot *et al*., 2016a). This work specifically suggests that abscondment plays an important role in reducing *Varroa* mite infestation levels in African honeybees. The two honeybee races, *A.m. scutellata*, and *A.m. adonsonii* which have been confirmed in Uganda (Kasangaki, 2016), abscond from beehives more frequently when disturbed than other races of *A. mellifera* (Hansen & Brodsgaard, 1997; Dietemann *et al*., 2009). During this study, abscondment occurred in 38 - 45% of colonies in the two AEZs of Uganda and accounted for the low *Varroa* mite infestation levels during the wet season. This behavioral trait could also result in disinfection in honeybee colonies formerly infected by parasites like *P. larvae* (Hansen and Brodsgaard 1997). Furthermore, the higher levels of hygienic and grooming behavior of African honeybees have been reported as key mechanisms for parasite resistance. Honeybee hygienic behavior may reduce the levels of AFB infection (Fries and Raina, 2003; Human *et al*., 2011) and *Varroa* mite infestation (Moretto, Guerra, & Bittencourt, 2006) resulting in higher resistance. In

addition, certain pests such as the wax moth, *Galleria mellonella* which is a very common pest in Africa and commonly affects weak colonies (Strauss *et al*., 2013) may reduce AFB pathogen infestation levels by destroying large amounts of infected combs after colony abscondment (Hansen and Brodsgaard, 1997; Human *et al*., 2011). The overall implication of this is that relatively very low AFB infection levels and extremely rare development of clinical symptoms are observed.

Despite the low infestation levels of some honeybee parasites and pathogens and the lack of clinical signs due to the higher levels of disease resistance in African honeybees (Human *et al*., 2011), the disease resistance trait may not persist long if pathogens accumulate in hives, especially with the increasing use of frame beehives. If honeybee breeding programs are not carefully done, the genes for resistance could be lost with far reaching consequences on honeybee health. Therefore, local beekeeping practices (e.g. the use of top-bar and traditional beehives) which are currently used and involve removal of the entire comb should not be abandoned since they help reduce pathogen accumulation in beehives.

In Europe and North America, *Varroa* mites (VanEngelsdorp *et al*., 2008; Neumann & Carreck, 2010), honeybee viruses (Cox-Foster *et al*., 2007; Meixner *et al*., 2014) and *N. ceranae* (Higes *et al*., 2008; Paxton, 2010) are associated with colony losses. *Varroa* mites vector honeybee viruses like DWV, KBV, BQCV and ABPV (Bowen-Walker *et al*., 1999; Chen *et al*., 2004; Shen *et al*., 2005; Le Conte *et al*., 2010). Moreover, synergistic interactions between honeybee parasites and pathogens e.g. *Varroa* and honeybee viruses undermines honeybee immunity and health (Di Prisco *et al*., 2016). The detection of these honeybee parasites and pathogens in Uganda clearly show the need for government to develop a honeybee health management plan to avert the likely food security risks associated with foreseen honeybee health threats.

6.1.2 Environmental factors

Currently, in Uganda beekeeping is practiced in a range of environments including protected areas (natural), agricultural and plantation forest (mostly *Eucalyptus*) landscapes (Chemurot *et*

al., 2016a). These beekeeping environments may have impacts on honeybee health. For instance, the number of honeybee virus infections (viral diversity) was correlated with elevation, height of hive placement and distances to potential water sources. Also, *Nosema* spore counts in colonies located in protected areas were significantly lower than those in farmlands and *Eucalyptus* plantations. From the regression models built, environmental factors (elevation, distances to potential water sources, nature of apiary slope and season) and human factors (agro-ecological zones, farming intensity and height of hive placement) to some extent explain honeybee parasite and pathogen presence and infestation levels. Specifically, the binary logistic regression models developed showed that 13.8%, 61.6% and 7.9% of the variance in viral, *Nosema* spore and *Varroa* mite presence respectively could be explained by the landscape and human factors explored. For honeybee viral diversity, *Nosema* spore and *Varroa* mite infestation levels; 8.8%, 4.6% and 5.3% respectively of the variance in infestation could be explained by the factors explored. The variation in the amount of variance explained by the factors investigated is likely due to differences in the parasite/pathogen life cycles and dependence on their hosts. For instance, *Varroa* mites lack free living stages and entirely depend on their hosts (Moretto & Leonidas, 2003; Rosenkranz *et al*., 2010), implying that the impacts of environmental factors act indirectly via the host. On the other hand, *Nosema* spp. is transmitted via fecal-oral routes (Smith, 2012) with possible direct effects of environmental factors on spore survival outside honeybee hosts.

These findings suggest that environmental factors such as climate and vegetation modulate honeybee parasite and pathogen transmission, infestation levels and maybe honeybee health in general. In the UK, the frequency among 76% of bumble bee forage plants declined between 1978 and 1998 (Carvell *et al*., 2006) raising concerns since floral changes are linked to pollinator dynamics (Carvell *et al*., 2006; Winfree *et al*., 2009).

Given the high deforestation rates in Uganda (Obua *et al*., 2010) that lead to ecological changes and their relative importance on honeybee health, these findings raise concerns on the health of honeybees and their likely impact on pollination services. First, these results suggest that some environmental factors that are influenced by anthropogenic factors may impact on honeybee

health. With the increasing landscape and climatic changes, honeybee health could be more affected. For instance climate influences flower development, nectar and pollen production, which are key components in honeybee nutrition (Le Conte and Navajas, 2008). Moreover, poor honeybee nutrition has been linked to increased parasite/pathogen infestation levels (Invernizzi *et al*., 2011). Second, these results show the potential vulnerability of the honeybees to their parasites and pathogens contrary to the popular believe that African honeybees are tolerant/resistant to most honeybee pathogens. For instance, this work shows that increase in the number of honeybee viruses leads to a reduction in colony performance. Also, intensification of monocultures in farms will impact on honeybee health through increased pathogen infestation levels as seen in *Nosema* spp. Importantly, factors including; parasites, pathogens, nutrient availability, agro-chemical exposure and climatic conditions can act individually or synergistically to affect honeybee health. For example, pesticide exposure increases honeybee pathogen abundance (Pettis *et al*., 2012; Doublet *et al*., 2015) and nutritional deficiencies provide adequate conditions for parasite multiplication (Invernizzi *et al*., 2011). Therefore, efforts are needed to ensure proper nutrition for both managed and wild colonies through protection of natural habitats and planting of melliferous crops.

The conceptual diagram (Figure 6.1) shows the range of variability of ‘‘Current honeybee environment’’ parameters for forage, land use change, natural enemies (alternative for parasites and pathogens) and pesticide use with only a small portion of the environment situation ‘‘space’’ currently exceeding the species-specific honeybee mortality threshold. ‘‘Future bee health’’ shows increases in extreme land use change, pesticide use and parasite/pathogen intensity events associated with foreseen anthropogenic/environmental changes, indicating increased risks of pesticide/pathogen/poor nutrition-induced die-off for current honeybee populations.

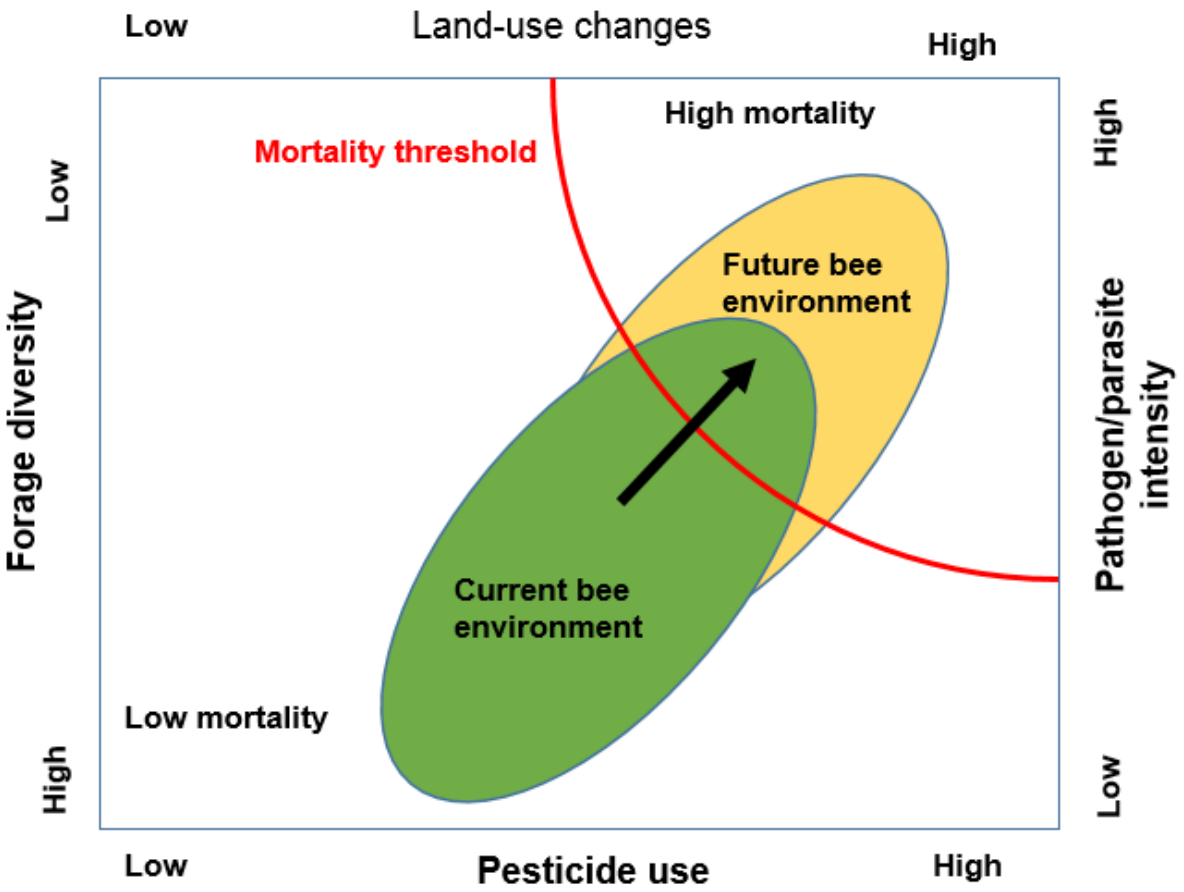

Figure 6.1: Honeybee vulnerability to changes in the environment in Africa.

6.1.3 Effects on colony performance

Honeybee colony strength is an important factor for honey production (Neupane *et al*., 2012) because it is correlated to the honey yields (Jevtić *et al*., 2009). From this study, the productivity of honeybee colonies in the eastern and western highlands AEZs of Uganda was generally low and comparable to the findings from the West Nile AEZ of Uganda (Chemurot, 2011). Different factors may explain such low productivity per beehive; notably, limited honeybee forage, inadequate colony management to prevent swarming, lack of knowledge and observance of apiary carrying capacity and pests which weaken colonies (Hussein, 2000).

Studies have shown that parasite infestation such as *Nosema* (Botías *et al*., 2013) and *Varroa* (Navajas *et al*., 2008; 2010; Rosenkranz *et al.*, 2010) can lower the strength and productivity of honeybee colonies. For instance, reduction in honey yields in Kenya between 2005 and 2007 was

reported and attributed to honeybee diseases (Muli *et al*., 2014). Importantly, the current study suggests that the performance of Ugandan honeybee colonies was negatively affected by viral and *Nosema* infestation. However, colony performance was not affected by *Varroa* mite infestation alone. Multiple pathogen infestation in some honeybee colonies was recorded. Honeybee parasites and pathogens together with pesticides and other honeybee health threats, have synergistic effects on honeybee health (Cornman *et al*., 2012; Doublet *et al*., 2015). The detection of these pathogens and parasites highlight the need for vigilance and development of strategies to manage them in the country.

6.2 Conclusions

This study confirmed the presence of five honeybee viruses (DWV, BQCV, ABPV, LSV and SBV), *P. larvae*, three *Nosema* spp. and *V. destructor* in Ugandan honeybee colonies. Apparently, increased honeybee viral diversity and *Nosema* are threats to the beekeeping sector while *Varroa* mites are not yet significantly affecting honeybee colonies in the two AEZs of Uganda. The composition of the parasite and pathogen complex of honeybees in the two AEZs of Uganda is somewhat similar but differs with landscape factors. For instance, the number of honeybee viruses declined with altitude and height of hive placement but increased with distances to potential water sources. Knowledge about the effects of honeybee parasites and pathogens on African honeybees has grown over the years (Strauss *et al*., 2014; Muli *et al*., 2014; Mumbi *et al*., 2014; Strauss *et al*., 2015). Unfortunately, our understanding of the effects of landscape factors on honeybee parasite/pathogen distribution and infestation levels remains scanty. In this study, the low number of honeybee viruses at higher altitudes and in apiaries near potential water sources suggests impacts of landscape factors such as vegetation and climate on parasite/pathogen transmission and infestation levels. Climatic factors and vegetation could affect conditions that enhance parasite/pathogen transmission and infestation levels. Such conditions can include multiplication of reservoir hosts/vectors for the parasites/pathogens. For the new *Nosema* spp. it is unknown whether there are other reservoir hosts. These findings widen our understanding of the honeybee parasite and pathogen distribution and potential factors involved in addition to expanding the geographical distribution of AFB, LSV and SBV in

Africa. Deeper studies on the new *Nosema* species and generally African honeybee health should be carried out in order to: 1) understand distribution, transmission, development, 2) identify associated effects on honeybees at individual and colony levels and 3) to reach a better understanding of the poorly known African honeybee pathogen complex especially in terms of the identities of pathogens present and survival mechanisms. Finally, since the honeybee is an important source of income and livelihood to several rural farmers in Uganda, based on this work, a national honeybee health strategy is required particularly as beekeeping evolves from traditional to modern beekeeping. The results should be kept in mind when making decisions concerning honeybee parasite/pathogen management.

6.3 Future perspectives

Although it is widely believed that African honeybees are relatively more resistant to honeybee parasites and pathogens than other races, they seem to become vulnerable with accumulating stresses. Since the start of this PhD work, our knowledge about honeybee microsporidian parasites has been greatly widened through documentation of a new species. However, this opens several unanswered questions such as; what is its epidemiology, distribution, mode of spread, development, host range and its impacts on honeybees at individual and colony levels. We have suggested that abscondment in African honeybees can be a successful survival mechanism that ensures low *Varroa* mite infestation. This mechanism has also been suggested to reduce AFB infection in colonies (Hansen & Brodsgaard, 1997). However, it remains to be investigated if this mechanism ensures survival from other parasites/pathogens as well. For example, could it be that as the honeybee colony absconds, generally unhealthy, diseased or heavily parasite-infested worker bees that would otherwise be the source of infection are either left in the old nest to die or they perish along the way to new nest sites? If this is the case, then only “healthy bees” reach new nest sites! In addition, this study suggests that landscape factors influence honeybee parasite/pathogen infestation levels. The exact ways through which such factors influence honeybee parasite/pathogen infestation levels should be studied in the future. The efforts made to explore honeybee parasites and pathogens summarized in this thesis relied on samples collected from only two out of 10 AEZs of Uganda. Notably also, the laboratory

analyses conducted were biased for specific parasites/pathogens and no viral titer quantification was done. Therefore, future studies should consider sampling AEZs of Uganda and using unbiased honeybee pathogen screening techniques in order to provide a complete understanding of the honeybee pathosphere. Quantifying the viral titer in Ugandan honeybees will be interesting especially since viruses were not detected in *Varroa* mites collected from virus-positive colonies. In general, new pathogens of honeybees will probably be discovered as more studies look at honeybee health in less investigated zones. This will present new challenges that require inquiry including their transmission, development, host range, impact on honeybee health and interaction with other species.

6.4 Recommendations

i. The Ministry of Agriculture, Animal Industry and Fisheries (MAAIF), Uganda should develop a national honeybee health management strategy that details practices and actions such as bee health monitoring, stricter controls on movement of bees, improved hygiene and parasite screening of colonies for breeding programs, provision of water in apiaries, honeybee forage planting around apiaries and regular capacity building for apicultural extension workers.

ii. Effort should be made to build the capacity of beekeeping extension workers on the honeybee parasites and pathogens in the country and how they can be managed so that they can provide beekeepers with up to date information and skills.

iii. The use of top-bar beehives and traditional beekeeping systems should be encouraged in order to keep in balance the honeybee parasites and pathogens such as *P. larvae* that can accumulate in beehives.

iv. Provision of water in apiaries during the dry season should be promoted in order to avoid long distances that increase chances of viral pathogen transmission.

v. Beekeepers should be encouraged to place their beehives at recommended heights (1m for top-bar, 1.5m for traditional (log) for ease of manipulation and to avoid possible pathogen infestation.

vi. Currently, beekeepers in the study sites should not use pesticides to control *Varroa* mites since the honeybees can survive without the need for treatment with no significant reduction in colony performance.

vii. Investigations on local remedies such as botanicals that can be used to control *Varroa* in case the situation changes should be considered.

viii. Studies should be conducted on African honeybee race pathosphere using unbiased techniques, impacts of land use change, nutrition and pesticide use on bee health and synergistic impacts of these threats in order to generate information to enable more effective prioritization of African honeybee conservation and management efforts.

ix. In this study, *Varroa* mite infestation, *Nosema* spores infection and *P. larvae* infection rates were relatively low. The mechanisms of honeybee parasite/pathogen resistance in local honeybee races should be investigated for informed decision making in the beekeeping industry.

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Curriculum vitae

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Education

2014-2017	Ph.D. candidate, Biology, Ghent University **PhD thesis**: The distribution, infestation levels and effects of honeybee parasites and pathogens on colony performance in two agro-ecological zones of Uganda. **Promoter:** Prof. Dr. Dirk de Graaf
2006-2008	Master in Science (MSc) Zoology, Makerere University **Master thesis**: Food consumption and rest time in chimpanzees (*Pan troglodytes*) of Kanyawara community, Kibale National Park, Uganda. **Supervisors:** Prof. Gilbert Isabirye-Basuta and Dr. Eric Sande
2001-2004	Bachelor in Science (BSc), Makerere University
1999-2000	Advanced level: Mbale Secondary School: Subjects: Biology, Chemistry, Geography and General Paper
1995-1998	Ordinary level: Sebei Secondary School

Work experience

Assistant lecturer, Makerere University, Department of Zoology, Entomology and Fisheries Sciences (August 2012 to date)

- Teaching undergraduate students
- Conducting practical exercises and field work

- ❖ Supervising undergraduate special projects

District entomologist, Adjumani District Local Government (2008 to August 2012)

- ❖ Led in designing and implementing plans to improve beekeeping and carried out environmental awareness
- ❖ Spearheaded tsetse control activities using environmentally friendly methods
- ❖ Actively involved in planning for entomology related activities in the district
- ❖ Coordinated Farm Income Enhancement and Forest Conservation (FIEFOC) Project

Research team member, British Petroleum (BP) Conservation Project, Rwanda (2004-2005)

- ❖ Collected data from protected and unprotected conservation sites
- ❖ Participated in environmental sensitization meetings
- ❖ Actively involved in production of research reports

Selected research projects

1. The Factors Influencing the Distribution of Honeybee Pathogens in Two Selected Agro-Ecological Zones in Uganda (Ghent University), PhD research funded by Erasmus mundus action II (Caribu project) November 2014-June 2017
2. Potential impact of municipal waste on the health of riverine aqua fauna of River Rwizi, Western Uganda (collaboration between Jane Yatuha, Mbarara University; Moses Chemurot (Makerere University) and Patrice Kasangaki (National Agricultural Research Organisation) funded by Tropical Biology Association in 2014
3. Food consumption and rest time in chimpanzees (*Pan troglodytes*) of Kanyawara community, Kibale National Park, Uganda (Makerere University) MSc. research, funded by Kibale Chimpanzee Project (July 2006-July 2007)

Scientific output

A1 peer reviewed publications

1. **Chemurot, M.,** Brunain, M., Akol, A.M., Descamps, T., de Graaf, D.C. 2016. First detection of *Paenibacillus larvae* the causative agent of American Foulbrood in a Ugandan honeybee colony. *Springerplus.* 5(1):1090. doi: 10.1186/s40064-016-2767-3.
2. **Chemurot, M.,** Akol, A.M., Masembe, C., De Smet, L., Descamps, T., de Graaf, D.C. 2016. Factors influencing the prevalence and infestation levels of *Varroa destructor* in honeybee colonies in two highland agro-ecological zones of Uganda. *Experimental and Applied Acarology.* 68(4):497-508. doi: 10.1007/s10493-016-0013-x.
3. Kasangaki, P., Otim, A. S., Abila, P. P. O., Angiro, C., **Chemurot, M.,** Kajobe, R. (2015). The presence of varroa in Uganda and knowledge about it by the beekeeping industry. *Journal of Apicultural Research*, *54*(4), 373–377. doi:org/doi.org/10.1080/00218839.2016.1159858

Other peer reviewed publications

1. Masette, M., Isabirye-Basuta, G., Baranga, D., & **Chemurot, M.** (2015). Levels of tannins in fruit diet of grey-cheeked mangabeys (*Lophocebus ugandae*, Groves) in Lake Victoria Basin forest reserves. *Journal of Ecology and The Natural Environment*, 7(5), 146-157.
2. **Chemurot, M.**, Kasangaki, P., Ojja, F., Sande, Eric. and Isabirye-Basuta, G. (2013) Beehive and honey losses caused by bush burning in Adjumani District, Uganda. *BeeWorld,* 90(2), 33-35.
3. **Chemurot, M.** (2012). Beekeeping in Adjumani District, Uganda. *BeeWorld,* 88 (3), 58-61.
4. **Chemurot, M**., Isabirye-Basuta, G. and Sande E. (2012). Amount of Plant Foods Eaten and Sexual Differences in Feeding among Wild Chimpanzees (*Pan troglodytes*) of Kanyawara Community. *International Scholarly Research Network, ISRN Zoology*, Volume 2012, Article ID 120250, 5 pages, doi:10.5402/2012/120250
5. Kahindo, C.M.N., Barakabuye, N., **Chemurot, M.,** Sande, E. and Nsabagasani, C. (2009). Status of the globally endangered Grauer's Rush Warbler (*Bradypterus graueri*) in Rugezi Marsh, Rwanda. In *Society for Conservation Biology (SCB), 1st Meeting of the Africa section.*

Book chapter

1. Kasangaki, P., **Chemurot, M.,** Sharma, D and R. K. Gupta (2014). Bee Hives in the World, In book: Beekeeping for Poverty Alleviation and Livelihood Security, Chapter: 4, Publisher: Springer, Editors: Gupta, R.K., Reybroeck, W, van Veen, J.W., Gupta, A.

Participation at conferences (selected)

1. **Chemurot, M**., De Smet, L., De Rycke, R. & de Graaf, D.C. 2016-Oral. New *Nosema* spp. detected in honeybee colonies from two highland agro-ecological zones of Uganda: is it an emerging threat to beekeeping? *Eurbee7* held in Cluj Napoca (Romania). 7th- 9th September 2016.
2. **Chemurot, M. 2015**– oral**.** Beekeeping as a Conservation tool: Opportunities and Challenges in Uganda. *First African Primatological Consortium Conservation Conference, Makerere University*. 15-16th December 2015.
3. **Chemurot, M**., De Smet, L., de Graaf, D.C. 2015– oral. Factors influencing the prevalence and incidence of two honeybee parasites in two agro-ecological zones of Uganda. *Belgian Society of Parasitology and Protistology.* Aula Janssens Institute of Tropical Medicine Antwerp. Thursday 26 November 2015.
4. **Chemurot, M**., Akol, A.M., De Smet, L., de Graaf, D.C. 2015– oral. New health threats to Uganda's honeybees. *44th APIMONDIA International Apicultural Congress* 2015, *Apimondia* 19th September 2015.p 214.
5. Kasangaki, P., Otim, A.S., Abila, P.P., Angiro, C., **Chemurot, M.** and Kajobe, R. (2015). The presence of *Varroa* in Uganda and knowledge on it by the beekeeping industry. *Second TAAG Students' Conference*, University of Ghana Accra, Ghana, 9th – 12th June 2015
6. **Chemurot, M**., Akol, A.M., Descamps, T., Masembe, C., de Graaf, D.C. 2015. Factors influencing the incidence of parasitic *Varroa* mites in honeybee colonies in two highland agro-ecological zones of Uganda–poster. *International Symposium on Crop Protection*, Ghent University. May 19th 2015. *Communications in Agricultural and Applied Biological Sciences Ghent University. 80(2)* p. 237.

7. **Chemurot, M.**, Kasangaki, P., Ojja, F., Sande, Eric. and Isabirye-Basuta, G. (2013) Beehive and honey losses caused by bush burning in Adjumani District, Uganda. *TAAG African Maiden Students' Conference*, National Museums of Kenya, 2nd- 4th July 2013

Participation at workshops and courses (selected)

1. Training on "Project management", Ghent University, January 2017.
2. Primate Research and Conservation Training, Kyoto University, Japan November-December 2016.
3. Training on "Effective image editing", Ghent University 26th October 2016.
4. Biogazelle qPCR course at Ghent University 20th - 21st October 2016.
5. Training on Academic posters, Ghent University 11th and 18th April 2016.
6. Workshop on Designing projects in the field – April 2012, Makerere University Biological Field Station, Fort Portal Uganda. (DRECA).
7. Beekeeping for Poverty Alleviation International Training Program, May-August 2012 Ghent University.
8. Communicating Research Results – June 2011 Kampala, Uganda (DRECA)
9. Appropriate Techniques in Tsetse and Trypanosomiasis Control –December 2009 Faculty of Veterinary Medicine, Makerere University.
10. Tools and Techniques in delivering Agricultural Advisory Services in Multi-stakeholder systems – September 2009 Faculty of Agriculture –Makerere University.