UNIVERSITEIT GENT

FACULTY OF SCIENCES

Exploring the involvement of honey bee pathogens in colony collapses from epidemiological data, pathogen genotyping and host immune responses.

Jorgen Ravoet

Promotor: Prof. Dr. Dirk de Graaf

Academic year 2014-2015

Thesis submitted in fulfilment of the requirements for the degree of Doctor in Sciences:

Biochemistry and Biotechnology

Department of Biochemistry and Microbiology

Supervisors

Promotor: **Prof. Dr. Dirk de Graaf**

Laboratory of Molecular Entomology and Bee Pathology (L-MEB)

Department of Biochemistry and Microbiology

Faculty of Science, Ghent University, Belgium

Examination committee

Prof. Dr. Savvas Savvides (Ghent University, Belgium, chairman) *

Prof. Dr. Dirk C. de Graaf (Ghent University, Belgium, secretary) *

Prof. Dr. Guy Smagghe (Ghent University, Belgium) *

Prof. Dr. Peter Geldhof (Ghent University, Belgium) *

Prof. Dr. Tom Wenseleers (KU Leuven, Belgium) *

Prof. Dr. Peter Neumann (University of Bern, Switzerland) *

Dr. Bach Kim Nguyen (University of Liege, Belgium) *

Dr. Lina De Smet (Ghent University, Belgium)

* Members of the reading committee

Dean: Prof. Dr. Herwig Dejonghe

Rector: Prof. Dr. Anne De Paepe

Please refer to this work as:

Ravoet, J. (2013) Exploring the involvement of honey bee pathogens in colony collapses from epidemiological data, pathogen genotyping and host immune responses. PhD thesis, Ghent University.

Funding:

This study was financially supported by Research Foundation-Flanders (FWO) (research Grant G.0628.11).

Author's email address:

jorgen.ravoet@gmail.be

Front cover

Drawings: Kathleen Hamaekers

DANKWOORD

Ongelooflijk wat er allemaal kan gebeuren op enkele jaren tijd. Vier jaar geleden kwam ik vol zenuwen op sollicitatiegesprek bij prof. Dirk de Graaf. Na enkele dagen kreeg ik het verlossende telefoontje dat ik was aangenomen. Nu kan ik met veel voldoening terug kijken op de periode sindsdien, met goede en helaas ook slechte tijden. Dit alles zou echter niet mogelijk zijn geweest zonder vele personen.

In de eerste plaats dank ik mijn promotor prof. Dirk de Graaf voor het vertrouwen, de steun en het gebruik van het onderzoekslabo. Ik zou zelf niet willen opdraaien voor alle offertes die ik op al die jaren met succes aan hem heb voorgesteld. Ook prof. Tom Wenseleers heeft als promotor van het FWO project '*Deformed wing virus: a contributory factor of Colony Collapse Disorder in honeybees?*', waarop ik vier jaar betaald ben, een belangrijke bijdrage heeft geleverd. Vanzelfsprekend is het FWO dan bedankt voor de financiële steun die dit doctoraat mogelijk heeft gemaakt.

Alle coauteurs van de verschillende hoofdstukken delen in mijn dank. Hun bijdragen waren dikwijls niet te onderschatten, en gaven de artikels mee vorm. Especially the efforts of Prof. Jay D. Evans and Dr. Ryan Schwarz to include us in their study of honey bee trypanosomatid taxonomy are much appreciated. This collaboration led to many fruitful discussions. Also Prof. Mariano Higes and Dr. Raquel Martin-Hernandez are thanked to allow a visit to their laboratory and to introduce me into experimental infections of *Nosema ceranae*.

Vanzelfsprekend wil ik ook de grote rol van mijn vriendin Els in de verf zetten. Ze toont steeds begrip voor de lange werkuren, de jaarlijkse congressen in het buitenland en de klachten in mindere periodes. Sinds kort kunnen we ons samen toeleggen op de zorgen van onze schattige tweeling, Lars en Nore. Dit resultaat zou er natuurlijk nooit geweest zijn zonder de steun van familie en vrienden. Ik wil dan ook graag mijn ouders, grootouders, zus, toekomstige schoonbroer en schoonouders bedanken.

Verder bedank ik de collega's van het Laboratorium voor Moleculaire Entomologie en Bijenpathologie voor het aangename gezelschap en de leuke werksfeer. Marleen, bedankt voor alle bestellingen, de mede-doctoraatsstudenten Ellen D, Ellen F, Matthias en Tine (bovendien mijn voormalige thesisstudente) voor de toffe gesprekken tussen de experimenten door, Lina voor de begeleiding in het labo, de vele besprekingen en het beantwoorden van een constante stroom aan vragen. Ook Dieter, waarmee ik het genoegen had om een bureau te delen, zal ik zeker nooit vergeten. Het personeel van het informatiecentrum van bijenteelt waren ook dikwijls een grote hulp. Jeroen, Mikalai, Dries, Wilfried en Patrick, van harte bedankt om vaak honingbijen ter beschikking te stellen.

Om af te sluiten wil ik nog graag alle leden van de jury bedanken voor hun opmerkingen die deze de eerste versie van doctoraatsthesis verder verfijnd hebben tot dit finale resultaat.

SUMMARY

Pollination is required for the cultivation in an agricultural context of many fruit, nut, vegetable and crops. European honey bees (*Apis mellifera*) are considered the most economically valuable pollinators for crop monocultures worldwide, followed by bumble bees (*Bombus* spp.). However, over the last decade unusually high honey bee colony losses have been reported. Several factors contribute to their decline, of which pathogens are the main culprits. We could confirm the presence of several parasites which were previously not known for Belgium. This included viruses (ALPV strain Brookings, AmFV, LSV and VdMLV), trypanosomatids (*Crithidia mellificae* and *Lotmaria passim*) and parasitic flies (*Apocephalus borealis*). Other pathogens were already reported from Belgian bumble bees, namely the neogregarine *Apicysis bombi* and the bacteria *Spiroplasma apis* and *Spiroplasma melliferum*, but remained ignored so far in honey bees.

In this work, the pathosphere of *Apis mellifera* was extensively exploited. After the initial examination, we selected a few parasites for further characterisation. Our knowledge about most of these novel or neglected parasites is limited. For instance only few nucleotide sequences are available for some of them, such as *A. bombi*, *A. borealis* and AmFV. The transmission routes are also not yet known. Nevertheless, vertical transmission was proved for the viruses ALPV strain Brookings, LSV and VdMLV since we detected them in honey bee eggs. This demonstrates a vertical transmission, in accordance with previous studies on other viruses. Numerous of these pathogens were also discovered in solitary bees, caught nearby an apiary. Although solitary bees can suffer from honey bee parasites, as demonstrated for DWV and *Nosema ceranae*, spillover appears to occur in both direction. Indeed we detected SBV solely in solitary bees and not in honey bees. Nevertheless, solitary bees can also be infected by their specific pathogens. For the moment only microsporidians and fungi are known.

The poor knowledge about some pathogens is demonstrated in this work since we could demonstrate that at least two trypanosomatids can infect honey bees and the presence of several novel LSV genotypes. Comprehensive characterisation revealed that a broad complex of related LSV

genotypes exists in honey bees. We were able to assemble almost the entire genome of four LSV strains and observed high strain diversity. Moreover, we could demonstrate several strains within single bee specimens. Soon after its initial report in the USA, LSVs were reported from other countries and even from other hosts. The negative strand intermediate, a marker for replication of ssRNA (+) viruses, was also detected in honey bees and the mason bee *Osmia cornuta*. Also replication of ALPV strain Brookings and VdMLV in honey bees was demonstrated for the first time, besides LSV replication in the solitary bee *O. cornuta*. Concluding, during this work it became clear that honey bees face more parasites, and in particular more viruses, than previously thought.

SAMENVATTING

Honingbijen worden wereldwijd ingezet voor de bestuiving van allerlei fruit en gewassen. De laatste decenia worden echter ongewoon hoge kolonie sterftes waargenomen op verscheidene continenten zonder een eenduidige oorzaak. Ook de Belgische bijenkolonies worden jaarlijks hard getroffen. Verschillende oorzaken dragen bij aan deze sterftes, maar vooral de bijenpathogenen spelen een belangrijke rol. In dit doctoraatswerk zijn een breed gamma aan pathogenen onderzocht in Belgische bijenkolonies. Dit leidde tot de ontdekking van parasieten die nog niet eerder vermeld werden voor België, waaronder virussen (ALPV stam Brookings, AmFV, LSV en VdMLV), trypanosomen (*Crithidia mellificae* en *Lotmaria passim*) en parasitaire vliegen (*Apocephalus borealis*). Sommige andere gevonden pathogenen werden al eerder in Belgische hommels gedetecteerd, zoals de neogregarine *Apicystis bombi* en de bacteriën *Spiroplasma apis* en *Spiroplasma melliferum*. Tot nu toe werden ze echter verwaarloosd als honingbij parasieten.

De pathosfeer, het totale scala aan pathogenen die de honingbij infecteren, werd in dit doctoraatswerk grondig bestudeerd. Na prevalentie onderzoeken in verscheidene stadia selecteerden we aan aantal van hen om verder te karakteriseren. Onze kennis is immers beperkt wanneer het gaat om een aantal verwaarloosde of nieuw ontdekte soorten. De manieren waarop deze zich verspreiden is ook niet goed gekend. Zo konden we de virussen ALPV stam Brookings, LSV en VdMLV aantonen in honingbij eitjes, wat dus verticale transmissie aantoont. Velen van de honingbij pathogenen werden eveneens aangetoond in solitaire bijen die we in de buurt van een bijenstand collecteerden. Voor een aantal parasieten zoals DWV en *Nosema ceranae* is aangetoond dat ze ook andere (solitaire) bijen kunnen aantasten. Toch blijkt de transmissie in beide richtingen te gebeuren. Zo konden we SBV enkel in solitaire bijen aantonen en niet in honingbijen. Behalve de honingbij parasieten blijken solitaire bijen ook een specifieke patosfeer te hebben, maar momenteel zijn echter enkel microsporidiën en schimmels gekend.

Onze gebrekkige kennis over sommige pathogenen is in dit werk aangetoond door de vondst van niet één, maar twee trypanosomen die honingbijen infecteren. Bovendien bleken er verscheidene LSV genotypen te bestaan. Door dit viraal complex verder te analyseren waren we in staat om het bijna volledige genoom van maar liefst vier stammen te achterhalen. Bovendien bleek er een hoge diversiteit te bestaan, zelfs in één enkele bij. Nadat we LSV in enkele solitaire bijensoorten hadden aangetoond, konden we zelfs replicatie van dit virus aantonen in honingbijen en in de gehoornde metselbij (*Osmia cornuta*). In de loop van dit doctoraatswerk werd dus duidelijke dat de pathosfeer van honingbijen uitgebreider is dan voordien verwacht.

TABLE OF CONTENTS

Part III: In-depth characterization of selected honey bee parasites

Part V: Discussion

LIST OF ABBREVIATIONS

AACV	Anopheline-associated C virus
ABPV	Acute Bee Paralysis Virus
ABV	Arkansas Bee Virus
AIC	Akaike information criterion
AIV	*Apis* Iridescent Virus
ALPV	Aphid Lethal Paralysis Virus
aLRT SH-like	approximate likelihood ratio test non-parametric branch support based on a Shimodaira-Hasegawa-like
AmFV	*Apis mellifera* Filamentous Virus
ATCC	American type cultures collection
BBPV	Berkeley Bee Picorna-like Virus
BHI	Brain Heart Infusion
BIC	Bayesian information criterion
BLAST	basic local alignment search tool
BQCV	Black Queen Cell Virus
BSRV	Big Sioux River Virus
BVX	Bee Virus X
BVY	Bee Virus Y
CBPV	Chronic Bee Paralysis Virus
CCD	Colony Collapse Disorder
cDNA	complementary deoxyribonucleic acid
CLSM	confocal laser scanning microscopy
CWV	Cloudy Wing Virus
cytb	cytochrome *b*
DAPI	4',6-diamidino-2-phenylindole
DIC	differential image contrast
DNA	desoxyribonucleic acid
dsRNA	double-stranded RNA
DWV	Deformed Wing Virus
gGAPDH	glycosomal glyceraldehyde-3-phosphate dehydrogenase
gp63	glycoprotein of 63 kilodalton
HRM	high resolution melting
IAPV	Israeli Acute Paralysis Virus

Imd	Immune Deficiency
ITS	internal transcribed spacer
Jak/STAT	Janus kinase/signal transducers and activators of transcription
Jnk	c-Jun N-terminal kinases
KBV	Kashmir Bee Virus
kDNA	kinetoplast DNA
KV	Kakugo Virus
LG	Le-Gascuel
Lp	Leader protein
LPO	left probe oligo
LSV	Lake Sinai Virus
ML	maximum likelihood
MLPA	multiplex ligation-dependent probe amplification
MMLV	Moloney Murine Leukemia Virus
NJ	Neighbor-Joining
ORF	open reading frame
PCR	polymerase chain reaction
qPCR	quantitative (real time) PCR
RdRP	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RNAi	RNA interference
RPO	right probe oligo
rRNA	ribosomal RNA
SBPV	Slow Bee Paralysis Virus
SBV	Sacbrood Virus
SE	slowly evolving
SEM	Scanning electron microscopy
siRNA	small interfering RNA
SNP	single-nucleotide polymorphism
SSU	small subunit ribosomal
TEM	Transmission electron microscopy
TRSV	Tobacco Ringspot Virus
UTR	untranslated region
VdMLV	*Varroa destructor* Macula-like Virus
VDV-1	*Varroa destructor* Virus-1

PART I

INTRODUCTION

CHAPTER 1:

GENERAL INTRODUCTION

1. Honey bee colony collapses

The famous quote “If the bee disappears from the surface of the earth, man would have no more than four years to live” is attributed to Albert Einstein. Although he probably never said this, honey bees (*Apis mellifera*) are considered the most valuable pollinators for crop monocultures, like fruit, nuts and vegetable crops. The annual worldwide value of insect pollination to agriculture is estimated at € 153 billion (Gallai et al., 2009).

In the last decade unusually high honey bee colony losses were reported in Europe (Potts et al., 2010) and North America (vanEngelsdorp et al., 2008). In addition, a recent European survey on honey bee health revealed that the Belgian colony mortality in winter 2012-2013 was the highest of all member states (Figure 1) (Chauzat et al., 2013). While beekeepers in the USA have suffered high losses since 2004, the colony collapse disorder (CCD) phenomenon was first reported in 2006 (Holden, 2006). A strict definition of CCD has been proposed (Cox-Foster et al., 2007; vanEngelsdorp et al., 2009): (i) rapid loss of adult workers as evidenced by large amounts of capped brood within a collapsing colony, (ii) when workers remain, they appear young and the queen is present, (iii) lack of dead workers within or proximal to collapsed colonies, (iv) ample food stores in collapsed colonies, (v) delayed invasion by other hive pests and (vi) collapsing colonies did not have damaging levels of the ectoparasitic mite *Varroa destructor* and/or the microsporidian endoparasite *Nosema* spp. at the time of collapse. At present, CCD has been only documented in the USA (Cox-Foster et al., 2007) and in Switzerland (Dainat et al., 2012b) although unusually high colony losses are observed in many countries across the Northern hemisphere. Initially, researchers thought to be dealing with only one ethiological agent but most known bee killers, like parasites and pesticides, could be eliminated

(Stokstad, 2007). Though, extensive colony losses have occurred repeatedly at different continents during the history of beekeeping. The first documented record of colony collapses appeared in 1869. Although many causes have been proposed in the past for these losses, none has been assigned with certainty (Underwood and vanEngelsdorp, 2007).

An unbiased metagenomic study revealed many pathogens in bee samples, and indicated Israeli Acute Bee Paralysis Virus (IAPV) as a marker for CCD (Cox-Foster et al., 2007). Nevertheless, this was not confirmed in a follow-up broad holistic screening (vanEngelsdorp et al., 2009). Several studies revealed a high pathogen load in samples from collapsed colonies (Cornman et al., 2012; Cox-Foster et al., 2007; vanEngelsdorp et al., 2009), but is not clear whether this is a primary or a secondary effect. In conclusion, there is a currently consensus that no single explanation can be given for these losses, and that there are several contributory factors. Causes can differ in the different continents because of the tradition of massive migratory beekeeping in the USA, in contrast to Europe.

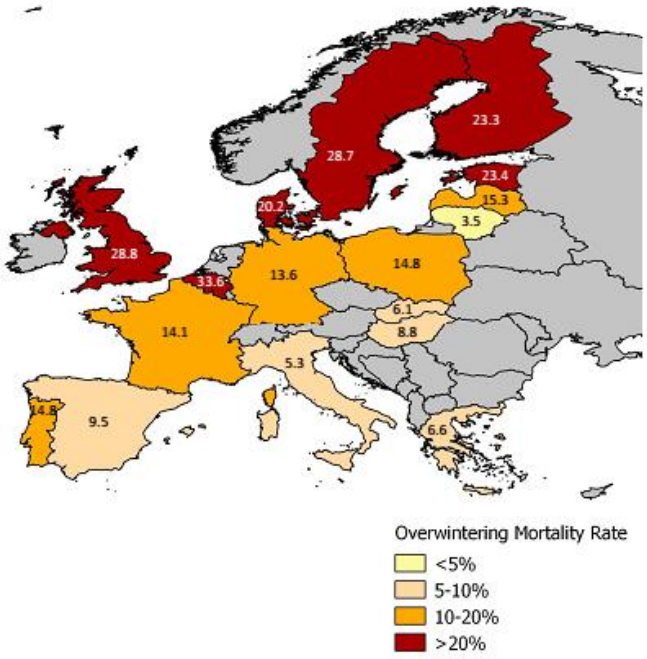


***Figure 1**: Winter mortality rates during 2012-2013 in the member states of the European Union (Chauzat et al., 2014).*

2. Pathogenic drivers of honey bee declines

Numerous parasites will be discussed in this section. A target species is included if it meets one of the following criteria: (1) known marker for honey bee mortality, (2) neglected parasite (which may contribute to the decline) and (3) parasite occurring in Europe. This implicates that several pathogens are not discussed, although they can be emerging threats to apiculture elsewhere like the small hive beetle (*Aethina tumida*) and *Tropilaelaps* mites.

2.1 Mites (Acari: Varroidae)

2.1.1 Varroa destructor

Publications before the year 2000 about *Varroa* mites on *A. mellifera* refer to *Varroa jacobsoni*, which appeared to be a complex of two related species. The 'true' *V. jacobsoni* is restricted to the Malaysia–Indonesia region, where it parasitizes on *Apis cerana*. On the other hand, *V. destructor* infests *A. cerana* in mainland Asia and *A. mellifera* almost worldwide (Anderson and Trueman, 2000). Currently, Australia is the only country free of *V. destructor*. The mites are also absent in parts of Norway (Dahle, 2010) and some islands of Hawaii and New Zealand (Martin et al., 2012; Mondet et al., 2014).

A female mite will enter a brood cell with a last instar honey bee larva. Approximately 70 hours after capping of the cell, the first egg is laid. This is unfertilised, so it develops into a male. Afterwards, fertilised female eggs will be laid in intervals of 30 hours. The male develops first and starts mating when the nymphal females are molted into adults, so reproduction occurs within the brood cell. One mite will reproduce approximately five eggs in worker brood and six eggs in drone brood (Figure 2). The offspring passes through two nymphal stages; protonymph and deutonymph, before molting into an adult (Rosenkranz et al., 2010).

The *Varroa* mite feeds on the hemolymph of last instar larvae, pupae and adult bees. This causes a serious weight loss and reduced life span (Rosenkranz et al., 2010). Moreover, this infestation has a severe impact on honey bee health by suppressing antimicrobial peptides and immunity-related enzymes (Navajas et al., 2008; Yang and Cox-Foster, 2005).

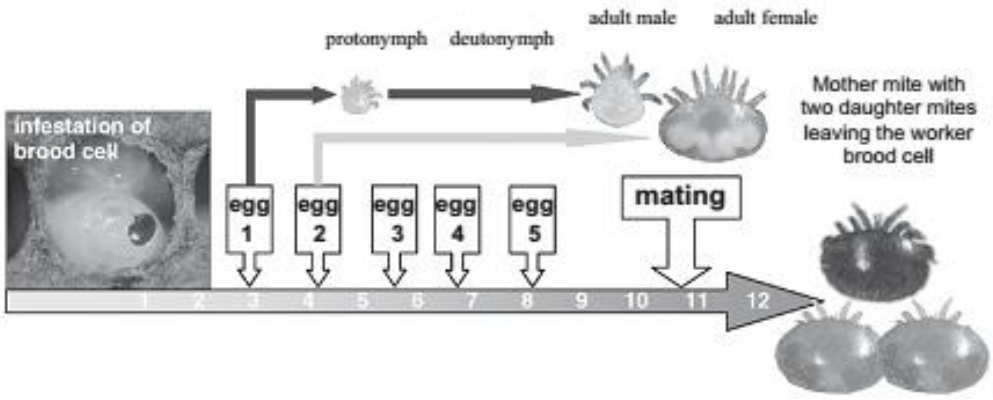


***Figure 2**: The reproductive cycle of V. destructor within the sealed honey bee worker brood cell (Rosenkranz et al., 2010).*

However, the most important consequence is that *V. destructor* can transmit several viruses (Chen and Siede, 2007). Even viral replication was demonstrated in *Varroa* mites (Di Prisco et al., 2011; Ongus et al., 2004).

Without treatment, a colony will collapse within two years (Rosenkranz et al., 2010). The *Varroa* mite has been indisputable assigned as a key factor in recent colony losses (Dainat et al., 2012a; Genersch et al., 2010; Guzman-Novoa et al., 2010; Schafer et al., 2010; van Dooremalen et al., 2012). Moreover, Norwegian beekeepers reported significantly fewer losses in regions without *Varroa* (Dahle, 2010).

2.1.2 Acarapis woodi

This mite is an internal parasite of the respiratory system, which feeds on the hemolymph. Infestation of *Acarapis woodi* can cause obstructions of the trachea, lesions and hemolymph depletion (Sammataro et al., 2013). It is considered to be associated with poor winter survival (McMullan and Brown, 2009; Otis and Scott-Dupree, 1992). *Acarapis* mites were associated with recent *A. cerana* collapses in Japan (Kojima et al., 2011). This mite has been reported almost worldwide (Ellis and Munn, 2005). Although it was thought to be diminishing due to *Varroa* treatments, *A. woodi* was recently detected in Japan (Kojima et al., 2011) and Spain (Garrido-Bailon et al., 2012).

2.2 Viruses

2.2.1 Introduction

Viruses can infect all organisms, from animals to bacteria. Those viruses infesting honey bees are positive sense single-stranded RNA viruses, with the exception of the DNA viruses *Apis mellifera* Filamentous Virus (AmFV) and *Apis* Iridescent Virus (only detected in *A. cerana*). The positive sense implicates that the RNA can be immediately translated into viral proteins. Replication requires an infected host cell, where the genome is copied to a negative-stranded intermediate. This serves as a template from which new viral genomes are copied. Several virus families can be distinguished, based mainly on the gene arrangement (Figure 3).

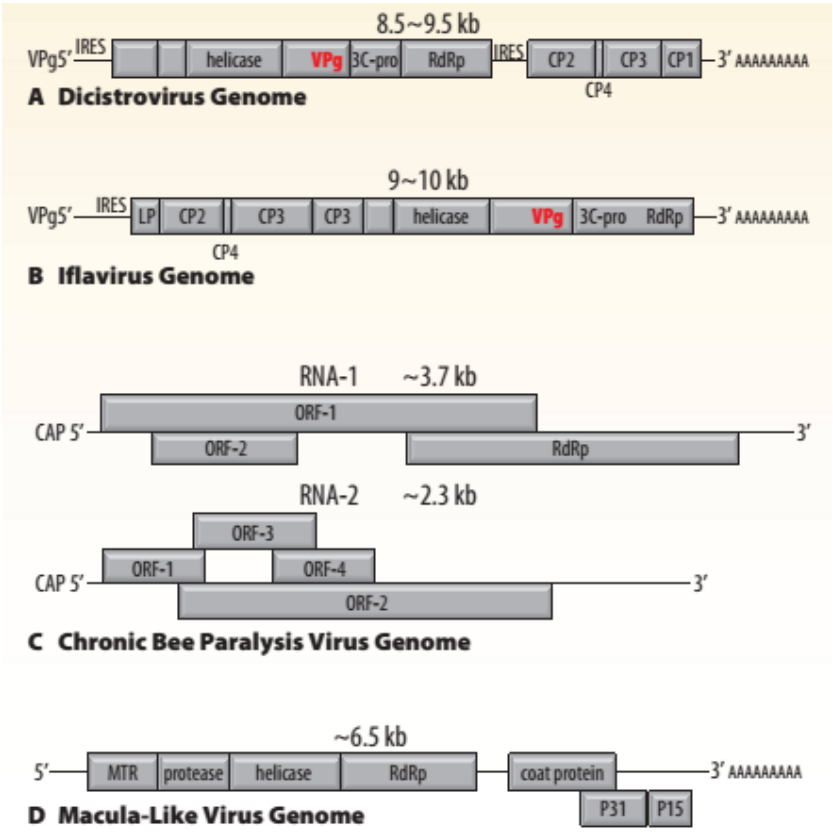


***Figure 3**: Genomic maps for the different virus families known to infect honey bees (deMiranda 2010).*

Dicistrovirises have a dicistronic RNA genome. This implicates that two non-overlapping open reading frames (ORFs) are separate from each other by an internal ribosomal entry site (IRES), in contrast to the monocistronic Iflaviruses. Their structural proteins are located at the N-terminus and

the non-structural proteins at the C-terminus. This order is reversed in the Dicistrovirus genome. Several functional domains were identified in both genomes, which led to the formation of the order Picornavirales, comprising the genera Iflavirus and Dicistrovirus, among others (Le Gall et al., 2008). The non-structural proteins include RNA-dependent RNA polymerase (RdRP), helicase, protease, genome-linked viral protein (VPg) and leader protein (Lp). They are involved in translation of viral proteins and virus replication, except Lp and Vpg. The latter is attached to the 5' end and is involved in the RNA stabilisation, like the CAP in the CBPV RNAs. The Lp has probably a protease function but is very variable in amino acid sequence. The genome of Picornavirales encodes four structural proteins (VP1-4). Three related proteins (VP1-3) determine the virion capsid. They are unrelated to the small protein VP4, which is probably cleaved from the polyprotein (Le Gall et al., 2008).

The *Varroa destructor* Macula-like Virus (VdMLV) is included in the Tymoviridae. The genome consists of around 6,500 nucleotides and encodes for one coat protein. Several replication associated proteins are also present in the genome, in addition with two proteins with unknown functions (deMiranda, unpublished information).

Chronic Bee Paralysis Virus (CBPV) is not officially assigned to a genus. Its genome contains two RNA strands: RNA 1 (3,674 nucleotides in length) and RNA 2 (2,305 nucleotides). These RNAs have a 5' CAP structure and are not polyadenylated at the 3' end, in contrast to the other honey bee viruses. Analysis of the RNA 1 and 2 sequences predicts respectively three and four overlapping open reading frames (ORFs). One of them, ORF 3 on RNA 1, encodes for a RdRP (Olivier et al., 2008). Lake Sinai Virus (LSV) has not been officially assigned to a genus either. Their genomes vary between 5,355 (LSV2, Genbank: HQ888865) and 5,508 nucleotides (LSV1, Genbank: HQ871931) (Runckel et al., 2011). They have overlapping ORFs, coding for RdRP, one capsid protein and one protein with unknown function (ORF1). Although originally no similarities were found for the unknown CBPV and LSV ORFs, homologues with Alphavirus methyltransferase-guanylyltransferase and virion proteins were recently reported (Kuchibhatla et al., 2014).

2.2.2 Transmission routes

Most bee viruses usually persist as 'covert' infections, without disease symptoms, in strong and healthy colonies. Due to external factors like parasite pressure, they can convert into 'overt' infections with high viral levels and obvious symptoms. Viruses can be transmitted both horizontally and vertically (figure 4). The spread between bees from the same generation is called horizontal transmission, which includes oral transmission and mite-vectored transmission. Transfer from the parents towards their offspring, via eggs or semen, is called vertical transmission. Several studies showed that honey bee viruses can be transmitted through both pathways (reviewed by deMiranda (deMiranda et al., 2011)). The different transmission routes can cause a rapid spread of these viruses in the dense colony. Although the *Varroa* mite is considered as an important transmitter, honey bees themselves spread viruses by feeding and cleaning. Although there are some reports about contact and airborne transmission (Bailey et al., 1983; Lighthart et al., 2005), little is known about this in comparison to the other routes. The queen can pass on viruses to her offspring when the ovaries or spermatheca become infected.

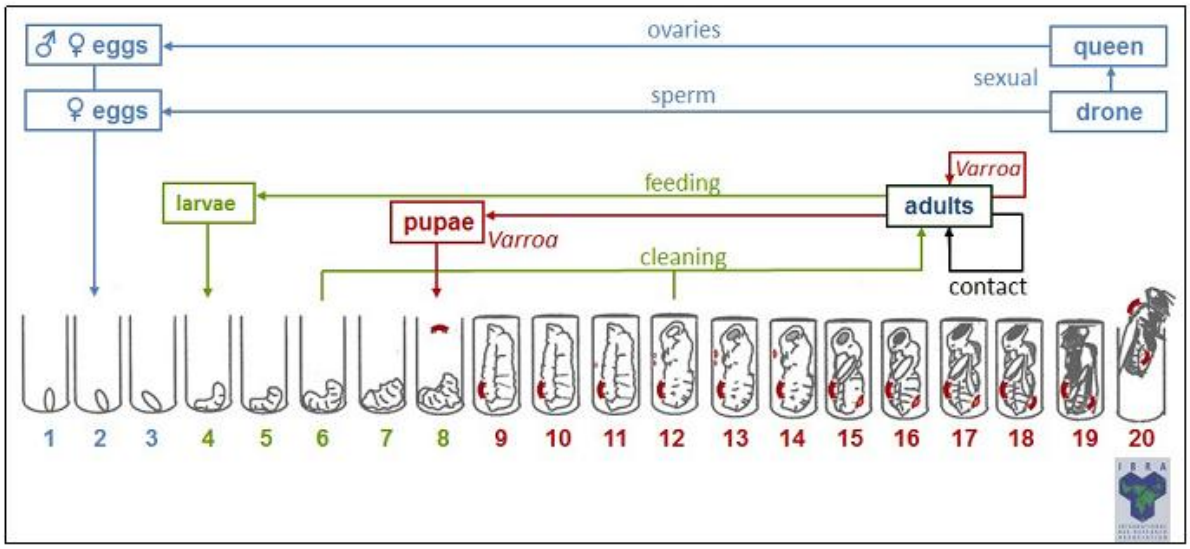


***Figure 4**: Diagram describing the different possible transmission routes for honey bee viruses (deMiranda et al., 2013).*

2.2.3 Deformed Wing Virus

Deformed Wing Virus (DWV) is part of a complex consisting of the three related viruses: DWV, Kakugo Virus (KV) and *Varroa destructor* Virus-1 (VDV-1) (deMiranda and Genersch, 2010). The genomes of these Iflaviruses consist of around 10,000 nt (Fujiyuki et al., 2006; Lanzi et al., 2006; Ongus et al., 2004). Since these viruses have a high sequence similarity, they are mostly considered as strains of one virus. Most nucleotide differences are located in the Lp gene, which is a variable region (deMiranda et al., 2010). Besides, several recombinants between VDV-1 and DWV have been reported (Moore et al., 2011; Wang et al., 2013; Zioni et al., 2011). A phylogenetic analysis of DWV samples from diverse geographical origins revealed a highly conserved genome (Berenyi et al., 2007). Nevertheless, the Lp gene was not included in this study. DWV has a high prevalence in many countries nowadays (De Smet et al., 2012; Tentcheva et al., 2004), but before the *Varroa* invasion it was mainly present as a covert virus. The recent infestation of *V. destructor* across parts of Hawaii demonstrated a huge decrease of DWV diversity and prevalence, leading to the predominance of one strain (Martin et al., 2012). Later on, the strain domination was experimentally confirmed after *Varroa*-mediated or oral transmission (Ryabov et al., 2014).

Figure 5*: Adult honey bee with deformed wings and a phoretic* Varroa *mite (Genersch and Aubert, 2010).*

Distinctive symptoms can be caused by DWV in adult bees. The high virus levels are associated with shrivelled wings (Figure 5), increased mortality and learning deficits (deMiranda et al., 2010). Wing deformities were also observed in a *Varroa*-free colony, although DWV was only present in low quantities (Forsgren et al., 2012). Nevertheless, several studies showed that winter colony collapses are strongly associated with the amount of DWV present in the bees (Dainat et al., 2012a; Genersch et al., 2010; Highfield et al., 2009)

2.2.4 Acute Bee Paralysis Virus

Acute Bee Paralysis Virus (ABPV) is one of the first described honey bee viruses, although it was serendipitously discovered during Chronic Bee Paralysis Virus (CBPV) research. ABPV, Kashmir Bee Virus (KBV) and Israeli Acute Paralysis Virus (IAPV) are closely related viruses, which are well separated in phylogenetic analyses. Still, several genetic 'groups' can be distinguished in particularly for IAPV (Chen et al., 2014; Palacios et al., 2008). The genomes of these Iflaviruses comprise around 9,500 nt and are polyadenylated (deMiranda et al., 2004; Govan et al., 2000; Maori et al., 2007).

An overt infection causes a rapid paralysis, trembling and inability to fly, eventually followed by death one day after paralysis. All members of this viral complex have been associated with *V. destructor* (deMiranda et al., 2010a; Di Prisco et al., 2011; Shen et al., 2005). Prior to the arrival of *Varroa*, ABPV rarely caused colony collapses. The transmission enables ABPV to quickly kill both adult bees and pupae, leading to an inadequate replacement of adult bees. ABPV appears to be the most prevalent in Europe and South America, KBV in North America and New Zealand, and IAPV in the Middle East and Australia (deMiranda et al., 2010a).

IAPV was initially identified as a predictive marker for colony losses in the USA (Cox-Foster et al., 2007). However, a retrospective study revealed that this virus was already present before the first colony collapse disorders were ever reported (Chen and Evans, 2007). In Europe, ABPV was linked with colony losses in Belgium, Germany and Switzerland (Berthoud et al., 2010; Genersch et al., 2010; Nguyen et al., 2011) .

2.2.5 Other common viruses

Several other honey bee viruses are commonly detected in surveys. Although most of them can cause clear clinical symptoms, colony collapses are not associated with these viruses.

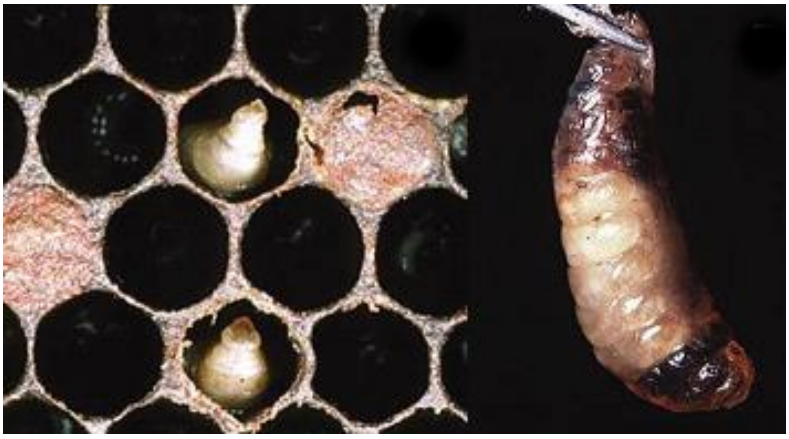


Figure 6*: Disease symptoms of honey bee larvae infected with SBV (deMiranda et al., 2011).*

Black Queen Cell Virus (BQCV) contains a 8,550 nt polyadenylated genome (Leat et al., 2000). This iflavirus is lethal for queen larvae and prepupae. The initially yellow larva will rapidly turn dark following death, eventually also discolouring the cell walls. Symptomatic drone pupae have also been reported. This virus persists as asymptomatic infections in worker bees and brood (reviewed in deMiranda 2010).

Sacbrood Virus (SBV) causes mortality in larvae, but adult bees can be asymptomatically infected. The infected larvae develops apparently normal, but they fail to pupate. During this molt, the larval skin will not be discarded. This causes an accumulation of fluid, so the larva will resemble a pale sac (Figure 6). Larvae which are not removed will dry out and form a scale. Infected adults forage earlier and have a reduced lifespan (deMiranda et al., 2011). Although this virus has no severe impact on *Apis mellifera*, several harmful genotypes have been described from *Apis cerana* (Choe et al., 2012; Nguyen and Le, 2013). SBV is one of the earliest sequenced honey bee viruses, with a genome of 8,832 nt (Ghosh et al., 1999).

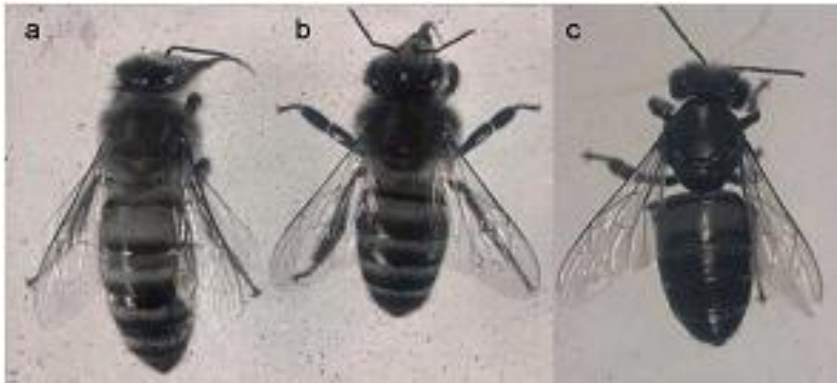


Figure 7*: Bees infected with CBPV type 1 (a) and type 2 (c), compared with a healthy individual (b) (Ribiere et al., 2008).*

CBPV causes paralysis of honey bees, like ABPV. Experimentally infected bees are symptomatic after 6 days, and die several days later. Two syndrome types have been reported (figure 7). Type 1 causes a paralysis, abnormal wing trembling and bloated abdomen. These bees are unable to fly and crawl together, which can cause masses of dead bees in front of the hive. Type 2 bees are apparently healthy, but they become hairless, flightless and paralysed after which they die soon after (Ribiere et al., 2010). The virions are irregularly shaped, mostly ellipsoidal, and contain two RNA fragments.

2.2.6 Enigmatic honey bee viruses

Several other honey bee viruses have been reported whose genomes are not completely characterized or are rarely found. This includes the Bee Virus X-Y complex, Arkansas and Berkeley Bee Virus and Cloudy Wing Virus (Bailey et al., 1980a; Bailey et al., 1980b; Bailey and Woods, 1974; Lommel et al., 1985).

In Europe, Slow Bee Paralysis Virus (SBPV) is only reported in Britain (Bailey et al., 1974) and Switzerland (deMiranda et al., 2010b). This is another lethal virus that causes paralysis of two pairs of legs after 12 days. SBPV has been associated with colony mortalities in Britain (Carreck et al., 2010). The genome of this Iflavirus is 9,500 nt and contains a polyadenylated 3' end (deMiranda et al., 2010b).

Genome sequences of *Varroa destructor* Macula-like virus (VdMLV), belonging to the Tymoviridae, were discovered during the characterisation of DWV. It is principally associated with *Varroa* mites, but appears to be very common in honey bees as well (deMiranda et al., 2011). The genome is not yet published but is around 6,500 nt.

Apis mellifera Filamentous Virus (AmFV) is one of the few DNA viruses infecting honey bees. The virion is very large and contains a coiled nucleoprotein. Severely infected bees have a milky-white hemolymph, but AmFV is not considered as harmful. The recently obtained AmFV genome (Hartmann et al., unpublished) suggests that this virus is related to both the baculo- and ascoviruses. It is probably very prevalent since AmFV is reported from several countries (Bailey et al., 1981; Clark, 1978a; Sitaropoulou et al., 1989).

By the use of unbiased molecular techniques, like next-generation sequencing, several novel honey bee viruses were discovered: Aphid Lethal Paralysis Virus strain Brookings, Big Sioux River Virus and Lake Sinai Virus (strains 1 and 2) (Runckel et al., 2011). Their pathology towards honey bees is not yet known. The canonical ALPV, isolated from an aphid, was characterised as a Dicistrovirus which is 9,812 nucleotides in length (van Munster et al., 2002). Later on, ALPV strains from honey bees and the pea aphid (*Acyrthosiphon pisum*) are proposed as isolates from a new species (Liu et al., 2014). BSRV is another Dicistrovirus, but only reported from the USA (Runckel et al., 2011). The LSVs are not assigned to a genus, and appear to be very divergent (Cornman et al., 2012).

2.2.7 Other hosts

Several studies have reported the presence of honey bee viruses in other Hymenopteran hosts, mainly in bumble bees and social wasps. Concerning the viruses, it involved ABPV complex (Anderson, 1991; Bailey and Gibbs, 1964; Singh et al., 2010), BQCV (Peng et al., 2011; Singh et al., 2010), CBPV (Celle et al., 2008), DWV (Furst et al., 2014; Genersch et al., 2006; Li et al., 2011; Singh et al., 2010) and SBV (Singh et al., 2010). Most of these viruses were also retrieved in non-Hymenopteran insects, like cockroaches (Blattodea) and earwigs (Dermaptera) (Levitt et al., 2013). Even replication could be demonstrated in some of them. However, these insects were sampled

nearby or within an apiary. The detection rates are smaller when urban or arable sites are studied (Evison et al., 2012).

2.3 Microsporidian pathogens

2.3.1 Introduction

An unusual group of eukaryotic, intracellular parasites is formed by the microsporidian. They are considered as the earliest diverging clade of fungi nowadays (Capella-Gutierrez et al., 2012; James et al., 2006). Microsporidia form solid spores which can survive outside the host cells. They contain several specialized structures like the polar tube and polaroplast involved in host cell invasion. The former is internally coiled and anchored at the apex of the spore (Figure 8A). The latter is a membranous structure that is incorporated in the polar tube during germination. In this stage the osmotic pressure builds up in the spore. This will cause the extrusion of the polar tube at a relative high speed which enables the parasite to pierce a nearby host cell, and delivers the sporoplasm intracellularly. At this merogonic stage, the parasite will reproduce vegetatively. Infected cells are enlarged by the proliferative cycles of the parasite. Later on, the spore walls and organelles like the polar tube will develop in the sporogonic phase. The spores are released by rupture of the host cell (figure 8B) (Keeling and Fast, 2002).

2.3.2 Microsporidia infecting honey bees

Honey bees can be infested by two microsporidian parasites, i.e. *Nosema apis* and *Nosema ceranae*. The latter was originally described as a parasite of *A. cerana* (Fries et al., 1996). Later on, it was reported as an *A. mellifera* pathogen (Higes et al., 2006; Huang et al., 2007). It is suggested that *N. ceranae* has jumped to another host and spread worldwide due to transportation (Klee et al., 2007). However, retrospective analyses revealed that *N. ceranae* infected *A. mellifera* decades ago (Chen et al., 2008; Invernizzi et al., 2009; Paxton et al., 2007; Teixeira et al., 2013). This microsporidian is also known to infect several honey bee (Chaimanee et al., 2010; Suwannapong et al., 2011) and bumble bee species (Furst et al., 2014; Graystock et al., 2013; Li et al., 2012; Plischuk et al., 2009). Lately, it

was demonstrated that *N. ceranae* isolated from honey bees can be virulent towards bumble bees (Furst et al., 2014; Graystock et al., 2013).

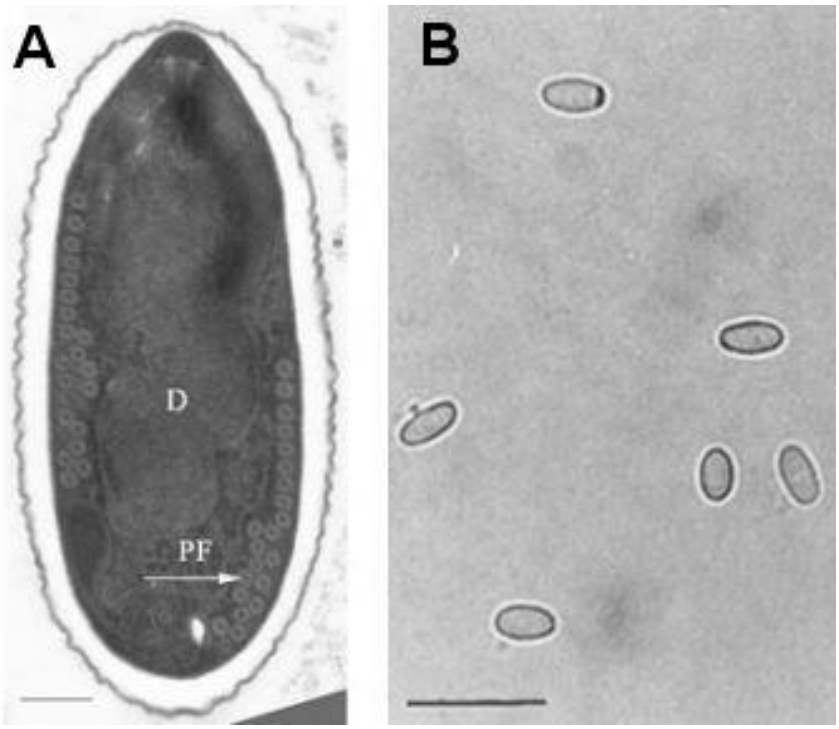


Figure 8*: A) The internal structure of a* N. ceranae *spore by electron microscopy. D: diplokaryon, PF: coils of the polar tube. Scale bar = 0.5 µm.(Fries, 2006) B) Light microscopy of fresh* Nosema *spores in water, scale bar = 10 µm (Fries, 2010).*

Several recent studies proposed an apparent displacement of *N. apis* by *N. ceranae* (Paxton 2007, Martin-Hernandez 2007). However, *N. ceranae* infections appear to dominate the warmer and temperate regions, whereas *N. apis* is more common in colder climates (Martin-Hernandez et al., 2012; Martin-Hernandez et al., 2007; Williams et al., 2008). Both species seem to have different seasonal patterns (Martin-Hernandez et al., 2012; Runckel et al., 2011).

The role of *N. ceranae* in causing colony collapse is still controversial (Fries, 2010; Higes et al., 2013). Sudden colony collapses in Spain were attributed to *N. ceranae* infection (Higes et al., 2008; Martin-Hernandez et al., 2012), but these observations were not confirmed in any other countries (Gisder et al., 2010; Invernizzi et al., 2009). At the individual level, infestation of this parasite can cause a decreased longevity (Martin-Hernandez et al., 2011) and several sub-lethal effects (Higes et al., 2013).

2.4 Protists

2.4.1 Introduction

Protists are defined as "eukaryotic organisms with unicellular, colonial, filamentous, or parenchyniatous organization that lack vegetative tissue differentiation, except for reproduction" (Adl et al., 2005). It is a large and very diverse group, whose higher level classification was recently revised (Adl et al., 2012). A few unrelated protists have been detected in honey bees.

2.4.2 Trypanosomatid parasites

The Trypanosomatida is an order of widespread parasites, belonging to the class Kinetoplastea (phylum Euglenozoa). Kinetoplastea are characterised by kinetoplast DNA, a network of condensed mitochondrial DNA (Adl et al., 2012). In addition, they contain some unusual features like RNA editing, trans-splicing and nucleotide modification (Simpson 2006 and references therein). Some groups complete their lifecycle in one host (monoxenous), others like the human parasites *Trypanosoma* spp. and *Leishmania* spp. require a second host (dixenous) (Lukes et al., 2014). Trypanosomatida contain one flagellum, which exits the cytoplasm through asmall invagination of the plasma membrane, the flagellar pocket. This is involved in many cell processes (Field and Carrington, 2009). Trypanosomatids can change their morphology during their life cycle. Monoxenous insect trypanosomatids are predominantly found in the gut, where they mostly attach to the wall (Wallace, 1966).

Honey bees can be parasitized by trypanosomatids, although these have been little studied. *Crithidia mellificae* (family Trypanosomatidae) was described in 1967 (Langridge and Mcghee, 1967), but remained neglected until molecular markers became available (Schmid-Hempel and Tognazzo, 2010). Investigations were further boosted by a recent cell culture (Runckel et al., 2011) and the corresponding draft genome (Runckel et al., 2014). Its current role in colony collapses is unclear, despite the fact that the related bumble bee pathogen *Crithidia bombi* is known to have serious effects, particularly under starvation conditions (Brown et al., 2000; Brown et al., 2003).

2.4.3 Neogregarine pathogens

Gregarines (subclass Gregarinasina) are protists belonging to the class Conoidasida, phylum Apicomplexa). This phylum is characterized by the apical complex, an assemblage at the apical end of the infectious stage. This structure is essential for gliding motility and invasion of the host cell (Gubbels and Duraisingh, 2012). Most apicomplexans also contain an apicoplast, consisting of circular extrachromosomal DNA. It encodes for genes of several essential metabolic pathways (McFadden, 2011). The ethiological agents of the human diseases malaria (*Plasmodium* spp.) and toxoplasmosis (*Toxoplasma gondii*) are apicomplexan pathogens. Gregarines on the other hand infect only invertebrates. Ingested oocysts release four or more sporozoites, which penetrate the gut into the body cavity and infect the appropriate tissue. They feed and develop into trophozoites. When a male and a female are matured, they pair up and develop into gamonts. They associate to form a gametocyst, which is released from the host. After the cell wall formation this gametocyst divide into numerous gametes. Pairs of them will fuse into zygotes, which will become oocysts (Kuriyama et al., 2005). The taxonomy was recently revised, which led to the invalidity of the former order Neogregarinorida (Cavalier-Smith, 2014).

The gregarine *Apicystis bombi* (family Lipotrophidae, order Arthrogregarida) is reported from several bumble bee species where it develops in the fat body (Lipa and Triggiani, 1996; Macfarlane et al., 1995). Although it was once found in honey bees in 1996 (Lipa et al., 1996), publication of the following report lasted until 2011 (Plischuk et al., 2011) after a molecular detection method became available (Meeus et al., 2010). This parasite can inhibit foraging, reduce the productiveness and increase queen mortality in bumble bees (Rutrecht and Brown, 2008). Little is known about its seasonality and virulence in honey bees.

2.5 Bacteria

Honey bees harbour an extensive range of commensal or beneficial bacteria, comprising a consistent microbiota in the gut (Martinson et al., 2011), with several phylotypes present in every honeybee (Moran et al., 2012). These bacteria belong to a diverse number of bacterial classes. Recently, a

diverse lactic acid bacterial flora was discovered within the honey stomach (Olofson 2008). Besides this microbiota, some pathogenic bacteria can be present in honey bees.

Paenibacillus larvae and *Melissocccus plutonius*, both belonging to the phylum Firmicutes, cause respectively American and European foulbrood which are lethal diseases of the larvae (Forsgren, 2010; Genersch, 2010). They are deleterious for the colony and notifiable in many countries. Honey bee colonies need to be certified that they are free of American foulbrood in the context of import and export.

Spiroplasmas are situated in the Mollicutes class, characterized by the absence of a cell wall. They are related to the genus Mycoplasma, which contains several human pathogens. Spiroplasmas infect the gut and can penetrate it to subsequently invade the hemolymph (Gasparich, 2010). Two spiroplasma species can lethally infect honey bees (Clark, 1978b; Mouches et al., 1984; Mouches et al., 1982), namely *Spiroplasma apis* (Mouches et al., 1983) and *Spiroplasma melliferum* (Clark et al., 1985). These spiroplasmas were both described decades ago, but a molecular detection method was only recently reported (Meeus et al., 2012). Despite the genome assembly of *S. melliferum* KC3 and the discovery of virulence factors in its proteome (Alexeev et al., 2012), its pathogenicity has not yet been made clear. Most of these bacteria appear to be commensals, but several arthropod diseases are attributed to spiroplasmas (Gasparich, 2010).

3. Non-pathogenic drivers

Although this PhD work is focussed on the relation between pathogens and honey bee colony collapses, some other factors can also contribute to these losses. They are here only briefly discussed, in contrast to the pathogenic drivers above.

3.1 Pesticides

Many pesticides are known to cause poisoning of honey bees by toxic dust or direct application. Their use is regulated by several laws and decrees to protect non-target species, so acute intoxications are seldom reported today. Nevertheless, there is growing evidence that they cause numerous severe

sub-lethal effects. Especially the effects of neonicotinoid insecticides have recently received considerate attention (reviewed by Blacquire and collegues (Blacquiere et al., 2012)). Apart from insecticides and fungicides from agricultural use, honey bees are also exposed to acarides. These pesticides originate mainly from beekeeping practices to control *Varroa* mites, but also from agricultural use. In some countries antibiotics are also used in beekeeping for treatment of nosemosis and foulbrood, but they are not permitted in the European Union (Reybroeck et al., 2012). The combination of all these chemicals can interact, causing a heightened toxicity (Johnson et al., 2013). Most of these pesticides accumulate in beeswax due to their lipophilic structure. This might cause sub-lethal effects on the queen (Collins and Pettis, 2013; Pettis et al., 2004) and the developing larvae (Wu et al., 2011; Wu et al., 2012).

3.2 Genetic variability

The introduction of non-native subspecies, monoandric insemination methods and massive propagation of selective bee breeds seems to have reduced the genetic variability of honey bees, although there is conflicting evidence concerning the latter (Harpur et al., 2012). It is important to note that adequate genetic variability within the colony is important for the overall fitness and productivity of that colony (Mattila and Seeley, 2007; Tarpy, 2003).

3.3 Nutrition

Poor nutrition may be involved in colony declines. Both the quality and diversity of pollen can shape the honey bee health and immunity (Di Pasquale et al., 2013). Adequate nutrition reduces the susceptibility to parasites (Basualdo et al., 2014; Foley et al., 2012). However, this cannot reverse the severe effects of *Varroa* infestation (Alaux et al., 2011).

4. Defence mechanisms against pathogens

4.1 General immunity

Defence levels against parasites are placed on different levels. Besides the individual defence pathways (humeral and cellular immunity), honey bees can interact behaviourally which results in a

'social immunity'. This includes grooming, hygienic behaviour and task differentiation (Evans and Spivak, 2010).

Since honey bees are susceptible to many pathogens, they have evolved several methods to control them. Insects have to rely on their innate immunity consisting of cellular and humoral immune defences. The honey bee genome encodes for four interconnected routes for responding to parasite exposure: the Toll, Immune Deficiency (Imd), Janus kinase/signal transducers and activators of transcription (Jak/STAT), and c-Jun N-terminal kinases (Jnk) pathways (Evans et al., 2006; Evans et al., 2010). However, the gene numbers of these pathways are severely reduced when compared with other insects like *Drosophila melanogaster* (Honeybee Genome Sequencing Consortium, 2006). An invading parasite can be recognized by microbe associated molecular patterns (MAMP) like peptidoglycan recognition proteins and Gram-negative binding protein. These proteins trigger the signaling pathways, leading to the activation of several effector molecules like antimicrobial peptides, prophenoloxidase (PPO) and thiolester-containing proteins (Evans et al., 2006).

Cellular immunity can also be activated, but less is known about the cellular defences. Several hemocytes circulate in the hemolymph of honey bees. Nevertheless, the different subsets were only recently profiled by flow cytometry (Marringa et al., 2014). These cells can phagocytose single bacteria and form nodulations around bacterial aggregations. Larger pathogens are also encapsulated. These nodules and capsules can be melanized by effectors like PPO. Hemocytes also forms clots in response to wounding (Govind, 2008; Lavine and Strand, 2002), such as punctures from *Varroa* mites.

4.2 Antiviral immunity

In addition to the above pathways, RNA interference (RNAi) is an important mechanism of antiviral immunity. RNAi is involved in three related pathways, from which the silencing RNA pathway regulates double-stranded RNA (dsRNA) derived from exogenous or endogenous sources (figure 9). This dsRNA, originated from a replicating virus or a viral RNA with a secondary structure, will be recognized by Dicer proteins. These endoribonucleases cleave the RNA in smaller fragments of 20-25

double-stranded nucleotides, called small interfering RNA (siRNA). They are loaded into the RNA-induced silencing complex (RISC), where the siRNA is unwinded and one strand is retained. This activated RISC will bind to complementary viral sequences, which are degraded by the Argonaute (AGO) RNases (Bronkhorst and van Rij, 2014; Kingsolver et al., 2013).

Several homologue genes for RNA interference have been discovered in the honey bee genome. This includes Dicer, SID-1 and several RISC components like AGO and R2D2 (Honeybee Genome Sequencing Consortium, 2006). Most work on honey bee immunity has been focused on bacterial pathogens (Cornman et al., 2013; Evans, 2004), but the immune response against other parasites like *N. ceranae* and *C. mellificae* were also elucidated (Chaimanee et al., 2012; Schwarz and Evans, 2013). Resistance mechanisms against viruses have recently received a lot of interest, mainly in *Drosophila*. Several studies indicated that the Imd, Jak/STAT and Toll cascades are activated in *Drosophila melanogaster* after viral infection (Costa et al., 2009; Dostert et al., 2005; Zambon et al., 2005), along the RNAi pathway (Sabin et al., 2010).

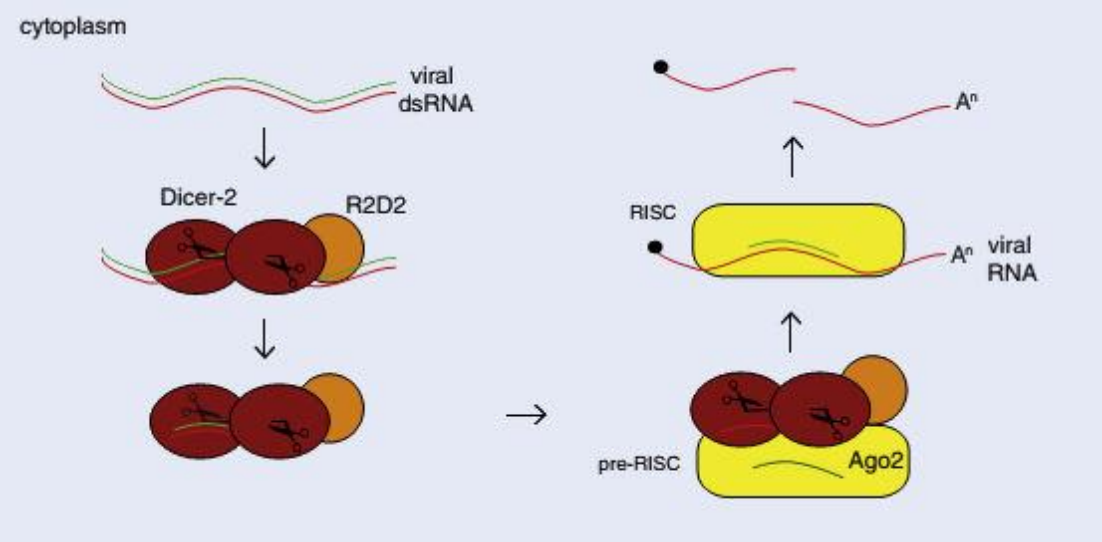


Figure 9*: Overview of the siRNA pathway. A Dicer protein will cleave viral dsRNA in smaller fragments. After unwinding, one strand will be selected. When this bounds to complementary RNA, the RISC cleaves it, consequently inhibiting viral replication (Kingsolver et al., 2013)).*

Although only few members of the silencing RNA pathway in honey bees have been described, viral siRNAs from DWV, KBV and IAPV were recently discovered (Chejanovsky et al., 2014).

Also, the oral uptake of specific dsRNAs reduced the infection of DWV and IAPV (Desai et al., 2012; Hunter et al., 2010). However, some viruses are able to evade the RNAi response. A putative viral interference motive was recently identified in all members of the ABPV complex (Chen et al., 2014).

5. References

Adl, S.M., Simpson, A.G., Farmer, M.A., Andersen, R.A., Anderson, O.R. and others, 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol 52**,** 399-451.

Adl, S.M., Simpson, A.G., Lane, C.E., Lukes, J., Bass, D. and others, 2012. The revised classification of eukaryotes. J Eukaryot Microbiol 59**,** 429-493.

Alaux, C., Dantec, C., Parrinello, H., Le, C.Y., 2011. Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. BMC Genomics 12**,** 496.

Alexeev, D., Kostrjukova, E., Aliper, A., Popenko, A., Bazaleev, N. and others, 2012. Application of Spiroplasma melliferum proteogenomic profiling for the discovery of virulence factors and pathogenicity mechanisms in host-associated spiroplasmas. J Proteome Res 11**,** 224-236.

Anderson, D.L., 1991. Kashmir bee virus - a relatively harmless virus of honey bee colonies. Am. Bee J. 131**,** 767-770.

Anderson, D.L., Trueman, J.W.H., 2000. Varroa jacobsoni (Acari: Varroidae) is more than one species. Exp Appl Acarol 24**,** 165-189.

Bailey, L., Ball, B.V., Carpenter, J.M., Woods, R.D., 1980a. Small virus-like particles in honey bees associated with Chronic Paralysis Virus and with a previously undescribed disease. J Gen Virol 46**,** 149-155.

Bailey, L., Carpenter, J.M., Govier D.A., Woods, R.D., 1980b. Bee Virus Y. J Gen Virol 51**,** 405-407.

Bailey, L., Ball, B.V., Perry, J.N., 1983. Honeybee Paralysis - Its natural spread and its diminished incidence in England and Wales. J Apicult Res 22**,** 191-195.

Bailey, L., Carpenter, J.M., Woods, R.D., 1981. Properties of a filamentous virus of the honey bee (Apis mellifera). Virology 114**,** 1-7.

Bailey, L., Gibbs, A.J., 1964. Acute infection of bees with paralysis virus. Journal of Insect Pathology 6**,** 395-407.

Bailey, L., Woods, R.D., 1974. Three previously undescribed viruses from the honey bee. J Gen Virol 25**,** 175-186.

Basualdo, M., Barragan, S., Antunez, K., 2014. Bee bread increases honeybee haemolymph protein and promote better survival despite of causing higher Nosema ceranae abundance in honeybees. Env Microbiol Rep 6**,** 396-400.

Berenyi, O., Bakonyi, T., Derakhshifar, I., Koglberger, H., Topolska, G. and others, 2007. Phylogenetic analysis of deformed wing virus genotypes from diverse geographic origins indicates recent global distribution of the virus. Appl Environ Microb 73**,** 3605-3611.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., Neumann, P., 2010. Virus infections and winter losses of honey bee colonies (Apis mellifera). J Apicult Res 49**,** 60-65.

Blacquiere, T., Smagghe, G., Van Gestel, C.A.M., Mommaerts, V., 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology 21**,** 973-992.

Bronkhorst, A.W., van Rij, R.P., 2014. The long and short of antiviral defense: small RNA-based immunity in insects. Current Opinion in Virology 7**,** 19-28.

Brown, M.J.F., Loosli, R., Schmid-Hempel, P., 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91**,** 421-427.

Brown, M.J.F., Schmid-Hempel, R., Schmid-Hempel, P., 2003. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. J. Anim. Ecol. 72**,** 994-1002.

Capella-Gutierrez, S., Marcet-Houben, M., Gabaldon, T., 2012. Phylogenomics supports microsporidia as the earliest diverging clade of sequenced fungi. BMC Biol. 10**,** 47.

Carreck, N.L., Bell, B.V., Martin, S.J., 2010. Honey bee colony collapse and changes in viral prevalence associated with Varroa destructor. J Apicult Res 49**,** 93-94.

Cavalier-Smith, T., 2014. Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of Sporozoa. Eur J Protistol 50**,** 472-495.

Celle, O., Blanchard, P., Olivier, V., Schurr, F., Cougoule, N. and others, 2008. Detection of Chronic bee paralysis virus (CBPV) genome and its replicative RNA form in various hosts and possible ways of spread. Virus Res. 133**,** 280-284.

Chaimanee, V., Chantawannakul, P., Chen, Y., Evans, J.D., Pettis, J.S., 2012. Differential expression of immune genes of adult honey bee (Apis mellifera) after inoculated by Nosema ceranae. J. Insect Physiol. 58**,** 1090-1095.

Chaimanee, V., Warrit, N., Chantawannakul, P., 2010. Infections of Nosema ceranae in four different honeybee species. J Invertebr Pathol 105**,** 207-210.

Chauzat, M.P., Cauquil, L., Roy, L., Franco, S., Hendrikx, P., Ribiere-Chabert, M., 2013. Demographics of the European apicultural industry. PLoS One 8**,** e79018.

Chauzat, M.P., Laurent, M., Riviere, M.P., Saugeon, C., Hendrikx, P., Ribiere-Chabert, M. A pan-European epidemiological study on honeybee colony losses 2012-2013. 2014.
Ref Type: Report

Chejanovsky, N., Ophir, R., Schwager, M.S., Slabezki, Y., Grossman, S., Cox-Foster, D., 2014. Characterization of viral siRNA populations in honey bee colony collapse disorder. Virology 454-455**,** 176-183.

Chen, Y., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. Nosema ceranae is a long-present and wide-spread microsporidian infection of the European honey bee (Apis mellifera) in the United States. J Invertebr Pathol 97**,** 186-188.

Chen, Y.P., Evans, J.D., 2007. Historical presence of Israeli acute paralysis virus in the United States. Am. Bee J. 147**,** 1027-1028.

Chen, Y.P., Pettis, J.S., Corona, M., Chen, W.P., Li, C.J. and others, 2014. Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health. Plos Pathog 10**,** e1004261.

Chen, Y.P., Siede, R., 2007. Honey bee viruses. Advances in Virus Research, Vol 70 70**,** 33-80.

Choe, S.E., Nguyen, L.T.K., Noh, J.H., Kweon, C.H., Reddy, K.E. and others, 2012. Analysis of the complete genome sequence of two Korean sacbrood viruses in the Honey bee, Apis mellifera. Virology 432**,** 155-161.

Clark, T.B., 1978a. Filamentous virus of honey bee. J Invertebr Pathol 32**,** 332-340.

Clark, T.B., 1978b. Honey bee spiroplasmosis - New problem for beekeepers. Am Bee J 118**,** 18-19.

Clark, T.B., Whitcomb, R.F., Tully, J.G., Mouches, C., Saillard, C. and others, 1985. Spiroplasma melliferum, a new species from the honeybee (Apis mellifera). Int J Syst Bacteriol 35**,** 296-308.

Collins, A.M., Pettis, J.S., 2013. Correlation of queen size and spermathecal contents and effects of miticide exposure during development. Apidologie 44**,** 351-356.

Cornman, R.S., Lopez, D., Evans, J.D., 2013. Transcriptional response of honey bee larvae infected with the bacterial pathogen Paenibacillus larvae. PLoS One 8**,** e65424.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D. and others, 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7**,** e43562.

Costa, A., Jan, E., Sarnow, P., Schneider, D., 2009. The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS One 4**,** e7436.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

Dahle, B., 2010. The role of Varroa destructor for honey bee colony losses in Norway. J Apicult Res 49**,** 124-125.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012a. Predictive markers of honey bee colony collapse. PLoS One 7**,** e32151.

Dainat, B., vanEngelsdorp, D., Neumann, P., 2012b. Colony collapse disorder in Europe. Environ Microbiol Rep 4**,** 123-125.

deMiranda, J.R., Bailey, L., Ball, B.V., Blanchard, P., Budge, G.E. and others, 2013. Standard methods for virus research in Apis mellifera. J Apicult Res 52.

deMiranda, J.R., Cordoni, G., Budge, G., 2010a. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J Invertebr Pathol 103**,** S30-S47.

deMiranda, J.R., Dainat, B., Locke, B., Cordoni, G., Berthoud, H. and others, 2010b. Genetic characterization of slow bee paralysis virus of the honeybee (Apis mellifera L.). J Gen Virol 91**,** 2524-2530.

deMiranda, J.R., Drebot, M., Tyler, S., Shen, M., Cameron, C.E. and others, 2004. Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. J Gen Virol 85**,** 2263-2270.

deMiranda, J.R., Gauthier, L., Ribière, M., Chen, Y., 2011. Honey bee viruses and their effect on bee and colony health. In: Sammataro, D., Yoder, J.A. (Eds.), Honey bee colony health: Challenges and sustainable solutions CRC Press, pp. 71-112.

deMiranda, J.R., Genersch, E., 2010. Deformed Wing Virus. J Invertebr Pathol 103 Suppl 1**,** S48-S61.

De Smet, L., Ravoet, J., deMiranda, J.R., Wenseleers, T., Mueller, M.Y. and others, 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One 7**,** e47953.

Desai, S.D., Eu, Y.J., Whyard, S., Currie, R.W., 2012. Reduction in deformed wing virus infection in larval and adult honey bees (Apis mellifera L.) by double-stranded RNA ingestion. Insect Molecular Biology 21**,** 446-455.

Di Pasquale, G., Salignon, M., Le, C.Y., Belzunces, L.P., Decourtye, A. and others, 2013. Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? PLoS One 8**,** e72016.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H.F., Evans, J.D., Chen, Y.P., 2011. Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. J Gen Virol 92**,** 151-155.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D. and others, 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nature Immunology 6**,** 946-953.

Ellis, J.D., Munn, P.A., 2005. The worldwide health status of honey bees. Bee World 86**,** 88-101.

Evans, J.D., 2004. Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, Paenibacillus larvae. J Invertebr Pathol 85**,** 105-111.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L. and others, 2006. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Molecular Biology 15**,** 645-656.

Evans, J.D., Spivak, M., 2010. Socialized medicine: individual and communal disease barriers in honey bees. J Invertebr Pathol 103 Suppl 1**,** S62-S72.

Evison, S.E., Roberts, K.E., Laurenson, L., Pietravalle, S., Hui, J. and others, 2012. Pervasiveness of parasites in pollinators. PLoS One 7**,** e30641.

Field, M.C., Carrington, M., 2009. The trypanosome flagellar pocket. Nature Reviews Microbiology 7**,** 775-786.

Foley, K., Fazio, G., Jensen, A.B., Hughes, W.O., 2012. Nutritional limitation and resistance to opportunistic Aspergillus parasites in honey bee larvae. J Invertebr Pathol 111**,** 68-73.

Forsgren, E., 2010. European foulbrood in honey bees. J Invertebr Pathol 103 Suppl 1**,** S5-S9.

Forsgren, E., Fries, I., deMiranda, J.R., 2012. Adult honey bees (Apis mellifera) with deformed wings discovered in confirmed varroa-free colonies. J Apicult Res 51**,** 136-138.

Fries, I., 2006. Natural infections of Nosema ceranae in European honey bees. J Apicult Res 45**,** 230-233.

Fries, I., 2010. Nosema ceranae in European honey bees (Apis mellifera). J Invertebr Pathol 103**,** S73-S79.

Fries, I., Feng, F., daSilva, A., Slemenda, S.B., Pieniazek, N.J., 1996. Nosema ceranae n sp (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee Apis cerana (Hymenoptera, Apidae). Eur J Protistol 32**,** 356-365.

Fujiyuki, T., Ohka, S., Takeuchi, H., Ono, M., Nomoto, A., Kubo, T., 2006. Prevalence and phylogeny of Kakugo virus, a novel insect picorna-like virus that infects the honeybee (Apis mellifera L.), under various colony conditions. J. Virol. 80**,** 11528-11538.

Furst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506**,** 364-366.

Gallai, N., Salles, J.M., Settele, J., Vaissiere, B.E., 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecol Econ 68**,** 810-821.

Garrido-Bailon, E., Bartolome, C., Prieto, L., Botias, C., Martinez-Salvador, A. and others, 2012. The prevalence of Acarapis woodi in Spanish honey bee (Apis mellifera) colonies. Experimental Parasitology 132**,** 530-536.

Gasparich, G.E., 2010. Spiroplasmas and phytoplasmas: microbes associated with plant hosts. Biologicals 38**,** 193-203.

Genersch, E., 2010. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. J Invertebr Pathol 103 Suppl 1**,** S10-S19.

Genersch, E., Aubert, M., 2010. Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). Vet Res 41**,** 54.

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C. and others, 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41**,** 332-352.

Genersch, E., Yue, C., Fries, I., deMiranda, J.R., 2006. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. J Invertebr Pathol 91**,** 61-63.

Ghosh, R.C., Ball, B.V., Willcocks, M.M., Carter, M.J., 1999. The nucleotide sequence of sacbrood virus of the honey bee: an insect picorna-like virus. J Gen Virol 80 (Pt 6)**,** 1541-1549.

Gisder, S., Hedtke, K., Mockel, N., Frielitz, M.C., Linde, A., Genersch, E., 2010. Five-Year cohort study of Nosema spp. in Germany: Does climate shape virulence and assertiveness of Nosema ceranae? Appl Environ Microb 76**,** 3032-3038.

Govan, V.A., Leat, N., Allsopp, M., Davison, S., 2000. Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. Virology 277**,** 457-463.

Govind, S., 2008. Innate immunity in Drosophila: Pathogens and pathways. Insect Science 15**,** 29-43.

Graystock, P., Yates, K., Darvill, B., Goulson, D., Hughes, W.O., 2013. Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. J Invertebr Pathol 114**,** 114-119.

Gubbels, M.J., Duraisingh, M.T., 2012. Evolution of apicomplexan secretory organelles. Int J Parasitol 42**,** 1071-1081.

Guzman-Novoa, E., Eccles, L., Calvete, Y., Mcgowan, J., Kelly, P.G., Correa-Benitez, A., 2010. Varroa destructor is the main culprit for the death and reduced populations of overwintered honey bee (Apis mellifera) colonies in Ontario, Canada. Apidologie 41**,** 443-450.

Harpur, B.A., Minaei, S., Kent, C.F., Zayed, A., 2012. Management increases genetic diversity of honey bees via admixture. Molecular Ecology 21**,** 4414-4421.

Higes, M., Martin, R., Meana, A., 2006. Nosema ceranae, a new microsporidian parasite in honeybees in Europe. J Invertebr Pathol 92**,** 93-95.

Higes, M., Martin-Hernandez, R., Botias, C., Bailon, E.G., Gonzalez-Porto, A.V. and others, 2008. How natural infection by Nosema ceranae causes honeybee colony collapse. Environ Microbiol 10**,** 2659-2669.

Higes, M., Meana, A., Bartolome, C., Botias, C., Martin-Hernandez, R., 2013. Nosema ceranae (Microsporidia), a controversial 21st century honey bee pathogen. Env Microbiol Rep 5**,** 17-29.

Highfield, A.C., El, N.A., Mackinder, L.C., Noel, L.M., Hall, M.J. and others, 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Appl Environ Microb 75**,** 7212-7220.

Holden, C., 2006. Ecology. Report warns of looming pollination crisis in North America. Science 314**,** 397.

Honeybee Genome Sequencing Consortium, 2006. Insights into social insects from the genome of the honeybee Apis mellifera. Nature 443**,** 931-949.

Huang, W.F., Jiang, J.H., Chen, Y.W., Wang, C.H., 2007. A Nosema ceranae isolate from the honeybee Apis mellifera. Apidologie 38**,** 30-37.

Hunter, W., Ellis, J., vanEngelsdorp, D., Hayes, J., Westervelt, D. and others, 2010. Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (Apis mellifera, Hymenoptera: Apidae). Plos Pathog 6**,** e1001160.

Invernizzi, C., Abud, C., Tomasco, I.H., Harriet, J., Ramallo, G. and others, 2009. Presence of Nosema ceranae in honeybees (Apis mellifera) in Uruguay. J Invertebr Pathol 101**,** 150-153.

James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V. and others, 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443**,** 818-822.

Johnson, R.M., Dahlgren, L., Siegfried, B.D., Ellis, M.D., 2013. Acaricide, fungicide and drug interactions in honey bees (Apis mellifera). PLoS One 8**,** e54092.

Keeling, P.J., Fast, N.M., 2002. Microsporidia: biology and evolution of highly reduced intracellular parasites. Annu Rev Microbiol 56**,** 93-116.

Kingsolver, M.B., Huang, Z., Hardy, R.W., 2013. Insect antiviral innate immunity: pathways, effectors, and connections. Journal of Molecular Biology. 425**,** 4921-4936.

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A. and others, 2007. Widespread dispersal of the microsporidian Nosema ceranae, an emergent pathogen of the western honey bee, Apis mellifera. J Invertebr Pathol 96**,** 1-10.

Kojima, Y., Toki, T., Morimoto, T., Yoshiyama, M., Kimura, K., Kadowaki, T., 2011. Infestation of Japanese native honey bees by tracheal mite and virus from non-native European honey bees in Japan. Microbial Ecol 62**,** 895-906.

Kuchibhatla, D.B., Sherman, W.A., Chung, B.Y., Cook, S., Schneider, G. and others, 2014. Powerful sequence similarity search methods and in-depth manual analyses can identify remote homologs in many apparently "orphan" viral proteins. J Virol 88**,** 10-20.

Kuriyama, R., Besse, C., Geze, M., Omoto, C.K., Schrevel, J., 2005. Dynamic organization of microtubules and microtubule-organizing centers during the sexual phase of a parasitic protozoan, Lecudina tuzetae (Gregarine, Apicomplexa). Cell Motility and the Cytoskeleton 62**,** 195-209.

Langridge, D.F., Mcghee, R.B., 1967. Crithidia mellificae n sp: An acidophilic trypanosomatid of honey bee Apis mellifera. J Protozool 14**,** 485-&.

Lanzi, G., deMiranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A. and others, 2006. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). J. Virol. 80**,** 4998-5009.

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochemistry and Molecular Biology 32**,** 1295-1309.

Le Gall, O., Christian, P., Fauquet, C.M., King, A.M., Knowles, N.J. and others, 2008. Picornavirales, a proposed order of positive-sense single-stranded RNA viruses with a pseudo-T = 3 virion architecture. Arch Virol 153**,** 715-727.

Leat, N., Ball, B., Govan, V., Davison, S., 2000. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. J Gen Virol 81**,** 2111-2119.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K. and others, 2013. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 176**,** 232-240.

Li, J., Peng, W., Wu, J., Strange, J.P., Boncristiani, H., Chen, Y., 2011. Cross-species infection of Deformed Wing Virus poses a new threat to pollinator conservation. J Econ Entomol 104**,** 732-739.

Li, J.L., Chen, W.F., Wu, J., Peng, W.J., An, J.D. and others, 2012. Diversity of Nosema associated with bumblebees (Bombus spp.) from China. Int J Parasitol 42**,** 49-61.

Lighthart, B., Prier, K.R.S., Bromenshenk, J.J., 2005. Flying honey bees adsorb airborne viruses. Aerobiologia 21**,** 147-149.

Lipa, J.J., Triggiani, O., 1996. Apicystis gen nov and Apicystis bombi (Liu, Macfarlane & Pengelly) comb nov (Protozoa: Neogregarinida), a cosmopolitan parasite of Bombus and Apis (Hymenoptera: Apidae). Apidologie 27**,** 29-34.

Liu, S., Vijayendran, D., Carrillo-Tripp, J., Miller, W.A., Bonning, B.C., 2014. Analysis of new aphid lethal paralysis virus (ALPV) isolates suggests evolution of two ALPV species. J Gen Virol 95**,** 2809-2819.

Lommel, S.A., Morris, T.J., Pinnock, D.E., 1985. Characterization of nucleic acids associated with Arkansas bee virus. Intervirology 23**,** 199-207.

Lukes, J., Skalicky, T., Tyc, J., Votypka, J., Yurchenko, V., 2014. Evolution of parasitism in kinetoplastid flagellates. Molecular and Biochemical Parasitology 195**,** 115-122.

Macfarlane, R.P., Lipa, J.J., Liu, H.J., 1995. Bumble bee pathogens and internal enemies. Bee World 76**,** 130-148.

Maori, E., Lavi, S., Mozes-Koch, R., Gantman, Y., Peretz, Y. and others, 2007. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. J Gen Virol 88**,** 3428-3438.

Marringa, W.J., Krueger, M.J., Burritt, N.L., Burritt, J.B., 2014. Honey bee hemocyte profiling by flow cytometry. PLoS One 9**,** e108486.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E. and others, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336**,** 1304-1306.

Martin-Hernandez, R., Botias, C., Bailon, E.G., Martinez-Salvador, A., Prieto, L. and others, 2012. Microsporidia infecting Apis mellifera: coexistence or competition. Is Nosema ceranae replacing Nosema apis? Environ Microbiol 14**,** 2127-2138.

Martin-Hernandez, R., Botias, C., Barrios, L., Martinez-Salvador, A., Meana, A. and others, 2011. Comparison of the energetic stress associated with experimental Nosema ceranae and Nosema apis infection of honeybees (Apis mellifera). Parasitology Research 109**,** 605-612.

Martin-Hernandez, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailon, E., Higes, M., 2007. Outcome of colonization of Apis mellifera by Nosema ceranae. Appl Environ Microb 73**,** 6331-6338.

Martinson, V.G., Danforth, B.N., Minckley, R.L., Rueppell, O., Tingek, S., Moran, N.A., 2011. A simple and distinctive microbiota associated with honey bees and bumble bees. Molecular Ecology 20**,** 619-628.

Mattila, H.R., Seeley, T.D., 2007. Genetic diversity in honey bee colonies enhances productivity and fitness. Science 317**,** 362-364.

McFadden, G.I., 2011. The apicoplast. Protoplasma 248**,** 641-650.

McMullan, J.B., Brown, M.J.F., 2009. A qualitative model of mortality in honey bee (Apis mellifera) colonies infested with tracheal mites (Acarapis woodi). Exp Appl Acarol 47**,** 225-234.

Meeus, I., de Graaf, D.C., Jans, K., Smagghe, G., 2010. Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. Journal of Applied Microbiology 109**,** 107-115.

Meeus, I., Vercruysse, V., Smagghe, G., 2012. Molecular detection of Spiroplasma apis and Spiroplasma melliferum in bees. J Invertebr Pathol 109**,** 172-174.

Mondet, F., deMiranda, J.R., Kretzschmar, A., Le, C.Y., Mercer, A.R., 2014. On the front line: quantitative virus dynamics in honeybee (Apis mellifera L.) colonies along a new expansion front of the parasite Varroa destructor. Plos Pathog 10**,** e1004323.

Moore, J., Jironkin, A., Chandler, D., Burroughs, N., Evans, D.J., Ryabov, E.V., 2011. Recombinants between Deformed wing virus and Varroa destructor virus-1 may prevail in Varroa destructor-infested honeybee colonies. J Gen Virol 92**,** 156-161.

Moran, N.A., Hansen, A.K., Powell, J.E., Sabree, Z.L., 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. PLoS One 7**,** e36393.

Mouches, C., Bove, J.M., Albisetti, J., 1984. Pathogenicity of Spiroplasma apis and other spiroplasmas for honeybees in Southwestern France. Ann Inst Pasteur Mic A135**,** 151-155.

Mouches, C., Bove, J.M., Albisetti, J., Clark, T.B., Tully, J.G., 1982. A spiroplasma of serogroup-IV causes a May-disease-like disorder of honeybees in Southwestern France. Microbial Ecol 8**,** 387-399.

Mouches, C., Bove, J.M., Tully, J.G., Rose, D.L., Mccoy, R.E. and others, 1983. Spiroplasma apis, a new species from the honeybee Apis mellifera. Ann Inst Pasteur Mic A134**,** 383-397.

Navajas, M., Migeon, A., Alaux, C., Martin-Magniette, M., Robinson, G. and others, 2008. Differential gene expression of the honey bee Apis mellifera associated with Varroa destructor infection. BMC Genomics 9**,** 301.

Nguyen, B.K., Ribiere, M., vanEngelsdorp, D., Snoeck, C., Saegerman, C. and others, 2011. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res 50**,** 195-202.

Nguyen, N.T., Le, T.H., 2013. Complete genome sequence of Sacbrood Virus strain SBM2, isolated from the honeybee Apis cerana in Vietnam. Genome Announcements 1.

Olivier, V., Blanchard, P., Chaouch, S., Lallemand, P., Schurr, F. and others, 2008. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus Res 132**,** 59-68.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. J Gen Virol 85**,** 3747-3755.

Otis, G.W., Scott-Dupree, C.D., 1992. Effects of Acarapis woodi on overwintered colonies of honey bees (Hymenoptera, Apidae) in New York. J. Econ. Entomol. 85**,** 40-46.

Palacios, G., Hui, J., Quan, P.L., Kalkstein, A., Honkavuori, K.S. and others, 2008. Genetic analysis of Israel acute paralysis virus: distinct clusters are circulating in the United States. J Virol 82**,** 6209-6217.

Paxton, R.J., Klee, J., Korpela, S., Fries, I., 2007. Nosema ceranae has infected Apis mellifera in Europe since at least 1998 and may be more virulent than Nosema apis. Apidologie 38**,** 558-565.

Peng, W.J., Li, J.L., Boncristiani, H., Strange, J.P., Hamilton, M., Chen, Y.P., 2011. Host range expansion of honey bee Black Queen Cell Virus in the bumble bee, Bombus huntii. Apidologie 42**,** 650-658.

Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, Apis mellifera. Apidologie 35**,** 605-610.

Plischuk, S., Martin-Hernandez, R., Prieto, L., Lucia, M., Botias, C. and others, 2009. South American native bumblebees (Hymenoptera: Apidae) infected by Nosema ceranae (Microsporidia), an emerging pathogen of honeybees (Apis mellifera). Env Microbiol Rep 1**,** 131-135.

Plischuk, S., Meeus, I., Smagghe, G., Lange, C.E., 2011. Apicystis bombi (Apicomplexa: Neogregarinorida) parasitizing Apis mellifera and Bombus terrestris (Hymenoptera: Apidae) in Argentina. Env Microbiol Rep 3**,** 565-568.

Potts, S.G., Roberts, S.P.M., Dean, R., Marris, G., Brown, M.A. and others, 2010. Declines of managed honey bees and beekeepers in Europe. J Apicult Res 49**,** 15-22.

Reybroeck, W., Daeseleire, E., De Brabander, H.F., Herman, L., 2012. Antimicrobials in beekeeping. Vet Microbiol 158**,** 1-11.

Ribiere, M., Ball, B., Aubert, M., 2008. Natural history and geographical distribution of honey bee viruses. In: Aubert, M., Ball, B., Fries, I., Moritz, R., Milani, N., Bernardinelli, I. (Eds.), Virology and the Honey Bee Office for Official Publications of the European Communities, Luxembourg, pp. 15-84.

Ribiere, M., Olivier, V., Blanchard, P., 2010. Chronic bee paralysis: a disease and a virus like no other? J Invertebr Pathol 103 Suppl 1**,** S120-S131.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. J Invertebr Pathol 103 Suppl 1**,** S96-119.

Runckel, C., DeRisi, J., Flenniken, M.L., 2014. A draft genome of the honey bee trypanosomatid parasite Crithidia mellificae. PLoS One 9**,** e95057.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Rutrecht, S.T., Brown, M.J.F., 2008. The life-history impact and implications of multiple parasites for bumble bee queens. Int J Parasitol 38**,** 799-808.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C. and others, 2014. A virulent strain of Deformed Wing Virus (DWV) of honeybees (Apis mellifera) prevails after Varroa destructor-mediated, or in vitro, transmission. Plos Pathog 10**,** e1004230.

Sabin, L.R., Hanna, S.L., Cherry, S., 2010. Innate antiviral immunity in Drosophila. Current Opinion in Immunology 22**,** 4-9.

Sammataro, D., de Guzman, L., George, S., Ochoa, R., Otis, G., 2013. Standard methods for tracheal mite research. J Apicult Res 52.

Schafer, M.O., Ritter, W., Pettis, J.S., Neumann, P., 2010. Winter losses of honeybee colonies (Hymenoptera: Apidae): The role of infestations aith Aethina tumida (Coleoptera: Nitidulidae) and Varroa destructor (Parasitiformes: Varroidae). J Econ Entomol 103**,** 10-16.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. J Eukaryot Microbiol 57**,** 337-345.

Schwarz, R.S., Evans, J.D., 2013. Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. Dev Comp Immunol 40**,** 300-310.

Shen, M., Cui, L., Ostiguy, N., Cox-Foster, D., 2005. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. J Gen Virol 86**,** 2281-2289.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA Viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5.

Sitaropoulou, N., Neophytou, E.P., Thomopoulos, G.N., 1989. Structure of the nucleocapsid of a filamentous virus of the honey bee (Apis mellifera). J Invertebr Pathol 53**,** 354-357.

Stokstad, E., 2007. Entomology. The case of the empty hives. Science 316**,** 970-972.

Suwannapong, G., Yemor, T., Boonpakdee, C., Benbow, M.E., 2011. Nosema ceranae, a new parasite in Thai honeybees. J Invertebr Pathol 106**,** 236-241.

Tarpy, D.R., 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proceedings of the Royal Society B: Biological Sciences 270**,** 99-103.

Teixeira, E.W., Santos, L.G., Sattler, A., Message, D., Alves, M.L. and others, 2013. Nosema ceranae has been present in Brazil for more than three decades infecting Africanized honey bees. J Invertebr Pathol 114**,** 250-254.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F. and others, 2004. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl Environ Microb 70**,** 7185-7191.

Underwood, R., vanEngelsdorp, D., 2007. Colony Collapse Disorder: Have we seen this before? Bee Culture 135**,** 13-18.

van Dooremalen, C., Gerritsen, L., Cornelissen, B., van der Steen, J.J.M., van Langevelde, F., Blacquiere, T., 2012. Winter survival of individual honey bees and honey bee colonies depends on tevel of Varroa destructor infestation. PLoS One 7**,** e36285.

van Munster, M., Dullemans, A.M., Verbeek, M., van den Heuvel, J.F.J.M., Clerivet, A., van der Wilk, F., 2002. Sequence analysis and genomic organization of Aphid lethal paralysis virus: a new member of the family Dicistroviridae. J Gen Virol 83**,** 3131-3138.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E. and others, 2009. Colony Collapse Disorder: A descriptive study. PLoS One 4**,** e6481.

vanEngelsdorp, D., Hayes, J., Jr., Underwood, R.M., Pettis, J., 2008. A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLoS One 3**,** e4071.

Wallace, F.G., 1966. The trypanosomatid parasites of insects and arachnids. Experimental Parasitology 18**,** 124-193.

Wang, H., Xie, J., Shreeve, T.G., Ma, J., Pallett, D.W. and others, 2013. Sequence recombination and conservation of Varroa destructor virus-1 and deformed wing virus in field collected honey bees (Apis mellifera). PLoS One 8**,** e74508.

Williams, G.R., Shafer, A.B., Rogers, R.E., Shutler, D., Stewart, D.T., 2008. First detection of Nosema ceranae, a microsporidian parasite of European honey bees (Apis mellifera), in Canada and central USA. J Invertebr Pathol 97**,** 189-192.

Wu, J.Y., Anelli, C.M., Sheppard, W.S., 2011. Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity. PLoS One 6**,** e14720.

Wu, J.Y., Smart, M.D., Anelli, C.M., Sheppard, W.S., 2012. Honey bees (Apis mellifera) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to Nosema (Microsporidia) infection. J Invertebr Pathol 109**,** 326-329.

Yang, X.L., Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. P Natl Acad Sci USA 102**,** 7470-7475.

Zambon, R.A., Nandakumar, M., Vakharia, V.N., Wu, L.P., 2005. The Toll pathway is important for an antiviral response in Drosophila. P Natl Acad Sci USA 102**,** 7257-7262.

Zioni, N., Soroker, V., Chejanovsky, N., 2011. Replication of Varroa destructor Virus 1 (VDV-1) and a Varroa destructor Virus 1-deformed wing virus recombinant (VDV-1-DWV) in the head of the honey bee. Virology 417**,** 106-112.

CHAPTER 2:

OBJECTIVES

The Laboratory of Molecular Entomology and Bee Pathology focuses on three research themes. The first is the study on the function and composition of venoms of bees and wasps. The second concentrates on the pathology of the honeybee due to viral, bacterial and parasitic agents and the last topic focuses on cellular and humoral immunity of insects. The study of honey bee pathology was launched more than ten years ago. This resulted in an important progress about our knowledge of American foulbrood, such as the taxonomy and diagnosis. Recently that research pillar was further extended to study the drivers that are responsible for the decline of honey bee colonies, and research-based corrective measurements for the beekeeping sector. This PhD is mainly situated in that last topic.

This PhD thesis had two general objectives. The first objective is to perform a comprehensive survey of honey bee pathogens in Flanders. The screened pathosphere included not only viruses, but also bacteria, protists and fungi. Secondly, we further explored two poorly studied pathogens, Lake Sinai Virus and *Crithidia mellificae*. The results addressing these topics are described in the following chapters, which were assembled in two parts.

The objective of **part 1** was the screening of numerous honey bee pathogens. In **chapter 3** we developed the BeeDoctor, a MLPA-based tool, that allowed us to screen for ten different honey bee viruses in one reaction. This BeeDoctor tool was used to survey the virus load in more than 360 honey bee colonies. The same sample set was more extensively studied in **chapter 4** to survey a parasitic fly (*Apocephalus borealis*), two well known microsoporidian parasites (*Nosema apis* and *Nosema ceranae*), newly described viruses (Aphid Lethal Paralysis Virus strain Brookings, Big Sioux River Virus, Lake Sinai Virus and *Varroa destructor* Macula-like Virus), neglected protists (*Apicystis*

bombi and *Crithidia mellificae*) and two infectious bacteria (*Spiroplasma apis* and *Spiroplasma melliferum*). These studies brought new insights in the Belgian honey bee pathosphere. We this knowlegede, we investigated their occurrence in solitary bees which is described in **chapter 5** while in **chapter 6** the vertical transmission of honey bee viruses was explored.

The aim of **part 2** was the in-depth characterization of some selected honey bee parasites. In **chapter 7** we investigated the diversity of trypanosomatids present in honey bees by genetic characterization, phylogenetics and ultrastructural analyses, which eventually led to the description of a novel species. Since no convenient identification method was available, high resolution melting and fragment length polymorphism were evaluated in **chapter 8** to identify these trypanosomatids without the need for sequencing. Finally, the objective of **chapter 9** was to gain insights in the diversity of LSV strains present in Belgium by genome sequencing, phylogenetics and negative strand detection.

PART II

SCREENING OF HONEY BEE PATHOGENS

CHAPTER 3

BEEDOCTOR, A VERSATILE MLPA-BASED DIAGNOSTIC TOOL FOR SCREENING BEE VIRUSES

The work presented in Chapter 3 was adapted from the following manuscript:

L. De Smet, J. Ravoet, J.R. deMiranda, T. Wenseleers, M.Y. Mueller, R.F.A. Moritz, D.C. de Graaf. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. Plos ONE, 2012, 7(10): e47953. doi: 10.1371/journal.pone.0047953.

Contributions

R.F.A. Moritz and D.C. de Graaf assisted with the study design. L. De Smet optimized the MLPA technique to detect honey bee viruses by designing RT-primers, probes and constructing of a specific ladder. J. Ravoet extracted the viral RNA and conducted the virus survey. J.R. deMiranda performed the association analyses. M.Y. Mueller analyzed the MLPA amplicons using the QIAxcel platform. The manuscript was written by L. De Smet and was assisted by the co-authors through the writing phase.

1. Abstract

The long-term decline of managed honey bee hives in the world has drawn significant attention to the scientific community and bee-keeping industry. A high pathogen load is believed to play a crucial role in this phenomenon, with the bee viruses being key players. Most of the currently characterized honey bee viruses (around twenty) are positive stranded RNA viruses. Techniques based on RNA signatures are widely used to determine the viral load in honey bee colonies. High throughput screening for viral loads necessitates the development of a multiplex polymerase chain reaction approach in which different viruses can be targeted simultaneously. A new multiparameter assay, called "BeeDoctor", was developed based on multiplex ligation-dependent probe amplification (MLPA) technology. This assay detects 10 honey bee viruses in one reaction. "BeeDoctor" is also able to screen selectively for either the positive strand of the targeted RNA bee viruses or the negative strand, which is indicative for active viral replication. Due to its sensitivity and specificity, the MLPA assay is a useful tool for rapid diagnosis, pathogen characterization, and epidemiology of viruses in honey bee populations. "BeeDoctor" was used for screening 363 samples from apiaries located throughout Flanders; the northern half of Belgium. Using "BeeDoctor", virus infections were detected in almost eighty percent of the colonies, with Deformed Wing Virus by far the most frequently detected virus and multiple virus infections were found in 26 percent of the colonies.

2. Introduction

Honey bees provide both honey and key pollination services to much of the world (Aizen and Harder, 2009; Bos et al., 2007; Garibaldi et al., 2011). Overall pollinator populations, including wild and feral honey bee (*Apis mellifera*) populations, have been declining consistently worldwide due to a variety of causes (Biesmeijer et al., 2006). Annual losses of managed honey bee populations have also increased significantly during the last decades, highlighted by the recent dramatic mass colony losses in the USA due to Colony Collapse Disorder (CCD; (VanEngelsdorp et al., 2009)), as well as increased winter colony losses and reduced honey bee and queen vitality, largely due to pathogens and

parasites (VanEngelsdorp and Meixner, 2010), including mites (*Varroa destructor*, *Acarapis woodi*, *Tropilaelaps* spp.), microsporidia (*Nosema* spp.), fungi (chalkbrood; *Ascosphaera apis*), bacteria (American foulbrood (*Paenibacillus larvae*), European foulbrood (*Melissococcus plutonius*)), viruses, and pests (large wax moth *Galleria melonella*, small hive beetle *Aethina tumidae*) (Evans and Schwarz, 2011; Genersch, 2010).

Recently the role of pathogenic viruses gained particular interest since they have been suspected to be important drivers of colony declines. Honey bees are host to between 12-20 viruses, depending on classification (Bailey L and Ball BV, 1991), most of which are positive strand RNA viruses belonging to the order Picornavirales (Mayo, 2002). Black Queen Cell Virus (BQCV) (Leat et al., 2000) and the Acute Bee Paralysis Virus complex (deMiranda et al., 2010a), which includes Acute Bee Paralysis Virus (ABPV) (Govan et al., 2000), Kashmir Bee Virus (KBV) (deMiranda et al., 2004) and Israeli Acute Paralysis Virus (IAPV) (Maori et al., 2007), belong to the Dicistroviridae family. Deformed Wing Virus (DWV) (Lanzi et al., 2006) and its close relatives *Varroa destructor* Virus-1 (VDV-1) (Ongus et al., 2004)) and Kakugo Virus (KV) (Fujiyuki et al., 2004), Slow Bee Paralysis Virus (SBPV) (deMiranda et al., 2010b) and Sacbrood Virus (SBV) (Ghosh et al., 1999) belong to the Iflaviridae family while Chronic Bee Paralysis Virus (CBPV) (Olivier et al., 2008) is related to the Nodaviridae (Runckel et al., 2011). Several other known viruses, including Bee Virus X and Y (BVX; BVY), Cloudy Wing Virus (CWV), *Apis mellifera* Filamentous Virus (*Am*FV), *Apis* Iridescent Virus (AIV); Arkansas Bee Virus (ABV) and Berkeley Bee Picorna-like Virus (BBPV) remain to be fully characterized molecularly (deMiranda et al., 2012), while on the other hand next-generation sequencing techniques have identified several novel viruses (some of which may be the same as the uncharacterized viruses named above) and microbes (Cox-Foster et al., 2007; Runckel et al., 2011; Singh et al., 2010) through which the honey bee pathosphere has been expanded and this is likely to continue in the near future. Symptoms associated with specific viruses include wing deformities (DWV), hairless, dark, shiny bees (CBPV), swollen yellow larvae and/or dark-brown larva carcasses in the cells of worker-bees (SBV) or in queen cells (BQCV). Many virus infections also cause behavioural aberrations, such as shivering, paralysis,

disorientation, aggression or altered foraging preferences or changes in brood care (Aubert, 2008). The appearance of symptoms generally requires high virus titres; the result of close transmission within the colony. Most commonly however, viral infections in honey bees are low-medium titre and thus asymptomatic. An accurate diagnosis of such asymptomatic virus infections therefore requires molecular techniques.

The detection of viral infections in honey bees is increasingly based on the detection of specific viral genomic nucleic acids. Since most (honey bee) viruses have RNA genomes, this means the detection of virus-specific RNA signatures. The most widely used method is reverse transcriptase quantitative PCR (RT-qPCR). Many individual RT-(q)PCR protocols have been described for the detection of specific honey bee viruses (review (deMiranda, 2008)) as well as several multiplex RT-PCR approaches (Chen et al., 2004; Grabensteiner et al., 2007; Meeus et al., 2010). Multiplex detection approaches, where several targets are detected and quantified simultaneously, are increasingly important, both for reducing costs and more importantly for studying the complex interactions between different targets, which can include important host genes as well as RNA-based pathogens. However, the optimization of multiplex RT-PCR can pose several difficulties, including poor sensitivity and specificity, and/or preferential amplification of certain specific targets (Markoulatos et al., 2002). Furthermore, real-time multiplex assays are mostly restricted to detection of up to four or five targets in a reaction, depending on the number of channels available in the used PCR machine. Multiplex Ligation-dependent Probe Amplification (MLPA) is an amplification technique that allows simultaneous detection of up to 45 different targets with the use of a single primer set (Schouten et al., 2002). MLPA is based on the ligation of two adjacent oligonucleotides hybridizing next to each other on a single-stranded target template. The ligated oligonucleotides (‘probe’) serve as template for PCR-based amplification and detection. Apart from virus-specific sequences, each oligonucleotide (‘half-probe’) contains a universal tag, for simultaneous PCR-based amplification of multiple targets with a single PCR primer pair, and a non-specific stuffer fragment for generating controlled size differences between different targets. The different targets are identified

by size using electrophoresis. Because honey bee viruses are RNA viruses, a reverse transcription step is added prior to MLPA (RT-MLPA). MLPA and RT-MLPA assays have recently been developed for the simultaneous detection of several virus species causing central nervous system infections (Wolffs et al., 2009). Another application called RespiFinder™ tests differentially for fifteen respiratory viruses (Reijans et al., 2008). MLPA is also used for the detection of other pathogens like *Mycobacterium tuberculosis (Bergval et al., 2008)*, bacterial species in oral biofilms (Terefework et al., 2008), *Penicillium marneffei* (Zhang et al., 2011) and different opisthorchid liver fluke species (Sun et al., 2011).

Replication in positive-strand RNA viruses, such as many honey bee viruses, proceeds via the production of a negative-strand intermediate. Strand-specific RT-PCR was first developed for detection of negative-strand RNAs of viruses (Gisder et al., 2009; Yue and Genersch, 2005). However, strand-specific RT-PCR is very sensitive to false-positive results, primarily due to mis-priming and self-priming of the RNA during reverse transcription (Haddad et al., 2007). These inadequacies have been addressed with a combination of additional steps, primarily by using tagged cDNA primers and purifying the cDNA from residual primer prior to PCR amplification (Boncristiani, Jr. et al., 2009; Boncristiani et al., 2009). The RT-MLPA is ideal for strand-specific detection of nucleic acids since it amplifies a probe (rather than the original target) that can only be produced in a strand-specific manner, through ligation of two oligonucleotide half-probes hybridizing to a complementary cDNA target. The ligase-65 used to ligate the two half-probes to each other is not active on RNA-DNA hybrids, thus avoiding possible false-positive results due to ligation of the half-probes that hybridize directly on target RNA of the same polarity as the cDNA to the opposite strand.

Our report here shows the application of RT-MLPA for simultaneously detecting 10 targeted honey bee viruses. Two MLPA probe sets were developed which are able to detect selectively the positive strand RNA or the replicative negative strand RNA intermediate. The possibility to screen easily for replication will be valuable for studying virus replication and pathogenesis in naturally infected hosts. Because of its high sensitivity and specificity, the RT-MLPA assay is also a useful tool

for prompt diagnosis and epidemiological studies of viruses in honey bee populations. At last, we used this newly developed method in an epidemiological survey of honey bee viruses based on adult bee samples collected in Flanders during the summer of 2011.

3. Material and Methods

3.1 Samples

All Flemish beekeepers were invited to participate in an epidemiological survey for virus screening; 170 beekeepers accepted the invitation and submitted a total of 363 samples of 30 adult bees, collected in July 2011 at the entrance of colonies that seemed healthy and that were not (yet) treated against *Varroa*. Generally, each beekeeper sent two samples from their apiary. The bees were immediately frozen at -20°C until their shipment to the laboratory, where upon arrival they were stored at -80°C until RNA extraction. Excess bees were archived for long-term -80°C storage.

3.2 Nucleic acid extraction

To detect the positive strand viral RNA was isolated by using the QiaAmp Viral RNA mini kit (Qiagen). Individual whole adult bees were ground in a mortar in 1ml ice-cold PBS per bee. The extract was centrifuged at 14 000 x g and RNA was extracted from 140 µl of the liquid supernatant according to the manufacturer's instructions, eluting the RNA in a final volume of 50 µl. In the negative strand detection mode the total RNA was isolated using the RNeasy lipid tissue mini kit (Qiagen) starting from one complete honey bee. The tissue was homogenized by mechanical agitation in a TissueLyser (Precellys) for 90 sec at 30Hz, in the presence of a pair stainless steel beads and 1ml QIAzol lysis reagent. The total RNA was isolated according to the recommendation of the manufacturer's protocol, eluting the RNA in a final volume of 50 µl. These RNA samples from individual bees were solely used to develop the MLPA analysis tool for positive- and negative-strand detection of multiple honey bee viruses.

For the Flanders virus survey, 10 bees per colony were homogenized in a total of 5 ml PBS by mechanical agitation in a TissueLyser for 90 sec at 30Hz, in the presence of glass beads.

Table 1. Primers and half-probes used for detecting either the positive or negative (replicative) strand of different honey bee viruses and virus species complexes through RT-MLPA. The PCR sequence tags on each halfprobe are in lower-case letters, the non-specific stuffer sequences (for generating PCR products with pre-determined sizes) are shown in upper-case letters and the target-specific sequences are shown in underlined upper-case letters. Each RPO probe is 5'-phosphorylated (indicated by $^{P-}$) to permit ligation of the 5' end of the RPO to the 3' end of the LPO.

VIRUS	STRAND	FUNCTION	SEQUENCE (5'-3')	SIZE (bp)
CBPV	**+ (pos)**	(-)cDNA	GCCCCGATCATATAAGCAAA	88
		(+)MLPA-LPO	gggttccctaagggttggaCCGTAGCTGTTTCTGCTGCGGT	
		(+)MLPA-RPO	$^{P-}$ACTCAGCTCAGCTCGACGCTCAGAtctagattggatcttgctggcac	
	- (neg)	(+)cDNA	GAACATCCGGAACAGACGAT	88
		(-)MLPA-LPO	gggttccctaagggttggaTCTGAGCGTCGAGCTGAGCTGAGT	
		(-)MLPA-RPO	$^{P-}$ACCGCAGCAGAAACAGCTACGGtctagattggatcttgctggcac	
DWV/KV VDV-1	**+ (pos)**	(-)cDNA	TCACATTGATCCCAATAATCAGA	95
		(+)MLPA-LPO	gggttccctaagggttggaTGACCGATTCTTTATGCAGCGAGCTCT	
		(+)MLPA-RPO	$^{P-}$TACGTGCGAGTCGTACTCCTGTGACAtctagattggatcttgctggcac	
	- (neg)	(+)cDNA	GTGTGGTGCATCTGGAATTG	95
		(-)MLPA-LPO	gggttccctaagggttggaGTTGTCACAGGAGTACGACTCGCA	
		(-)MLPA-RPO	$^{P-}$CGTAAGAGCTCGCTGCATAAAGAATCGGTtctagattggatcttgctggcac	
ABPV KBV IAPV	**+ (pos)**	(-)cDNA (ABPV)	CAATGTGGTCAATGAGTACGG	104
		(-)cDNA (KBV&IAPV)	TCAATGTTGTCAATGAGAACGG	
		(+)MLPA-LPO	gggttccctaagggttggaCTCACTTCATCGGCTCGGAGCATGGATGAT	
		(+)MLPA-RPO	$^{P-}$ACGCACAGTATTATTCAGTTTTTACAACGCCCtctagattggatcttgctggcac	
	- (neg)	(+)cDNA	TGAAACGGAACAAATCACCA	104
		(-)MLPA-LPO	gggttccctaagggttggaCGAGCCGATGAAGTGTCTTGAGCCATGG	
		(-)MLPA-RPO	$^{P-}$GGGTATTGATCCTATTTGGAGTTTCCACATCATGtctagattggatcttgctggcac	
BQCV	**+ (pos)**	(-)cDNA	CGGGCCTCGGATAATTAGA	122
		(+)MLPA-LPO	gggttccctaagggttggaCTTCATGTTGGAGACCAGGTTTGTTTGCCGACTTACGGAA	
		(+)MLPA-RPO	$^{P-}$TGTCGTTAAACTCTAGGCTTTCCGGATGGCTTCTTCATGGtctagattggatcttgctggcac	

	- (neg)	(+)cDNA	TTAAAAGCCCCGTATGCTTG	122
		(-)MLPA-LPO	gggttccctaagggttggaTCAGCGCAACAGAAGCCATCCGGAAAGCCTAGAGTTTAACG	
		(-)MLPA-RPO	P-ACATTCCGTAAGTCGGCAAACAAACCTGCCTTATCTGGTtctagattggatcttgctggcac	
SBPV	**+ (pos)**	(-)cDNA	CGCAAACACGACGAATTTTA	131
	+ (pos)	(+)MLPA-LPO	gggttccctaagggttggaCGTTCAATGGTCGAGATAGAAGCCACAGTAGAAGTATTACGCGCT	
		(+)MLPA-RPO	P-TCTTGTGTTTTGGCTTATGGGCGTGGGCCTGATCTTCATTCAGCtctagattggatcttgctggcac	
	- (neg)	(+)cDNA	GGTGTCATAAACAGAATGACGAG	131
		(-)MLPA-LPO	gggttccctaagggttggaTCAGCGCAACACTCAGGCCCACGCCCATAAGCCAAAACACAAGAA	
		(-)MLPA-RPO	P-GCGCGTAATACTTCTACTGTGGCTTCTATCTCGCCTTATCTGGTtctagattggatcttgctggcac	
SBV	**+ (pos)**	(-)cDNA	TGGACATTTCGGTGTAGTGG	140
		(+)MLPA-LPO	gggttccctaagggttggaCGTTGATCCAATGGTCAGTGGACTCTTATACCGATTTGTTTAATGGTTGG	
		(+)MLPA-RPO	P-GTTTCTGGTATGTTTGTTGACAAGAACGTCCACCTTCAGCCATTCAGCtctagattggatcttgctggcac	
	- (neg)	(+)cDNA	CCTTACCTCTAGTAAGAAGACATTTGA	140
		(-)MLPA-LPO	gggttccctaagggttggaTAAAAAACTACCGTGTAGTGGACGTTCTTGTCAACAAACATACCAGAAA	
		(-)MLPA-RPO	P-CCCAACCATTAAACAAATCGGTATAAGAGTCCACTGAAAAGTCGGTGGAtctagattggatcttgctggcac	
β-Actin	**+ (pos)**	(-)cDNA	TTTCATGGTGGATGGTGCTA	182
		(+)MLPA-LPO	gggttccctaagggttggaGCAGGAAGTCGTTACCACCTGGCCCACGGAGCCAATTTCTCATGCTTGCCAACACTGTCCTTTCTGGAGGT	
		(+)MLPA-RPO	P-ACCACCATGTATCCTGGAATCGCGAAAACGTGGTGTACCGGCTGTCTGGTATGTATGAGTTTGTGGTGAtctagattggatcttgctggcac	
RPL8	**+ (pos)**	(-)cDNA	TGCTTTACCAATATGTTGATGATT	168
		(+)MLPA-LPO	gggttccctaagggttggaTCGGTGAGACGTGGGAGGCGAAAATTGGCGTGTTGGCCTAAGGTTCGTGGTGTTGCTATGAAC	
		(+)MLPA-RPO	P-CCTGTTGAACATCCACACGGTGGTGGTAATCATAACGTCCGGATGCTGAAGTGATGGCAGAGCtctagattggatcttgctggcac	
PCR		PCR-Forward	Gggttccctaagggttgga	n.a.
		PCR-Reverse	Gtgccagcaagatccaatctaga	

The extract was centrifuged at 14 000 x g and RNA was extracted from 140 µl of the liquid supernatant using the QiaAmp Viral RNA mini kit according to the manufacturer's instructions, as outlined above.

3.3 Probe design

MLPA probes and RT-primers were designed for 6 virus targets, covering the 10 most common honey bee viruses, and for two honey bee internal reference genes; β-actin and ribosomal protein 8 (RPL8), as positive controls for the quality of the RNA samples. For each virus or virus-complex a pair of probes was designed following the guidelines described in the manual "Design synthetic MLPA probes" (MRC Holland, Amsterdam, The Netherlands). All probe pairs contain the same universal binding sites for the reverse PCR primer on the right probe oligo (RPO) and for the forward PCR primer on the left probe oligo (LPO). The probes were designed using the AlleleID software (PREMIER Biosoft) against the most conserved regions within each virus or virus family as determined by aligning all available gene sequences in the Genbank using Clustal X program. An additional selection criterion was the absence of mismatches within 5 nucleotides from the ligation site. The uniqueness of our selected probe sequences was inspected by BLAST analysis at the NCBI website (www.ncbi.nlm.nih.gov). The primers for cDNA synthesis were positioned immediately adjacent to the MLPA probe, with no more than 15 nucleotides between the last nucleotide of the RT primer and the first nucleotide of the probe sequence, and with a maximum overlap of 7 nucleotides. The RT primers were designed with Primer 3 software (http://primer3.sourceforge.net). All primers and probes were synthesized by Integrated DNA Technologies (Leuven, Belgium). The RPO should be 5' phosphorylated and synthesized with 'ultramers' quality. The RT-primers, MLPA half-probes (LPO and RPO) and PCR amplification primers used in the experiments are listed in Table 1.

3.4 MLPA reaction

MLPA analysis was performed essentially as described earlier (Schouten et al., 2002). All the MLPA reagents were obtained from MRC-Holland (Amsterdam, the Netherlands). All reaction steps were performed in a thermocycler with heated lid (105°C) using 0.2 ml thin-walled PCR tubes. Briefly, 1 µl

RNA (between 10 – 500 ng total RNA), unless otherwise mentioned, was reverse transcribed using 30 U MMLV reverse transcriptase (Promega) in a 6 µl reaction with 0.5 µl RT primer/dNTP mix consisting of 5 pmol/µl of the RT primer for each target virus and 5 mM dNTPs. After 1 min 80°C and 5 min 45°C the reverse transcriptase was added to reaction and was incubated for 15 min at 37°C and deactivated for 2 min at 98°C. A probe mix containing all half-probe oligos for either positive–strand detection or negative-strand detection was prepared containing 1.33 fmol/µl of each oligo.

A mixture of 1.5 µl of the probe-mix and 1.5 µl of MLPA buffer was added to each RT reaction and hybridized overnight at 60°C after 1 min denaturation at 95°C. The hybridized probes were ligated together using the Ligase-65 enzyme in a 40 µl reaction at 54°C for 15 min followed by ligase inactivation at 98°C for 5 min. Subsequently, 10 µl of the ligation reaction was used as template for the PCR reaction, using the universal forward and reverse PCR primers (Table 1) in a total reaction volume of 50 µl. PCR amplification was performed for 35 cycles (30 s – 95°C, 30 s – 55°C and 1 min – 72°C) with a final extension step at 72°C for 20 min.

3.5 Analysis of PCR products

The amplified MLPA products were analyzed on different detection platforms. The MLPA was optimized by analysis 10 µl of the MLPA reaction on 4% high resolution agarose gel electrophoresis. As alternative an aliquot of 10 µl was also analysed via capillary electrophoresis using a High Resolution gel cartridge on a QIAxcel platform (Qiagen, Hilden, Germany). For the Flanders virus survey study the MLPA reactions were analyzed using 4% high resolution agarose gel electrophoresis.

3.6 Cloning and construction of specific MLPA ladder

Fragments generated by the RT-MLPA reactions were desalted with MSB Spin PCRapace (Invitek) and subsequently cloned into pCR4-TOPO vector from TOPO TA Cloning Kit for sequencing (Invitrogen, USA) according to manufacturer's instructions. The cloned inserts were sequenced on a ABI 3130XL platform using the vector primers. Positive constructs were used as template in a standard PCR reaction to amplify the expected MLPA reaction products. The concentrations of the different products were determined using a nanodrop ND-1000 spectrophotometer (Thermo Scientific). The

different products were mixed in equal amounts and 10 ng of each fragment was loaded on a gel as marker to simplify the interpretation of the results.

4. Results and discussion

The MLPA is a popular technique in the human genetics but, according to our knowledge, it has not yet been used in the field of veterinary virology. The multiplexing capacity of the technique is much higher than for PCR assays but far below the capability of microarrays. This medium-scale (1-40-fold) multiplexing ability makes MLPA extremely useful for the simultaneous screening of all honey bee viruses and its simplicity can facilitate widespread acceptance of the technique even in small size molecular laboratories.

We designed an RT-MLPA approach to detect 6 targets simultaneously covering 10 common honey bee viruses: ABPV, BQCV, IAPV, KBV, DWV, KV, VDV-1, SBPV, SBV and CBPV. We opted for detection by agarose gel electrophoresis, although this part of the protocol can be easily transferred to other platforms, such as capillary electrophoresis or the Agilent Bioanalyzer. A spacer in the RT-MLPA probes was included to adjust the final length of the specific RT-MLPA products so that they are separated by 7-9 nucleotide increments, for unambiguous identification of the fragments after electrophoresis (Figure 1A). For DWV, KV and VDV-1 we were not able to design specific probes as their sequences are too related and therefore a consensus probe set was developed for the entire DWV-complex. Similarly, a single consensus probe set was developed for the ABPV-complex of viruses (ABPV, KBV and IAPV). An overview of the probes is given in Table 1.

The diagnostic capacity of the RT-MLPA-assay was tested on (RT-PCR) proven virus positive samples (Figure 1A). All amplicons were cloned and sequenced to confirm their identity. The specificity of the primers and the probes were tested by running all RT primers and MLPA probes in either a monoplex or a multiplex MLPA reaction with different samples. There was no cross-reactivity among the different probes and/or primers.

We constructed an MLPA ladder by mixing equimolar amounts from the different expected RT-MLPA amplicons each corresponding to a specific virus. These were amplified in a PCR reaction using the different pCR4-cloned RT-MLPA amplicons as templates. The use of this marker greatly facilitates the interpretation of the MLPA results after electrophoresis (Figure 1A). The sensitivity of the MLPA was tested using synthetic templates for DWV and BQCV. A serial dilution of these templates in total RNA from a non-infected honey bee showed that as few as 1000 copies can be detected with clearly discernible signals.

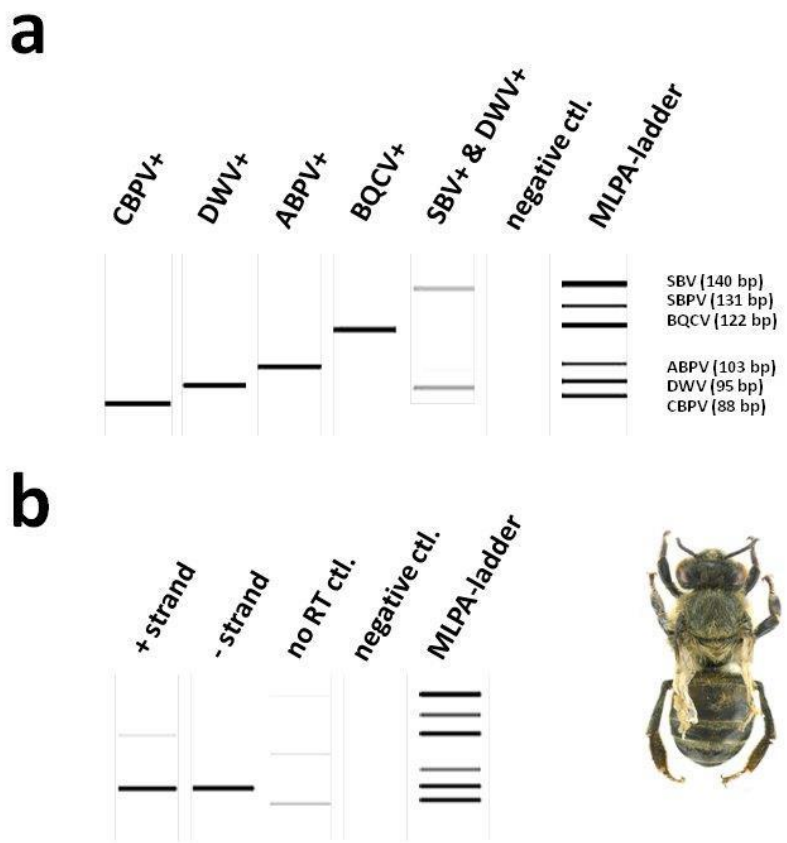


Figure 1. *High resolution analysis of MLPA amplicons using the Qiaxcel platform.* ***A*** *The result of a MLPA reaction on different samples from which the status was determined by RT-PCR. The status is indicated on top of each lane. The MLPA amplicons were analyzed via capillary electrophoresis using a High Resolution gel cartridge on a QIAxcel platform. Different amplicons of the MLPA ladder are indicated at the right site of panel A.* ***B*** *The result of a MLPA reaction on samples with clinical signs of DWV. Both strands, positive and negative strand intermediate could be determined (marked by arrow head). In lane 1 and 3 some weak nonspecific bands are present. In the RT-free control some non-specific products were amplified.* ***C*** *A bee with DWV symptoms.*

This detection limit is in accordance with the detection limit obtained in other studies (Reijans et al., 2008; Wolffs et al., 2009; Zhang et al., 2011). However, too much template in the MLPA reaction can lead to lower detection signals due to inhibition of the PCR reaction (Schouten et al., 2002). Therefore it is recommended to reduce the initial amount of RNA in the RT-MLPA reaction to 100 ng, in order to minimize the chance of false-negative results.

Well-designed MLPA probes have the ability to discriminate between single-nucleotide polymorphism (SNP) (Schouten et al., 2002). This means that the viral RT-MLPA probes require a more generic design, as viruses, especially RNA viruses, are generally highly variable due to their very high mutation rates (Schouten et al., 2002). Therefore, a major concern in the design of the RT-MLPA probes was the compatibility of virus-specific probes with as many known strains of each virus as possible. At the same time, however this generic design should not compromise the specificity of the probe. The probes were therefore positioned in well-conserved regions and no mismatch within 5 nucleotides from the ligation site was tolerated. Although MLPA is widely used to detect SNPs, we tested the robustness of our MLPA technique for the presence of mismatches at the ligation site. We synthesized different templates which mimic the DWV target sequences but with either one or two mutations at the ligation point (i.e. the last nucleotide of the LPO and/or the first nucleotide of the RPO). These synthetic templates were used in the MLPA assay, using 5 ng template. Clear positive MLPA results were obtained with the template containing one mutation. The template with two mutations, either side of the ligation site gave very faint signals and the signal was lost completely when the ligation time was shortened from 15 min to 3 min, or when the final amplification step was prematurely aborted at 26 cycles. Other parameters of the MLPA which could influence the detection of mutant templates were also investigated. A positive result was obtained for all mutated templates when the amplification reaction was run for at least 30 cycles. Raising the hybridization temperature up to 66°C did not influence the results. For this particular diagnostic purpose, the detection of honey bee viruses, the insensitivity of the RT-MLPA reaction to nucleotide variations in the target

should be seen as an advantage. This makes the diagnostic power of the RT-MLPA approach even stronger.

"BeeDoctor" is optimized to detect various honey bee viruses simultaneously. MLPA is able to multiplex up to 45 targets. Probes used in MLPA usually range between 80 to 400 nucleotides in length. Accurate chemical synthesis of MLPA probes is possible up to a length of approximately 180 nucleotides (Stern et al., 2004). As we are using synthetic probes the number of targets in our approach will be limited to 15 to 20. Anyhow the multiplex power of this technique rises far beyond the multiplex power of a real-time quantitative PCR approach which is typically limited to four or five targets depending on the platform used. In order to test the quantitative potential of the assay, we selected two reference genes which were used frequently in honey bee virus research: β-actin and RPL8. A dilution series of DWV synthetic template was spiked into the RNA of non infected honey bees. Unfortunately we observed strong competition between the simultaneously amplified MLPA probes in this case β-actin and DWV probes and hence failed to establish this technique in a quantitative way. However this technique can be widely used in high throughput screening studies. By mixing synthetic templates mimicking the binding sites for DWV en BQCV we could show that the competition problem will not generate false negative results when multiple infected honey bees would be screened. Only the intensities of the generated product are influenced which makes quantification difficult.

All positive-strand RNA viruses replicate and express their genomes through negative-strand RNA intermediates that are used as templates for the production of positive-strand progeny RNAs that are then packaged in new virion particles. Therefore, the presence of negative-strand RNA intermediates is a reliable marker for active virus replication in infected honey bees. It is also a very effective means to distinguish between active infections and the non-infectious, passive presence of virus particles, which is an important epidemiological distinction. RT-MLPA is the ideal technique to selectively detect the positive strand genomic RNA or the negative-strand intermediate RNA, since an amplifiable probe can only be generated in a strand-specific manner, through the ligation of the two

***Table 2**: Prevalence, co-infection rates and the results of the association analysis of honey bee viruses in Flemish apiaries.*

			FREQUENCY	TOTAL PREVALENCE	ASSOCIATION ANALYSIS
ZERO VIRUSES		**TOTAL 78**	**21,5%**		n.a.
ONE VIRUS	ABPV	1	0,3%	3,3%	n.a.
	BQCV	14	3,9%	13,5%	n.a.
	CBPV	2	0,6%	1,7%	n.a.
	DWV	164	45,2%	69,4%	n.a.
	SBV	10	2,8%	19,0%	n.a.
	SBPV	0	0,0%	0,0%	n.a.
		TOTAL 191	**52,6%**		
					$\chi^2_{(1)}$
TWO VIRUSES	ABPV-BQCV	0	0,0%	-	$0,06^{n.s.}$
	ABPV-CBPV	0	0,0%	-	$0,06^{n.s}$
	ABPV-DWV	9	2,5%	-	$0,04^{n.s.}$
	ABPV-SBV	1	0,3%	-	$0,03^{n.s.}$
	BQCV-CBPV	0	0,0%	-	$0,02^{n.s.}$
	BQCV-DWV	23	6,3%	-	$1,79^{n.s.}$
	BQCV-SBV	5	1,4%	-	$1,11^{n.s.}$
	CBPV-DWV	2	0,6%	-	$0,00^{n.s.}$
	CBPV-SBV	0	0,0%	-	$0,00^{n.s.}$
	DWV-SBV	45	12,4%	-	$2,19^{n.s.}$
		TOTAL 85	**23,4%**		
					$\chi^2_{(3)}$
THREE VIRUSES	ABPV-BQCV-CBPV	0	0,0%	-	$0,53^{n.s.}$
	ABPV-BQCV-DWV	0	0,0%	-	$4,47^{n.s.}$
	ABPV-BQCV-SBV	0	0,0%	-	$1,10^{n.s.}$
	ABPV-CBPV-DWV	1	0,3%	-	$0,42^{n.s.}$
	ABPV-CBPV-SBV	0	0,0%	-	$0,24^{n.s.}$
	ABPV-DWV-SBV	0	0,0%	-	$6,91^{P<0.10}$
	BQCV-CBPV-DWV	0	0,0%	-	$1,86^{n.s.}$
	BQCV-CBPV-SBV	0	0,0%	-	$1,12^{n.s.}$
	BQCV-DWV-SBV	7	1,9%	-	$5,33^{n.s.}$
	CBPV-DWV-SBV	1	0,3%	-	$1,94^{n.s.}$
		TOTAL 9	**2,5%**		
					$\chi^2_{(9)}$
FOUR VIRUSES	ABPV-BQCV-CBPV-DWV	0	0,0%	-	$3,09^{n.s.}$
	ABPV-BQCV-CBPV-SBV	0	0,0%	-	$2,22^{n.s.}$
	ABPV-BQCV-DWV-SBV	0	0,0%	-	$9,04^{n.s.}$
	ABPV-CBPV-DWV-SBV	0	0,0%	-	$5,94^{n.s.}$
	BQCV-CBPV-DWV-SBV	0	0,0%	-	$4,81^{n.s.}$
		TOTAL 0	**0,0%**		
					$\chi^2_{(21)}$
FIVE VIRUSES	ABPV-BQCV-CBPV-DWV-SBV	0	0,0%	-	$7,32^{n.s.}$
	TOTAL	**363**	**100,0%**		

half-probes hybridizing next to each other on a single-stranded cDNA target. Strand specific probes were developed (Table 1) and tested on the total RNA extracted from DWV infected honey bees showing clinical symptoms. In order to have a better recovery of negative strand intermediates from replicating viruses, RNA was isolated with RNeasy Lipid Tissue Mini kit. Samples from honey bees with deformed wings tested positive for the presence of the negative and positive strand (Figure 1B). No band of the correct size was obtained in the RT-free controls, for either the positive-strand or negative-strand MLPA reaction (Figure 1B), showing that the NAD-dependent ligase-65 used in RT-MLPA cannot ligate DNA probe oligonucleotides that are hybridized to RNA.

The newly developed technique, “BeeDoctor”, was used in a survey of the prevalence and distribution of the targeted viruses in Flemish apiaries. This survey revealed that almost 80% of the samples were positive for at least one of the viruses screened for by “BeeDoctor” (Table 2). No virus was detected in 21.5% of samples, 52.6% of samples had only a single virus detected, with DWV the most common virus; 23.4% of samples had double infections, with DWV-SBV the most common combination, and 2.5% of samples had 3 viruses detected. There was no regional variation in prevalence for any of the viruses. Association studies (Table 2) shows that the double, triple, fourfold and fivefold infections are totally predictable from the individual prevalences of the different viruses. The occurrence of each virus is thus independent from the other viruses and this on all virus levels.

The most prevalent virus was DWV, with 69.4% of colonies screened being positive for the presence of this virus. The high occurrence of DWV in *A. mellifera* has also been reported in several other countries (Antunez et al., 2006; Baker and Schroeder, 2008; Tentcheva et al., 2004). On the other hand, Spain had in 2006 and 2007 a very low prevalence of 18.6 and 5.9% respectively (Antunez et al., 2012).

BQCV was detected in 13.5% of the colonies and is reported to have a variable prevalence in different colonies. The prevalence changes from 10 to 90% across Europe (Antunez et al., 2012; Antunez et al., 2006; Forgach et al., 2008; Tentcheva et al., 2004). SBV was present in 19% of the Flemish colonies which is high in comparison with 2% in Hungary, 1.4% in England and 1.1% in Spain

(Antunez et al., 2012; Baker et al., 2008; Forgach et al., 2008). However, in France and Uruguay detection rates of respectively 86% and 100% were reported (Antunez et al., 2006; Tentcheva et al., 2004). CBPV was detected in only 1.7% of the samples which is in correspondence with the findings of Tentcheva et al. in France, who found a maximum frequency in colonies of 4%. These low frequency rates can be explained by the finding that CBPV might persist at undetectable levels in healthy colonies (Tentcheva et al., 2004). SBPV could not be detected which confirms the low natural prevalence of SBPV across a large part of Europe (deMiranda et al., 2010b).

The prevalence of the virus complex ABPV, IAPV and KBV is also very low, with only 3.3% of the Flemish colonies infected. These three viruses are closely related and were detected simultaneously. The infection rate of 3.3% for the ABPV family is low as each of the viruses separately have higher prevalences in other European countries. ABPV is present in 29% of the colonies sampled in England while KBV was not detected (Baker et al., 2008). In Spain, 13% of the colonies was infected with IAPV in 2006 and 25,7% in 2007, while KBV was very low abundant in both years (<1%) (Antunez et al., 2012). In France ABPV was present in 58% of the colonies and KBV in 17% (Tentcheva et al., 2004).

In conclusion, in this study we developed an RT-MLPA approach to diagnose for the most common honey bee viruses in one single procedure. We were also able to develop a strand specific assay in which we can specifically screen for the negative strand intermediate as marker for effective virus replication. The multiplex power is an enormous advantage in comparison with other well established RT-PCR approaches. The “BeeDoctor” can easily be expanded with probes for additional pathogens and/or markers for honey bee health and disease. Moreover, the “BeeDoctor” and RT-MLPA in general also works well even with highly degraded RNA, since it requires only very short fragments of intact RNA, since the probe-specific RT primers can partly overlap with their corresponding probe and have to be elongated by only 50 nucleotides. Proper sample preservation is often difficult to achieve in practical beekeeping and sampling in the field, and often is a limiting factor for many other screening techniques (Dainat et al., 2011).

The “BeeDoctor” assay was used to screen 363 apparently healthy colonies from randomly selected apiaries throughout Flanders. This survey showed that almost 80% of colonies are infected with at least one virus, and many with multiple infections, showing that virus infections in apiaries are quite common, even in the absence of clinical symptoms.

5. Acknowledgment

The authors wish to thank the Flemish beekeeping association and beekeepers for supplying the samples. We also want to thank Víteslav Maňák for the photo of the DWV infected honey bee.

6. References

Aizen, M.A., Harder, L.D., 2009. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr Biol 19**,** 915-918.

Antunez, K., Anido, M., Garrido-Bailon, E., Botias, C., Zunino, P. and others, 2012. Low prevalence of honeybee viruses in Spain during 2006 and 2007. Res Vet Sci 93**,** 1441-1445.

Antunez, K., D'Alessandro, B., Corbella, E., Ramallo, G., Zunino, P., 2006. Honeybee viruses in Uruguay. J Invertebr Pathol 93**,** 67-70.

Aubert, M., 2008. Impact of virus infection in honey bees. (Eds.), 233-253.

Bailey L, Ball BV, 1991. Honeybee pathology (2nd Ed.). Academic Press, London, pp. pp113.

Baker, A., Schroeder, D., 2008. Occurrence and genetic analysis of picorna-like viruses infecting worker bees of Apis mellifera L. populations in Devon, South West England. J Invertebr Pathol 98**,** 239-242.

Bergval, I.L., Vijzelaar, R.N., Dalla Costa, E.R., Schuitema, A.R., Oskam, L. and others, 2008. Development of multiplex assay for rapid characterization of Mycobacterium tuberculosis. J Clin Microbiol 46**,** 689-699.

Biesmeijer, J.C., Roberts, S.P., Reemer, M., Ohlemuller, R., Edwards, M. and others, 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313**,** 351-354.

Boncristiani, H.F., Jr., Di, P.G., Pettis, J.S., Hamilton, M., Chen, Y.P., 2009. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. Virol J 6**,** 221.

Boncristiani, H.F., Rossi, R.D., Criado, M.F., Furtado, F.M., Arruda, E., 2009. Magnetic purification of biotinylated cDNA removes false priming and ensures strand-specificity of RT-PCR for enteroviral RNAs. J Virol Methods 161**,** 147-153.

Bos, M.M., Veddeler, D., Bogdanski, A.K., Klein, A.M., Tscharntke, T. and others, 2007. Caveats to quantifying ecosystem services: fruit abortion blurs benefits from crop pollination. Ecol Appl 17**,** 1841-1849.

Chen, Y., Zhao, Y., Hammond, J., Hsu, H.T., Evans, J., Feldlaufer, M., 2004. Multiple virus infections in the honey bee and genome divergence of honey bee viruses. J Invertebr Pathol 87**,** 84-93.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

Dainat, B., Evans, J.D., Chen, Y.P., Neumann, P., 2011. Sampling and RNA quality for diagnosis of honey bee viruses using quantitative PCR. J Virol Methods 174**,** 150-152.

deMiranda, J.R., 2008. Diagnostic techniques for virus detection. Virology and the honey bee 121-232.

deMiranda, J.R., Cordoni, G., Budge, G., 2010a. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J Invertebr Pathol 103 Suppl 1**,** S30-S47.

deMiranda, J.R., Dainat, B., Locke, B., Cordoni, G., Berthoud, H. and others, 2010b. Genetic characterization of slow bee paralysis virus of the honeybee (Apis mellifera L.). J Gen Virol 91**,** 2524-2530.

deMiranda, J.R., Drebot, M., Tyler, S., Shen, M., Cameron, C.E. and others, 2004. Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. J Gen Virol 85**,** 2263-2270.

deMiranda, J.R., Gauthier, L., Ribiere, M., Chen, Y.P., 2012. Honey bee viruses and their effect on bee and colony death. Honey Bee Colony Health: Challenges and Sustainable solutions (D Sammatro & YA Yoder Eds) Taylor & Francis Group Ltd, Oxford, England, pp. 71-102.

Evans, J.D., Schwarz, R.S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol.

Forgach, P., Bakonyi, T., Tapaszti, Z., Nowotny, N., Rusvai, M., 2008. Prevalence of pathogenic bee viruses in Hungarian apiaries: situation before joining the European Union. J Invertebr Pathol 98**,** 235-238.

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T. and others, 2004. Novel insect picorna-like virus identified in the brains of aggressive worker honeybees. J Virol 78**,** 1093-1100.

Garibaldi, L.A., Steffan-Dewenter, I., Kremen, C., Morales, J.M., Bommarco, R. and others, 2011. Stability of pollination services decreases with isolation from natural areas despite honey bee visits. Ecol Lett 14**,** 1062-1072.

Genersch, E., 2010. Honey bee pathology: current threats to honey bees and beekeeping. Appl Microbiol Biotechnol 87**,** 87-97.

Ghosh, R.C., Ball, B.V., Willcocks, M.M., Carter, M.J., 1999. The nucleotide sequence of sacbrood virus of the honey bee: an insect picorna-like virus. J Gen Virol 80 (Pt 6)**,** 1541-1549.

Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites (Varroa destructor). J Gen Virol 90**,** 463-467.

Govan, V.A., Leat, N., Allsopp, M., Davison, S., 2000. Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses. Virology 277**,** 457-463.

Grabensteiner, E., Bakonyi, T., Ritter, W., Pechhacker, H., Nowotny, N., 2007. Development of a multiplex RT-PCR for the simultaneous detection of three viruses of the honeybee (Apis mellifera L.): Acute bee paralysis virus, Black queen cell virus and Sacbrood virus. J Invertebr Pathol 94**,** 222-225.

Haddad, F., Qin, A.X., Giger, J.M., Guo, H., Baldwin, K.M., 2007. Potential pitfalls in the accuracy of analysis of natural sense-antisense RNA pairs by reverse transcription-PCR. BMC Biotechnol 7**,** 21.

Lanzi, G., deMiranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A. and others, 2006. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). J Virol 80**,** 4998-5009.

Leat, N., Ball, B., Govan, V., Davison, S., 2000. Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees. J Gen Virol 81**,** 2111-2119.

Maori, E., Lavi, S., Mozes-Koch, R., Gantman, Y., Peretz, Y. and others, 2007. Isolation and characterization of Israeli acute paralysis virus, a dicistrovirus affecting honeybees in Israel: evidence for diversity due to intra- and inter-species recombination. J Gen Virol 88**,** 3428-3438.

Markoulatos, P., Siafakas, N., Moncany, M., 2002. Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal 16**,** 47-51.

Mayo, M.A., 2002. Virus taxonomy - Houston 2002. Arch Virol 147**,** 1071-1076.

Meeus, I., Smagghe, G., Siede, R., Jans, K., de Graaf, D.C., 2010. Multiplex RT-PCR with broad-range primers and an exogenous internal amplification control for the detection of honeybee viruses in bumblebees. J Invertebr Pathol 105**,** 200-203.

Olivier, V., Blanchard, P., Chaouch, S., Lallemand, P., Schurr, F. and others, 2008. Molecular characterisation and phylogenetic analysis of Chronic bee paralysis virus, a honey bee virus. Virus Res 132**,** 59-68.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. J Gen Virol 85**,** 3747-3755.

Reijans, M., Dingemans, G., Klaassen, C.H., Meis, J.F., Keijdener, J. and others, 2008. RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. J Clin Microbiol 46**,** 1232-1240.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, F., Pals, G., 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30**,** e57.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA viruses in hymenopteran pollinators: evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5**,** e14357.

Stern, R.F., Roberts, R.G., Mann, K., Yau, S.C., Berg, J., Ogilvie, C.M., 2004. Multiplex ligation-dependent probe amplification using a completely synthetic probe set. Biotechniques 37**,** 399-405.

Sun, J., Xu, J., Liang, P., Mao, Q., Huang, Y. and others, 2011. Molecular identification of Clonorchis sinensis and discrimination with other opisthorchid liver fluke species using multiple Ligation-depended Probe Amplification (MLPA). Parasit Vectors 4**,** 98.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F. and others, 2004. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl Environ Microbiol 70**,** 7185-7191.

Terefework, Z., Pham, C.L., Prosperi, A.C., Entius, M.M., Errami, A. and others, 2008. MLPA diagnostics of complex microbial communities: relative quantification of bacterial species in oral biofilms. J Microbiol Methods 75**,** 558-565.

VanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E. and others, 2009. Colony collapse disorder: a descriptive study. PLoS One 4**,** e6481.

VanEngelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 103 Suppl 1**,** S80-S95.

Wolffs, P.F., Vink, C., Keijdener, J., Habek, B., Reijans, M. and others, 2009. Evaluation of MeningoFinder, a novel multiplex ligation-dependent probe amplification assay for simultaneous detection of six virus species causing central nervous system infections. J Clin Microbiol 47**,** 2620-2622.

Yue, C., Genersch, E., 2005. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). J Gen Virol 86**,** 3419-3424.

Zhang, J.M., Sun, J.F., Feng, P.Y., Li, X.Q., Lu, C.M. and others, 2011. Rapid identification and characterization of Penicillium marneffei using multiplex ligation-dependent probe amplification (MLPA) in paraffin-embedded tissue samples. J Microbiol Methods 85**,** 33-39.

CHAPTER 4

COMPREHENSIVE BEE PATHOGEN SCREENING IN BELGIUM REVEALS *CRITHIDIA MELLIFICAE* AS A NEW CONTRIBUTORY FACTOR TO WINTER MORTALITY

The work presented in Chapter 4 was adapted from the following manuscript:

J. Ravoet, J. Maharramov, I. Meeus, L. De Smet, T. Wenseleers, G. Smagghe, D.C. de Graaf. Comprehensive Bee Pathogen Screening in Belgium Reveals *Crithidia mellificae* as a New Contributory Factor to Winter Mortality, Plos ONE, 2013, 8(8): e72443.

doi: 10.1371/journal.pone.0072443

Contributions

D.C. de Graaf and G. Smagghe assisted with the study design. J. Ravoet collected the samples, extracted the RNA and executed the experiments concerning viruses, microsporidians supported by L. De Smet. J. Maharramov performed the experiments concerning protists and spiroplasmas, assisted by I. Meeus. J. Ravoet and T. Wenseleers carried out the statistical analyses, the former tested the multiple-kind lottery model and the latter tested the correlation of pathogen prevalences with winter mortality. D.C. de Graaf and J. Ravoet wrote the manuscript, assisted by all co-authors.

1. Abstract

Since the last decade, unusually high honey bee colony losses have been reported mainly in North-America and Europe. Here, we report on a comprehensive bee pathogen screening in Belgium covering 363 bee colonies that were screened for 18 known disease-causing pathogens and correlate their incidence in summer with subsequent winter mortality. Our analyses demonstrate that, in addition to *Varroa destructor*, the presence of the trypanosomatid parasite *Crithidia mellificae* and the microsporidian parasite *Nosema ceranae* in summer are also predictive markers of winter mortality, with a negative synergy being observed between the two in terms of their effects on colony mortality. Furthermore, we document the first occurrence of a parasitizing phorid fly in Europe, identify a new fourth strain of Lake Sinai Virus (LSV), and confirm the presence of other little reported pathogens such as *Apicystis bombi,* Aphid Lethal Paralysis Virus (ALPV), *Spiroplasma apis*, *Spiroplasma melliferum* and *Varroa destructor* Macula-like Virus (VdMLV). Finally, we provide evidence that ALPV and VdMLV replicate in honey bees and show that viruses of the LSV complex and Black Queen Cell Virus tend to non-randomly co-occur together. We also noticed a significant correlation between the number of pathogen species and colony losses. Overall, our results contribute significantly to our understanding of honey bee diseases and the likely causes of their current decline in Europe.

2. Introduction

Pollination is vital to the functioning of natural ecosystems, boosting the reproduction of wild plants, on which many other organisms depend. Likewise, many fruit, nut, vegetable and seed crops cultivated in an agricultural context depend on pollination. Honey bees (*Apis mellifera*) are considered the most economically valuable pollinators for crop monocultures worldwide (UNEP, 2010).

However, over the last decade unusually high honey bee colony losses have been reported, mainly in North-America (Vanengelsdorp and Meixner, 2010) and Europe (Potts et al., 2010). There is

a consensus nowadays that no single explanation can be given for these losses, and that there are several contributory factors to their decline, including pathogens, pesticides, nutrition and limited genetic diversity (Evans and Schwarz, 2011; Vanengelsdorp et al., 2010).

The ectoparasitic mite *Varroa destructor* is almost certainly a key player in causing the observed elevated colony losses (Dahle, 2010; Dainat et al., 2012b; Genersch et al., 2010; Guzman-Novoa et al., 2010; Schafer et al., 2010; van Dooremalen et al., 2012). This mite jumped from the Asian honey bee (*Apis cerana*) to the European honey bee (*Apis mellifera*) more than fifty years ago and has since become an almost cosmopolitan pest (Anderson and Trueman, 2000). The mite weakens the bees by sucking hemolymph from both adult bees and pupae (Garedew et al., 2004). In addition, they can transmit many of the known honey bee viruses (Bowen-Walker et al., 1999; Chen et al., 2004; Di Prisco et al., 2011; Genersch and Aubert, 2010; Shen et al., 2005) and cause a reactivation of covert virus infections due to host immune suppression (Yang and Cox-Foster, 2005). The mite destabilizes the within-host dynamics of viruses due to this immune suppression, which can then reach lethal levels (Nazzi et al., 2012). Further, *V. destructor* and Deformed Wing Virus (DWV) will reduce the life span of winter bees, which can cause a colony collapse (Dainat et al., 2012a).

So far, only three viruses have been correlated with colony losses: DWV, Acute Bee Paralysis Virus (ABPV) and Israeli Acute Bee Paralysis Virus (IAPV). ABPV and IAPV are members of a complex of closely related Dicistroviridae (de Miranda et al., 2010). IAPV was initially identified as a predictive marker for colony losses in the USA (Cox-Foster et al., 2007). An expanded study could not confirm this result (Vanengelsdorp et al., 2009). Moreover, a retrospective study revealed that this virus was already present before the first colony collapse disorders were ever reported (Chen and Evans, 2007). In Europe, ABPV was linked with colony losses in Belgium (Nguyen et al., 2011), Germany (Genersch et al., 2010) and Switzerland (Berthoud et al., 2010). Furthermore, DWV has been linked to winter mortality in both Switzerland (Berthoud et al., 2010) and Germany (Genersch et al., 2010).

The role of the Microsporidian fungus *Nosema ceranae*, another parasite that originates from the Asian honey bee (Fries et al., 1996), in causing colony collapse is still controversial. Sudden colony

collapses in Spain were attributed to *N. ceranae* infection (Higes et al., 2008; Higes et al., 2009), but these observations could not be confirmed by later independent studies in and outside Spain (Cox-Foster et al., 2007; Fernandez et al., 2012; Gisder et al., 2010; Invernizzi et al., 2009; Vanengelsdorp et al., 2010).

Recently, a prospective study revealed the presence of the little reported pathogens *Crithidia mellificae, Spiroplasma apis* and *Spiroplasma melliferum* in large-scale migratory beekeeping operations in the USA. Furthermore, the novel viruses (Aphid Lethal Paralysis Virus (ALPV) strain Brookings, Big Sioux River Virus (BSRV), Lake Sinai Virus (LSV) 1 and 2) and the phorid fly *Apocephalus borealis* were discovered as honey bee pathogens (Runckel et al., 2011). Earlier descriptions of spiroplasmas in honey bees go back to the early eighties (Clark et al., 1985; Mouches et al., 1983). Also *C. mellificae* has been little studied since its first description in 1967 (Langridge and Mcghee, 1967), even though the related *Crithidia bombi* is known to have serious effects on bumble bees, particularly under starvation conditions (Brown et al., 2000; Brown et al., 2003). The prevalence of these and other new pathogens and their potential correlation with winter losses in Europe, where no large-scale migratory commercial beekeeping occurs, is at present unknown.

In 2011, we performed an epidemiological study of the most common honey bee viruses in Belgium (De Smet et al., 2012). As shortly afterwards several neglected and new honey bee pathogens were described in the USA (Runckel et al., 2011), we decided to re-examine these samples in order to type them for several of these other known honeybee pathogens. In addition, we examine whether the presence of these pathogens in the summer can be used as a predictor of later winter mortality, and study possible associations in the prevalence of these pathogens.

3. Materials and methods

3.1 Honey bee sampling and preparation

For detailed description of the worker bee sampling procedure we refer to our previous paper (De Smet et al., 2012). In brief, in July 2011 around 30 bees were randomly sampled at the hive entrance

of 363 colonies. RNA was extracted from 10 bees per colony for the molecular detection of pathogens. In addition, the natural *Varroa* drop was monitored by placing a sheet of paper under the open mesh floor during one week, and counting the mites in the laboratory. Optimization of the PCR for VdMLV was done on mites collected at the apiary of Ghent University, campus Sterre. This is a newly discovered virus in both mites and honey bees (Gauthier et al., 2011).

3.2 MLPA analysis

BeeDoctor (De Smet et al., 2012), a 'multiplex-ligation probe dependent amplification' (MLPA) based method capable of detecting CBPV, DWV complex, ABPV complex, BQCV, SBPV and SBV, was expanded with probes to detect the positive and the negative strand each of BSRV, ALPV strain Brookings and viruses of the LSV complex (Table S1). Because of the high similarities of the two described LSV strains (Runckel et al., 2011), we were unable to differentiate between them. This new prototype of BeeDoctor was used for screening purposes in the present study. All probes were synthesized by Integrated DNA Technologies (Leuven, Belgium). BeeDoctor analysis was performed as described before (De Smet et al., 2012), starting from 1 µl RNA. All the MLPA reagents were obtained from MRC-Holland. The amplified MLPA products were analysed using 4% high resolution agarose gel electrophoresis.

3.3 PCR analysis

Five µl RNA (variable concentration) was retro-transcribed using random hexamer primers with the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific), according to the manufacturer's instructions. All PCR reaction mixtures contained 2 µM of each primer; 1.5 mM $MgCl_2$; 0.2 mM dNTP; 1.25 U Hotstar Taq DNA polymerase (Qiagen) and 1 µl cDNA product. The primers used are shown in Table S1. Temperature cycles for slowly-evolving trypanosomatids and neogregarines were as described (De Smet et al., 2012), but PCRs were performed in their uniplex mode. Samples that were positive for trypanosomatids were sequenced to confirm the presence of *C. mellificae*. Positive samples of neogregarines were subsequently re-analyzed with *A. bombi*-specific primers. Fifteen amplicons were sequenced for verification. Spiroplasmas were detected as described (Meeus et al.,

2012), based on the 16S ribosomal RNA (rRNA) sequence. Due to unspecific bands from *N. ceranae* rRNA around 700 bp, only universal *Spiroplasma* primers were used. Amplicons around 1kb were extracted using the GeneJET Gel Extraction Kit (Thermo Scientific) and sequenced for *S. apis* and *S. melliferum* differentiation. For the differentiation of *Nosema apis* and *N. ceranae*, PCR conditions described by (Chen et al., 2008; Chen et al., 2009) were used. Samples negative with these primers but positive for *Nosema* spore counting, were re-analyzed with primers specific for Microsporidia. A subset of the amplicons was sequenced for verification.

In order to detect the LSV strain 1 and 2, we followed the procedure described by (Runckel et al., 2011). However, when other strains appeared to be present we developed a PCR to detect a partial sequence of the Orf1 and RNA-dependent RNA polymerase genes of any known member of the LSV complex (strain 1, 2 and 3 at that time) using a degenerated primer set and the following cycling conditions: 94°C for 15 min; 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min; 35 cycles; final elongation 72°C for 10 min; hold at 4°C. Amplicons around 600 bp were extracted and sequenced. Temperature cycles for Microsporidia, Phoridae, ALPV and VdMLV were as described above, but with the annealing temperature set at respectively 60°C, 59°C, 60°C and 51°C. All PCR products were electrophoresed in 1.4% agarose gels, stained with ethidium bromide and visualised under UV light.

We developed a positive control for the Phoridae PCR and the MLPA-based detection of ALPV and BSRV by synthesizing 486-mer, 160-mer and 190-mer oligonucleotides respectively in a pIDTSmart vector (done by Integrated DNA Technologies). In other cases, the first positive sample detected in preliminary screenings served as a positive control.

3.4 Cloning and sequencing

Amplicons of ALPV strain Brookings, LSV complex, VdMLV and Phoridae were cloned into the pCR4 TOPO vector from TOPO TA Cloning Kit for sequencing (Invitrogen, USA) according to manufacturer's instructions. The cloned inserts were sequenced on an ABI 3130XL platform using M13 primers after isolation of the plasmids with the GeneJet™ Plasmid Miniprep kit (Thermo Scientific).

DNA sequences obtained by direct sequencing of amplicons or by sequencing cloned PCR products were BLAST-searched at http://blast.ncbi.nlm.nih.gov/. Alignments of the LSV amplicons and strict consensus sequences (100% threshold) from LSV 1 (Genbank: HQ871931), LSV 2 (Genbank: HQ888865) and LSV 3 (Genbank: JQ480620) RNA polymerases and Orf1 genes were generated with Geneious 5.6.4.

3.5 *Nosema* spore counting

We determined the *Nosema* spore levels in the extracts from 10 bees using light microscopy and a haemocytometer according to Cantwell (Cantwell, 1970). This extract was diluted when necessary.

3.6 Statistics

The multiple-kind lottery model of (Janovy et al., 1995) was used to infer the theoretical distribution of pathogens in surviving and collapsed colonies. By use of the individual infection percentage of each pathogen (n = 16) the model calculates the expected pathogen distribution or the number of colonies infected with 0 to 16 pathogens. As described earlier (Rutrecht and Brown, 2008), significant deviations between the observed and theoretically predicted pathogen distributions imply an interaction between different pathogens in this multi-pathogen host system. By means of a Pearson Chi-square test ($P < 0.05$) with SPSS 21.0 we compared if the observed pathogen distribution differed from the theoretical distribution. The same approach was followed to infer which interaction between pathogen pairs occurred within this multi-pathogen host system.

Pathogen prevalence was correlated with winter mortality using a binomial generalized linear model with probit link function using function *glm* in package *stats* in *R* 2.16. This analysis was performed with a subset of the samples (229), since we excluded colonies for which the beekeepers did not provide any data on winter mortality, as well as colonies that had undergone queen supersedure. To select the most parsimonious model we used an exhaustive search based on the Akaike Information Criterion (AIC). This was done using R package *glmulti*, based on a set of predictor variables which either included all main effects (but excluding pathogens *S. apis*, CBPV and ABPV, since they occurred in fewer than 10 out of 229 colonies), or one which also considered possible first

order interaction effects, and which included the pathogens which in a full main effects model had probit coefficients > 0.2 (*N. ceranae, C. mellificae, V. destructor, S. melliferum* and BQCV) as well as DWV, which had been linked to winter mortality before (Berthoud et al., 2010; Genersch et al., 2010) . In addition, we also ran a model in which all main effects were included as well as a first and third order polynomial model in which the total number of detected pathogens was used as a predictor of winter mortality. Significance was assessed using Type III likelihood ratio tests using function Anova in R package *car*. In all cases, one-sided *p*-levels were used, since pathogens a priori are expected to increase colony mortality. The predictive power of our resulting models was assessed using function CVbinary in R package DAAG.

4. Results

4.1 Survey of pathogens

An overview of the prevalences of the investigated pathogens is given in Table 1. The natural *Varroa destructor* drop ranged from 0 to more than 500 mites per week. A value equal to 0 does not necessarily imply that the colony is uninfected, only that the *Varroa* drop is below the detection limit. Within the boundaries, a prevalence of 93.7% (313/334) was found. *Nosema* spores were found in 75.2% (273/363) of the samples, and ranged from 10^5 till 10^9 per bee. PCR-based detection reveals 10.2% *N. apis* infection (37/363) and 92.6% *N. ceranae* infection (336/363), accounting for a total *Nosema* prevalence of 93.9% (341/363). Mixed infections, single *N. apis* and *N. ceranae* infection occurred in respectively 8.8% (32/363), 1.4% (5/363) and 83.7% (304/363) of the samples.

Amplicons had an almost complete nucleotide similarity with sequences of *N. apis* (Genbank: U97150) or *N. ceranae* (Genbank: DQ486027).

While an ALPV strain and different LSV strains were fairly abundant, with prevalences of 56.2% (204/363) and 14.6% (43/363) in our studied colonies, BSRV could not be detected. ALPV amplicons shared 97% nucleotide identity with two strains isolated from honey bees (Genbank: HQ871932; JX045858) and 89% with the canonical ALPV sequence (Genbank: AF536531). At the

amino acid level, our isolates (Genbank: KC880119) appeared to be identical to ALPV strain Brooking and 99% identical to a Spanish strain (Genbank: JX045858), caused by one substitution of valine to isoleucine. Moreover, we could show that ALPV and VdMLV are replicating in honey bees by demonstrating the presence of their negative strand intermediate, a marker for replication of positive sense single stranded RNA viruses, by a strand specific MLPA reaction. Surprisingly, VdMLV was detected in the majority of our bee samples (84.3%; 306/363). The Belgian strain (Genbank: KC880120) showed high sequence homology (97% on nucleotide level, 99% on amino acid level) with a French strain (Genbank: HQ916350).

The spiroplasmas *S. apis* and *S. melliferum* were found only in respectively 0.3% (1/363) and 4.4% (16/363) of the tested samples. One sequence appeared 100% identical to the *S. apis* strain ATCC 33834 (Genbank: GU993267); all others matched to *S. melliferum* IPMB4A (Genbank: JQ347516) (4 sequences with 100% identity and 6 sequences with only a single nucleotide substitution).

The little reported trypanosomatid *C. mellificae* was found in 70.5% (256/363) of the samples. The amplicons showed 100% sequence identity with a partial sequence of the small subunit ribosomal RNA of *C. mellificae* (Genbank: AB745488). We also found molecular evidence that the neogregarine *A. bombi*, primarily known as a bumble bee parasite (Lipa and Triggiani, 1996), was present in 40.8% (148/363) of our samples. The 15 sequenced amplicons showed 100% identity with a partial small subunit ribosomal RNA sequence of *A. bombi* (Genbank: FN546182).

Unexpectedly, we were also able to demonstrate the molecular presence of phorid flies in 31.1% (118/363) of the samples. These amplicons fully matched a partial *A. borealis* 18S ribosomal RNA sequence (Genbank: JF808447).

4.2 Identification of the LSV strains

In order to determine which LSV strains we had found by MLPA, we re-investigated the positive samples by PCR using the primers specific for LSV 1 and LSV 2 (Runckel et al., 2011). These specific primers did not work on our samples and therefore a degenerated primer set was developed. The

sequence of the amplicons generated with the degenerated primer set revealed one sample (Genbank: KC880123) with high resemblance to a known strain (Genbank: JQ480620) (96% nucleotide and 94% amino acid identity with LSV 3, a third LSV type that was described in the meanwhile (Cornman et al., 2012)), while others gave only moderate similarity to any of them (Genbank: KC880121, KC880122, KC880124-KC880126). Amplicons from six apiaries had the same trimmed sequence, which aligned very well with the consensus sequence of the RNA-dependent RNA polymerases of the three different strains (Figure S1). We designated this sequence representative for a new fourth strain of LSV (Genbank: JX878492). The LSV Orf1 sequences showed a high degree of sequence divergence (data not shown) but the majority of the conserved Orf1 amino acids were also retrieved in LSV 4 and our other sequences.

4.3 Effect of pathogens on colony winter losses

Overall, 46.5% of the sampled colonies were reported to be lost over the winter of 2011-2012. Combined with our data on the prevalence of 16 known honeybee pathogens in these colonies in summer (July 2011), including several little reported ones detected in the present paper, but also the more common viruses detected previously in these samples (De Smet et al., 2012), we decided to test whether these winter losses could be predicted on the bases of the presence or absence of these pathogens (Table 1). Based on a probit binomial model in which only main effects were considered, an exhaustive model search showed that *V. destructor* and *C. mellificae* contributed most to explaining winter mortality (AIC=317.21) (*C. mellificae*: p=0.03, marginal odds ratio=1.3; *V. destructor*: p=0.07, marginal odds ratio=1.3, Table 2a). Nevertheless, if we also included first order interaction effects and carried out an exhaustive search we obtained a model with slightly better explanatory power (AIC=316.11). *C. mellificae*, *N. ceranae*, *V. destruc*tor and as well as the interaction effect *C. mellificae* x *N. ceranae*, significantly contributed to explaining winter mortality in this model (p=0.01, 0.02, 0.07 and 0.03, respectively; Table 2 and Figure 1). The significant interaction effect was due to a negative synergy between *C. mellificae* and *N. ceranae* on winter mortality (Figure 1).

Table 1. *Honey bee pathogen incidences. Prevalences of honey bee pathogens found in Belgian honey bee colonies, the relationships between these pathogens and the effect of the occurrence of each pathogen on colony winter losses. * These data includes a subset of the samples (229), since 25% of the beekeepers did not provide data about winter losses of the monitored colonies*

Pathogen	**Type**	**Prevalences**			**Associations**	
		Overall	**Surviving colonies***	**Collapsed colonies***	**Between pathogens**	**With winter losses***
ABPV	Dicistroviridae	3.3% (12/363)	3.3% (4/122)	3.7% (4/107)		No
ALPV	Dicistroviridae	56.2% (204/363)	59.0% (72/122)	54.2% (58/107)	*Nosema* spores (p = 0.011)	No
Apicystis bombi	Ophryocystidae	40.8% (148/363)	41.8% (51/122)	41.1% (44/107)		No
Apocephalus borealis	Phoridae	31.1% (118/363)	32.8% (40/122)	33.6% (36/107)		No
BQCV	Dicistroviridae	13.5% (49/363)	10.7% (13/122)	14.0% (15/107)	LSV complex (p = 0.009)	No
CBPV	Unclassified RNA virus	1.7% (6/363)	0.0% (0/122)	1.9% (2/107)		No
Crithidia mellificae	Trypanosomatidae	70.5% (256/363)	71.3% (87/122)	81.3% (87/107)		Yes (p = 0.03)
DWV	Iflaviridae	69.4% (252/363)	61.5% (75/122)	67.3% (72/107)		No
LSV complex	Unclassified RNA virus	14.6% (43/363)	17.2% (21/122)	15.0% (16/107)	BQCV (p = 0.009)	No
Nosema apis	Nosematidae	10.2% (37/363)	13.1% (16/122)	10.3% (11/107)		No
Nosema ceranae	Nosematidae	92.6% (336/363)	89.3% (109/122)	94.4% (101/107)	VdMLV (p < 0.001)	No
Nosema spores	Nosematidae	75.2% (273/363)	71.3% (87/122)	72.9% (78/107)	ALPV (p = 0.011)	No
SBV	Iflaviridae	19.0% (69/363)	17.2% (21/122)	21.5% (23/107)		No
Spiroplasma apis	Spiroplasmataceae	0.3% (1/363)	0.0% (0/122)	0.0% (0/107)		No
Spiroplasma melliferum	Spiroplasmataceae	4.4% (16/363)	3.3% (4/122)	6.5% (7/107)		No
Varroa destructor	Varroidae	93.7% (313/334)	91.0% (111/122)	95.3% (102/107)		Yes (p=0.07)
VdMLV	Tymoviridae	84.3% (306/363)	79.5% (97/122)	84.1% (90/107)	*N. ceranae* (p < 0.001)	No

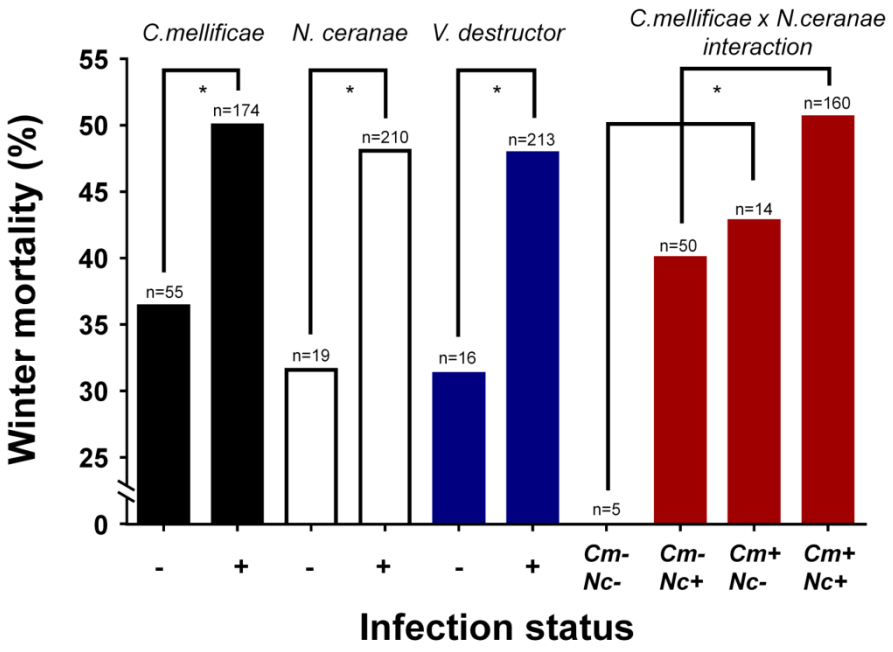


Figure 1. *Effect of* Crithidia mellificae*,* Nosema ceranae *and* Varroa destructor *on honey bee colony winter losses. The presence of* C. mellificae*,* N. ceranae *and* V. destructor *in summer all increase later winter mortality (binomial probit model, Table 2b, p=0.01, 0.03 and 0.07, respectively). In addition, there is a synergistic effect of* C. mellificae *and* N. ceranae *on winter mortality (Table 2b, p=0.03). Cm =* C. mellificae*, Nc =* N. ceranae.

Table 2. *Effects of the screened pathogens in summer on the observed honeybee winter mortality, based on probit models and exhaustive model searches in which the Akaike Information Criterion (AIC) was minimized.*

Pathogen	**Probit coefficients**	**Marginal odds ratios**	**LR χ^2**	***p* value**
(a) Best model based on main effects only[a] (AIC 317.21)				
Intercept	-0.82			
Crithidia mellificae	0.37	1.3	3.57	0.03
Varroa destructor	0.49	1.4	2.11	0.07
(b) Best model based on most important main effects and their 1st order interactions[b] (AIC 316.11)				
Intercept	-5.70			
Crithidia mellificae	5.09	1.3	6.16	0.01
Nosema ceranae	4.97	1.4	4.88	0.03
Varroa destructor	0.49	1.4	2.10	0.07
Crithidia mellificae x *Nosema ceranae*	-4.80	0.8	3.37	0.03

[a] Based on an exhaustive search with the following set of predictor variables: presence or absence of *N. apis, N. ceranae, C. mellificae, A. bombi, S. melliferum, A. borealis,* ALPV, DWV, BQCV, SBV, LSV, VDMLV*, V. destructor* as well as the natural *V. destructor drop* and *Nosema* spore load.

[b] Based on an exhaustive search, including all pathogens which occurred in more than 10 out of 229 colonies and which in a full main effects model had probit coefficients >0.2 (*N. ceranae, C. mellificae, V. destructor, S. melliferum* and BQCV) and DWV, which has been linked to winter mortality before (Berthoud et al., 2010; Genersch et al., 2010) , as well as their first order interaction effects.

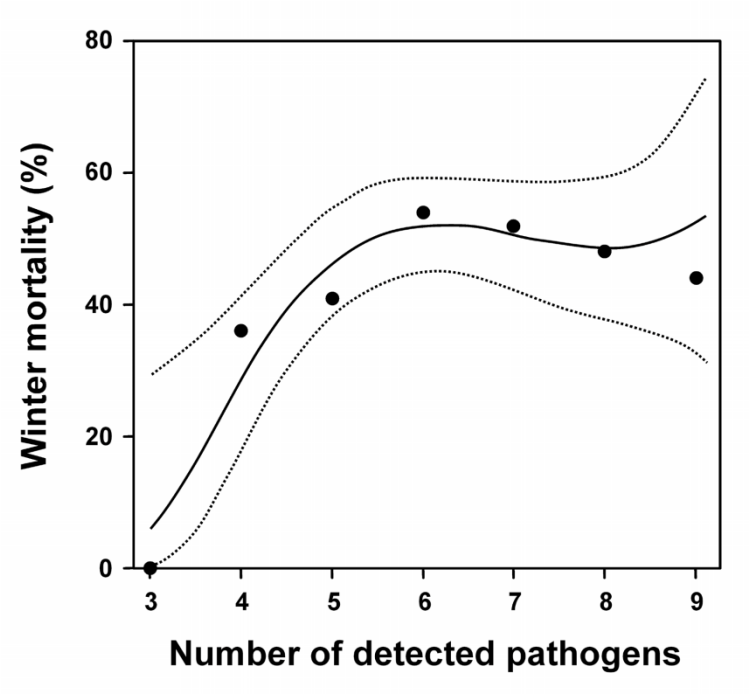


Figure 2. *Effect of the number of detected pathogens on winter mortality, based on a third order binomial probit model. The predicted winter mortality goes up markedly when the number of detected pathogens increase from 3 to 6 (from 5.9% to 52%), but then stabilizes around 50% when colonies have higher total numbers of pathogens.*

It means that the combination of both pathogens has a lesser output than the sum of each pathogen. Nevertheless, a clear enhancing effect can still be observed. Based on this model, the accuracy of the prediction of whether a colony would die or not in the winter was 55% using internal estimates, or 52% using cross-validation. Overall, higher numbers of detected pathogens in summer also resulted in a significantly increased winter mortality, as shown by a first order probit model (AIC=316.93, p=0.03).

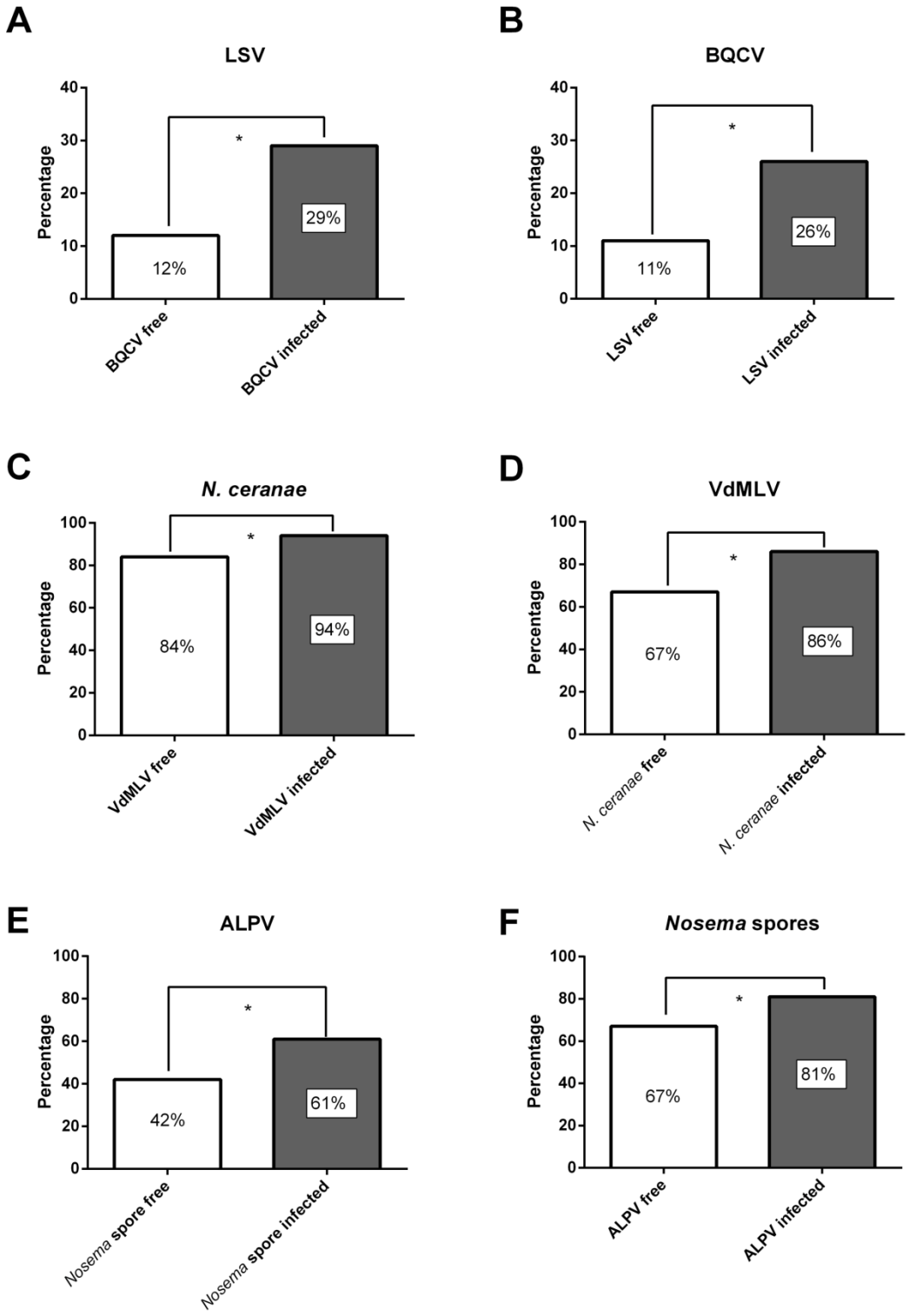


Figure 3. *Graphical representation of significant pathogen correlations. LSV complex is significantly associated with BQCV (p = 0.009) (**A** and **B**), VdMLV with N. ceranae (p < 0.001) (**C** and **D**) and ALPV strain Brookings with* Nosema *spores (p = 0.011) (**E** and **F**).*

In addition, the use of a third order probit model further increased the accuracy of the fit to the data (AIC=316.12), and resulted in a significantly positive first order effect (p=0.02) and a significantly negative second order effect (p=0.03) of the number of detected pathogens on winter mortality (Figure 2). When the amount of detected pathogens increases from 3 to 6 (from 5.9% to 52%), the predicted winter mortality goes up markedly but stabilizes around 50% at higher numbers of pathogen species.

4.4 Relationships between pathogens

As determined by a Pearson Chi-square test, we found evidence for positive associations between different pathogens ($p < 0.05$). LSV was significantly associated with BQCV ($\chi^2 = 9.41$, df = 2, $p = 0.009$), ALPV with *Nosema* spores ($\chi^2 = 9{,}087$, df = 2, $p = 0.011$) and VdMLV with *N. ceranae* ($\chi^2 = 28.067$, df = 2, $p < 0.001$). These pathogen associations are presented graphically in Figure 3.

5. Discussion

Overall, our data represent among the most comprehensive prevalence studies of honey bee pathogens carried out to date in Europe. The recent discovery of new bee viruses and neglected parasites in several countries highlighted the narrow window of pathogens that are the subject of many monitoring programs. As a result, we decided to re-investigate samples from July 2011 and statistically analyze whether the detected pathogens in summer had any effect on the winter mortality.

Our analysis confirmed the importance of *V. destructor* in summer as a marker for colony collapses (Dainat et al., 2012b) (Figure 1). Importantly, our analysis also demonstrated a large effect of the occurrence of *C. mellificae* in summer on later winter losses, even enhanced through *N. ceranae* co-infection (Figure 1). The protozoan *C. mellificae* has been ignored for a long time, but the current data highlight it as a new putative key player in honey bee colony declines. This trypanosome has probably a cosmopolitan distribution since it has been reported in Australia (Langridge et al., 1967), China (Yang et al., 2013), France (Dainat et al., 2012c), Japan (Morimoto et al., 2013),

Switzerland (Schmid-Hempel and Tognazzo, 2010) and USA (Runckel et al., 2011). Besides, the related *C. bombi*, also reported from Asian honey bees (Li et al., 2012), has serious effects on the survival of bumble bees under stress conditions (Brown et al., 2000). Recently, complex dynamic immune responses to *C. mellificae* infection were reported, with a distinct response when individuals were infected with *C. mellificae* and *N. ceranae* simultaneously (Schwarz and Evans, 2013). In addition, an association between both pathogens was reported in the USA (Runckel et al., 2011). Possibly, the controversial role of *N. ceranae* (Cox-Foster et al., 2007; Higes et al., 2009; Higes et al., 2013) might be explained by the synergistic effect of *N. ceranae* and *C. mellificae* on colony mortality. We also observed a significant correlation between the number of detected pathogens and colony losses, as was likewise reported in the USA (Vanengelsdorp et al., 2009). Collapsing colonies, induced by e.g. *V. destructor* and *C. mellificae*, are probably more vulnerable to a diverse set of parasites (Cornman et al., 2012), which elucidate this correlation. Moreover, it appeared that several pathogens can act synergistically and eventually cause a collapse of the honey bee colony (Cornman et al., 2012). The outcome of these pathogen interactions can vary between regions (Cornman et al., 2012), probably because of the multifactorial origin of colony losses and the interplay between different stressors.

Additionally, our results confirm that Lake Sinai Viruses are a viral complex (Figure S1). Diverse viral sequences are reported in the USA (Cornman et al., 2012; Runckel et al., 2011) and Spain (Granberg et al., 2013). We could also confirm the presence of one known American LSV strain in Belgium, namely LSV 3. Another strain, designated LSV 4 (Genbank: JX878492), was retrieved in several independent samples. An ALPV strain was detected for the first time in Belgium. This virus was also detected in American (Runckel et al., 2011) and Spanish honey bees (Granberg et al., 2013). Remarkable was its rather high incidence in the present study (56.2%; 204/363), akin to similar observations in different regions in the USA (Runckel et al., 2011). We could detect the presence of the ALPV negative strand intermediate, demonstrating that it is a replicating virus in honey bees. It is associated with the presence of *Nosema* spores, being indicative for a common oral transmission

route. Another less known virus, VdMLV, was suggested to be a virus of *V. destructor*, which can be transmitted to honey bees (de Miranda et al., 2011). Surprisingly, our study indicates a high prevalence in honey bees and a correlation with *N. ceranae*. The impact of this virus on honey bees remains unclear, but might well be significant since it replicates in honey bees.

The bacteria, *S. apis* and *S. melliferum*, are known as honey bee pathogens for a long time (Clark et al., 1985; Mouches et al., 1983), including Asian honey bees (Ahn et al., 2012). They seem to be uncommon in honey bees, with a sudden incidence in the summer (Runckel et al., 2011) which may be related to transmission via flowers.

Another unexpected discovery was the detection of phorid flies. Since the found amplicons had a 100% nucleotide similarity, we have strong molecular evidence that *A. borealis* or a similar phorid fly also infects honey bees outside the USA. To our knowledge this is the first description of a parasitizing phorid fly in honey bee samples in a Palaearctic region. This phorid fly was recently described as a new honey bee pathogen which alters the host behaviour by hive abandonment, eventually causing death (Core et al., 2012).

Besides viruses, bacteria and fungi, honey bees can also be parasitized by neogregarines. Our study revealed a high prevalence of *Apicystis bombi* in honey bees. This parasite is believed to be highly virulent in bumble bee spring queens, but re-emerges later on in worker bumble bees (Rutrecht et al., 2008). However, real empirical data is missing to describe the pathology of *A. bombi*. After its detection in honey bees in Finland (Lipa et al., 1996), *A. bombi* was also reported in honey bees in Japan (Morimoto et al., 2013) and Argentina (Plischuk et al., 2011).

The presence in Argentina is probably induced by spillover from invasive *Bombus terrestris* (Arbetman et al., 2013), an introduced pollinator outside the West Palaearctic area (Rasmont et al., 2008). The high prevalence (40.8%; 148/363) of *A. bombi*, without correlation with winter losses, indicates that it is not highly virulent in honey bees. Surprisingly, unbiased molecular studies in the USA did not report the occurrence of this pathogen (Cornman et al., 2012; Cox-Foster et al., 2007).

Besides pathogens, other factors like air quality, electromagnetic radiation, food deficiency and meteorological conditions can have an influence on colony losses. Later on, multiple linear regression modelling was applied on all combinations of explanatory variables and revealed no additional correlations with winter losses (BeeHappy, unpublished information).

6. Conclusions

Colony winter losses in Belgium seem to be associated with (1) *V. destructor* and (2) the detection of *C. mellificae* and *N. ceranae* in summer, with an enhancing effect on colony mortality being observed between the latter two. Thus, the present study not only extends the number of pathogens bees are exposed to in Europe, but also assigned the trypanosomatid parasite *C. mellificae* as a new contributory factor to explain winter losses, in addition to the parasitic mite *V. destructor* and the microsporidian parasite *N. ceranae*. Moreover, the present study describes the occurrence of 6 new pathogens in Belgian honey bee: ALPV strain Brookings, VdMLV, viruses of the LSV complex, *S. melliferum*, *A. bombi* and *A. borealis*. This phorid fly and *S. melliferum* were hitherto not reported as honey bee pathogens in Europe before. From LSV a new fourth strain was discovered. Screening for negative strand intermediate of these viruses demonstrated replication of ALPV and VdMLV in honey bees, which had never been demonstrated before. Furthermore, we found associations between viruses of the LSV complex and BQCV, between VdMLV and *N. ceranae*, and between an ALPV strain and *Nosema* spores. The latter might indicate a common oral route of transmission. We did not found a correlation between *V. destructor* and DWV. This can be caused since previous studies used quantitative data (Gisder et al., 2009; Martin et al., 2012), in contrast to our binomial dataset. Nevertheless, it seems advisory to look at a broader range of pathogens in nationwide monitoring programs.

7. Acknowledgments

The authors wish to thank the participating Flemish beekeepers.

8. Corrigendum

As demonstrated by Schwarz and colleagues (Schwarz et al., 2015), two trypanosomatid species can infect honey bees, namely *C. mellificae* and *Lotmaria passim*. It appears that nearly all of the *C. mellificae* identified the Belgian samples should be redubbed *L. passim* (J. Ravoet, unpublished information; Chapter 8).

9. References

Ahn, J.H., Hong, I.P., Bok, J.I., Kim, B.Y., Song, J., Weon, H.Y., 2012. Pyrosequencing analysis of the bacterial communities in the guts of honey bees Apis cerana and Apis mellifera in Korea. J Microbiol 50**,** 735-745.

Anderson, D.L., Trueman, J.W.H., 2000. Varroa jacobsoni (Acari : Varroidae) is more than one species. Exp Appl Acarol 24**,** 165-189.

Arbetman, M.P., Meeus, I., Morales, C.L., Aizen, M.A., Smagghe, G., 2013. Alien parasite hitchhikes to Patagonia on invasive bumblebee. Biological Invasions 15**,** 489-494.

Baker, M.D., Vossbrinck, C.R., Didier, E.S., Maddox, J.V., Shadduck, J.A., 1995. Small-Subunit Ribosomal DNA Phylogeny of Various Microsporidia with Emphasis on Aids-Related Forms. J Eukaryot Microbiol 42**,** 564-570.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., Neumann, P., 2010. Virus infections and winter losses of honey bee colonies (Apis mellifera). J Apicult Res 49**,** 60-65.

Bowen-Walker, P.L., Martin, S.J., Gunn, A., 1999. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite Varroa jacobsoni Oud. J Invertebr Pathol 73**,** 101-106.

Brown, M.J.F., Loosli, R., Schmid-Hempel, P., 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91**,** 421-427.

Brown, M.J.F., Schmid-Hempel, R., Schmid-Hempel, P., 2003. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. Journal of Animal Ecology 72**,** 994-1002.

Cantwell, G.E., 1970. Standard methods for counting nosema spores. Am Bee J 110**,** 222-223.

Chen, Y., Evans, J.D., Smith, I.B., Pettis, J.S., 2008. Nosema ceranae is a long-present and wide-spread microsporidian infection of the European honey bee (Apis mellifera) in the United States. J Invertebr Pathol 97**,** 186-188.

Chen, Y., Evans, J.D., Zhou, L., Boncristiani, H., Kimura, K. and others, 2009. Asymmetrical coexistence of Nosema ceranae and Nosema apis in honey bees. J Invertebr Pathol 101**,** 204-209.

Chen, Y.P., Evans, J.D., 2007. Historical presence of Israeli acute paralysis virus in the United States. Am Bee J 147**,** 1027-1028.

Chen, Y.P., Pettis, J.S., Evans, J.D., Kramer, M., Feldlaufer, M.F., 2004. Transmission of Kashmir bee virus by the ectoparasitic mite Varroa destructor. Apidologie 35**,** 441-448.

Clark, T.B., Whitcomb, R.F., Tully, J.G., Mouches, C., Saillard, C. and others, 1985. Spiroplasma melliferum, A New Species from the Honeybee (Apis mellifera). International Journal of Systematic Bacteriology 35**,** 296-308.

Core, A., Runckel, C., Ivers, J., Quock, C., Siapno, T. and others, 2012. A New Threat to Honey Bees, the Parasitic Phorid Fly Apocephalus borealis. Plos One 7**,** e29639.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D. and others, 2012. Pathogen webs in collapsing honey bee colonies. Plos One 7**,** e43562.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

Dahle, B., 2010. The role of Varroa destructor for honey bee colony losses in Norway. J Apicult Res 49**,** 124-125.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012a. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. Appl Environ Microbiol 78**,** 981-987.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012b. Predictive Markers of Honey Bee Colony Collapse. Plos One 7**,** e32151.

Dainat, B., Evans, J.D., Gauthier, L., Neumann, P. Crithidia mellifcae is widespread in Europe and can be used as a predictive marker of honeybee colony losses. Barth, B., Scharpenberg, H., and Moritz, R. F. A. 5th European Conference of Apidology . 2012c. Halle an der Saale.
Ref Type: Conference Proceeding

de Miranda, J.R., Cordoni, G., Budge, G., 2010. The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex. J Invertebr Pathol 103**,** S30-S47.

de Miranda, J.R., Gauthier, L., Ribière, M., Chen, Y., 2011. Honey Bee Viruses and Their Effect on Bee and Colony Health. In: Sammataro, D., Yoder, J.A. (Eds.), Honey Bee Colony Health: Challenges and Sustainable Solutions CRC Press, pp. 71-112.

De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y. and others, 2012. BeeDoctor, a Versatile MLPA-Based Diagnostic Tool for Screening Bee Viruses. Plos One 7**,** e47953.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H.F., Evans, J.D., Chen, Y.P., 2011. Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. J Gen Virol 92**,** 151-155.

Evans, J.D., Schwarz, R.S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol 19**,** 614-620.

Fernandez, J.M., Puerta, F., Cousinou, M., Dios-Palomares, R., Campano, F., Redondo, L., 2012. Asymptomatic presence of Nosema spp. in Spanish commercial apiaries. J Invertebr Pathol 111**,** 106-110.

Fries, I., Feng, F., daSilva, A., Slemenda, S.B., Pieniazek, N.J., 1996. Nosema ceranae n sp (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee Apis cerana (Hymenoptera, Apidae). Eur J Protistol 32**,** 356-365.

Garedew, A., Schmolz, E., Lamprecht, I., 2004. The energy and nutritional demand of the parasitic life of the mite Varroa destructor. Apidologie 35**,** 419-430.

Gauthier, L., Ravallec, M., Tournaire, M., Cousserans, F., Bergoin, M. and others, 2011. Viruses Associated with Ovarian Degeneration in Apis mellifera L. Queens. Plos One 6**,** e16217.

Genersch, E., Aubert, M., 2010. Emerging and re-emerging viruses of the honey bee (Apis mellifera L.). Veterinary Research 41**,** 54.

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C. and others, 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41**,** 332-352.

Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites (Varroa destructor). J Gen Virol 90**,** 463-467.

Gisder, S., Hedtke, K., Mockel, N., Frielitz, M.C., Linde, A., Genersch, E., 2010. Five-Year Cohort Study of Nosema spp. in Germany: Does Climate Shape Virulence and Assertiveness of Nosema ceranae? Appl Environ Microb 76**,** 3032-3038.

Granberg, F., Vicente-Rubiano, M., Rubio-Guerri, C., Karlsson, O.E., Kukielka, D. and others, 2013. Metagenomic detection of viral pathogens in spanish honeybees: co-infection by aphid lethal paralysis, Israel acute paralysis and lake sinai viruses. Plos One 8**,** e57459.

Guzman-Novoa, E., Eccles, L., Calvete, Y., Mcgowan, J., Kelly, P.G., Correa-Benitez, A., 2010. Varroa destructor is the main culprit for the death and reduced populations of overwintered honey bee (Apis mellifera) colonies in Ontario, Canada. Apidologie 41**,** 443-450.

Higes, M., Martin-Hernandez, R., Botias, C., Bailon, E.G., Gonzalez-Porto, A.V. and others, 2008. How natural infection by Nosema ceranae causes honeybee colony collapse. Environ Microbiol 10**,** 2659-2669.

Higes, M., Martin-Hernandez, R., Garrido-Bailon, E., Gonzalez-Porto, A.V., Garcia-Palencia, P. and others, 2009. Honeybee colony collapse due to Nosema ceranae in professional apiaries. Env Microbiol Rep 1**,** 110-113.

Higes, M., Meana, A., Bartolome, C., Botias, C., Martin-Hernandez, R., 2013. Nosema ceranae (Microsporidia), a controversial 21st century honey bee pathogen. Env Microbiol Rep 5**,** 17-29.

Invernizzi, C., Abud, C., Tomasco, I.H., Harriet, J., Ramallo, G. and others, 2009. Presence of Nosema ceranae in honeybees (Apis mellifera) in Uruguay. J Invertebr Pathol 101**,** 150-153.

Janovy, J., Clopton, R.E., Clopton, D.A., Snyder, S.D., Efting, A., Krebs, L., 1995. Species Density Distributions As Null Models for Ecologically Significant Interactions of Parasite Species in An Assemblage. Ecol Model 77**,** 189-196.

Langridge, D.F., Mcghee, R.B., 1967. Crithidia mellificae n sp: An Acidophilic Trypanosomatid of Honey Bee Apis mellifera. J Protozool 14**,** 485-&.

Li, J.L., Qin, H.R., Wu, J., Sadd, B.M., Wang, X.H. and others, 2012. The Prevalence of Parasites and Pathogens in Asian Honeybees Apis cerana in China. Plos One 7**,** e47955.

Lipa, J.J., Triggiani, O., 1996. Apicystis gen nov and Apicystis bombi (Liu, Macfarlane & Pengelly) comb nov (Protozoa: Neogregarinida), a cosmopolitan parasite of Bombus and Apis (Hymenoptera: Apidae). Apidologie 27**,** 29-34.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E. and others, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336**,** 1304-1306.

Meeus, I., de Graaf, D.C., Jans, K., Smagghe, G., 2010. Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. Journal of applied microbiology 109**,** 107-115.

Meeus, I., Vercruysse, V., Smagghe, G., 2012. Molecular detection of Spiroplasma apis and Spiroplasma melliferum in bees. J Invertebr Pathol 109**,** 172-174.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B. and others, 2013. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. Env Microbiol Rep 5**,** 74-77.

Mouches, C., Bove, J.M., Tully, J.G., Rose, D.L., Mccoy, R.E. and others, 1983. Spiroplasma apis, A New Species from the Honeybee Apis mellifera. Ann Inst Pasteur Mic A134**,** 383-397.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G. and others, 2012. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. Plos Pathog 8**,** e1002735.

Nguyen, B.K., Ribiere, M., Vanengelsdorp, D., Snoeck, C., Saegerman, C. and others, 2011. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res 50**,** 195-202.

Plischuk, S., Meeus, I., Smagghe, G., Lange, C.E., 2011. Apicystis bombi (Apicomplexa: Neogregarinorida) parasitizing Apis mellifera and Bombus terrestris (Hymenoptera: Apidae) in Argentina. Env Microbiol Rep 3**,** 565-568.

Potts, S.G., Roberts, S.P.M., Dean, R., Marris, G., Brown, M.A. and others, 2010. Declines of managed honey bees and beekeepers in Europe. J Apicult Res 49**,** 15-22.

Rasmont, P., Coppee, A., Michez, D., De Meulemeester, T., 2008. An overview of the Bombus terrestris (L. 1758) subspecies (Hymenoptera : Apidae). Annales de la Societe Entomologique de France 44**,** 243-250.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal Analysis of the Honey Bee Microbiome Reveals Four Novel Viruses and Seasonal Prevalence of Known Viruses, Nosema, and Crithidia. Plos One 6**,** e20656.

Rutrecht, S.T., Brown, M.J.F., 2008. The life-history impact and implications of multiple parasites for bumble bee queens. Int J Parasitol 38**,** 799-808.

Schafer, M.O., Ritter, W., Pettis, J.S., Neumann, P., 2010. Winter Losses of Honeybee Colonies (Hymenoptera: Apidae): The Role of Infestations With Aethina tumida (Coleoptera: Nitidulidae) and Varroa destructor (Parasitiformes: Varroidae). Journal of Economic Entomology 103**,** 10-16.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular Divergence Defines Two Distinct Lineages of Crithidia bombi (Trypanosomatidae), Parasites of Bumblebees. J Eukaryot Microbiol 57**,** 337-345.

Schwarz, R.S., Evans, J.D., 2013. Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. Dev Comp Immunol 40**,** 300-310.

Shen, M.Q., Yang, X.L., Cox-Foster, D., Cui, L.W., 2005. The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virology 342**,** 141-149.

UNEP, 2010. Global Honey Bee Colony Disorder and Other Threats to Insect Pollinators. UNEP Emerging Issues.

van Dooremalen, C., Gerritsen, L., Cornelissen, B., van der Steen, J.J.M., van Langevelde, F., Blacquiere, T., 2012. Winter Survival of Individual Honey Bees and Honey Bee Colonies Depends on Level of Varroa destructor Infestation. Plos One 7**,** e36285.

Vanengelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E. and others, 2009. Colony Collapse Disorder: A Descriptive Study. Plos One 4**,** e6481.

Vanengelsdorp, D., Meixner, M.D., 2010. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J Invertebr Pathol 103**,** S80-S95.

Vossbrinck, C.R., Woese, C.R., 1986. Eukaryotic Ribosomes That Lack A 5.8S RNA. Nature 320**,** 287-288.

Yang, B., Peng, G.D., Li, T.B., Kadowaki, T., 2013. Molecular and phylogenetic characterization of honey bee viruses, Nosema microsporidia, protozoan parasites, and parasitic mites in China. Ecology and Evolution 3**,** 298-311.

Yang, X.L., Cox-Foster, D.L., 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. P Natl Acad Sci USA 102**,** 7470-7475.

9. Supplementary information

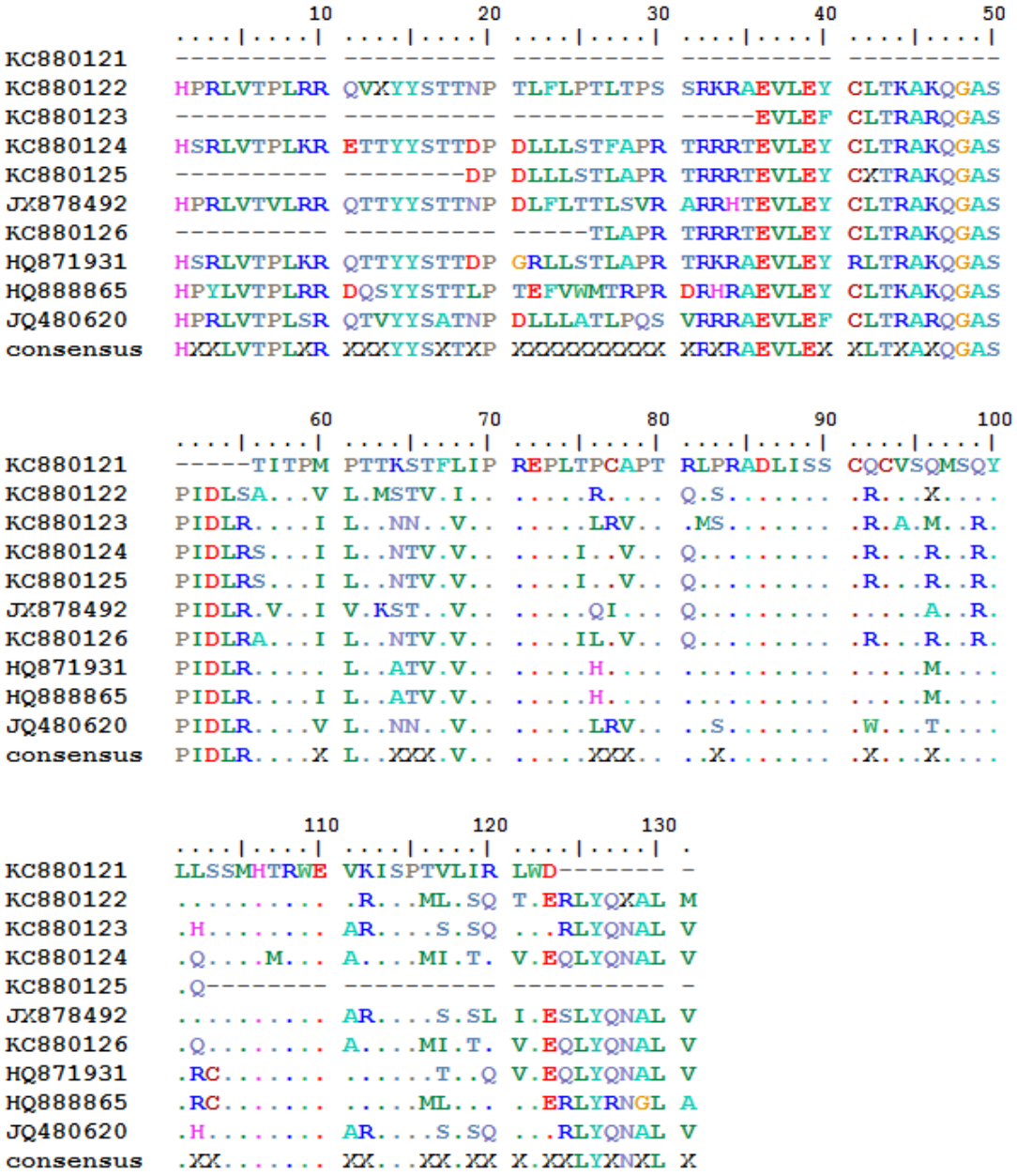


Figure S1. *Sequence variability of Lake Sinai Virus RNA-dependent RNA polymerase. Amino acid alignment of a consensus sequence (generated from LSV 1, 2 and 3) with known and new Lake Sinai Virus RNA-dependent RNA polymerase sequences.*

Table S1. *Primers and MLPA probes used in this study. Half-probes used for detecting different honey bee viruses or virus species complexes through RT-MLPA and primers used for detecting honey bee viruses or other pathogens. Each RPO probe is 5'- phosphorylated (indicated by $^{P-}$) to permit ligation of the 5' end of the RPO to the 3' end of the LPO. The PCR sequence tags on each halfprobe are in lower-case letters, the non-specific stuffer sequences (for generating PCR products with pre-determined sizes) are shown in upper-case letters and the target-specific sequences are shown in underlined upper-case letters.*

Target	Primers	Sequence (5' - 3')	Size (bp)	Reference
MLPA detection				
ALPV-Br (- strand)	MLPA_ALPV_rep_LPO	gggttccctaagggttggaTCCGTGGATTTATCATGCATAGCCAGTTCGGTTAATCC	119	This work
	ALPV_rep_RPO	$^{P-}$GCCGCTGATTGTGTCAACACAGATACGTAGAGGTAGTTGtctagattggatcttgctggcac		
	RT_ALPV_rep	CCTAACTGGGTACGTGTTGG		
ALPV-Br (+ strand)	MLPA_ALPV_LPO	gggttccctaagggttggaTCTGACCTTTCACATCTGGACAGCCAACTACCTCTACGTATCTGTGTTGACACAATCAGC	160	This work
	MLPA_ALPV_RPO	$^{P-}$GGCGGATTAACCGAACTGGCTATGCATGATAAAGTACAAGCCCGTTCAGCACCTGGGTtctagattggatcttgctggcac		
	RT_ALPV_new	TCATCTTAGACCTCCATTTAGAATCC		
BSRV	BSRV-LPO	gggttccctaagggttggaGTGAGCAGTCAGGTGGCGTGATACGTGGTGTTTTTGATGACCTTGATCGAGTTCCAAAAGCACTGAGTGGCATG	190	This work
	BSRV-RPO	$^{P-}$GAGAGTATGTGGAAACGCATAGACTCCGTGTCACTGAAAATATCAGCTATGCCGGACAGGGCGTGCGCGTTGAAtctagattggatcttgctggcac		
	RT_BSRV	CCATCCAATTCCATAGTACAGTTG		
LSV complex	LSV-LPO	gggttccctaagggttggaGACTCCCAGCTGGACCGCTACGAAATGCGCGTATCCTCGTGCGGACCTCATTTCTTCATGTCAGTGT	175	This work

	LSV-RPO	[P-]GTGAGCATGATGAGTCAATACCTACGGTGTTCCGGGGATGGGGGCCGGGTGAGGA AAGCTGGCTGATCTAGATTGGATCTTGCTGGCACtctagattggatcttgctggcac		
	RT_LSV	CCACCGAGTGTGCATGG		
PCR detection				
ALPV-Br	ALP-Br-F-2936	AACGTCGTATGCTACGATGAACTCG	464	Runckel et al., 2011
	ALP-Br-R-3400	GGGTTAAATTCAATTCCAGTACCACGG		
A. bombi	ApBF1	CGTACTGCCCTGAATACTCCAG	~ 511	Meeus et al., 2010
	ApUR2	TTTCTCATTCTTCAGATGATTTGG		
A. borealis	Phorid_rRNA 1F	GTACACCTATACATTGGGTTCGTACATTAC	486	Runckel et al., 2011
	Phorid_rRNA 1R	GAGRGCCATAAAAGTAGCTACACC		
LSV complex	LSVdeg-F	GCCWCGRYTGTTGGTYCCCCC	578	This work
	LSVdeg-R	GAGGTGGCGGCGCSAGATAAAGT		
LSV 1	LSV1-F-2294	TTATCTCGCGCCGCCACCTC	672	Runckel et al., 2011
	LSV1-R-2966	ATCGCCGCTGCAACGTGACC		
LSV 2	LSV2-F-3954	CGGCCGGTCTAGCGTGGTTG	558	Runckel et al., 2011
	LSV2-R-4512	TGGCAAGCTGTGACGAATCCCT		
Neogregarines	NeoF:	CCAGCATGGAATAACATGTAAGG	258	Meeus et al., 2010
	NeoR:	GACAGCTTCCAATCTCTAGTCG		
Microsporidia	V1F	CACCAGGTTGATTCTGCCTGAC	~ 406	Vossbrinck and Woese, 1986

	530R	CCGCGGCTGCTGGCAC		Baker et al., 1995
Nosema apis	Napis-sense	CCATTGCCGGATAAGAGAGT	269	Chen et al., 2009
	Napis-antisense	CCACCAAAAACTCCCAAGAG		
Nosema ceranae	NceranaeF	CGGATAAAAGAGTCCGTTACC	250	Chen et al., 2008
	NceranaeR	TGAGCAGGGTTCTAGGGAT		
Spiroplasma spp.	BS1-F	AAGTCGAACGGGGTGCTT	976	Meeus et al., 2012
	BS1-R	TGCACCACCTGTCTCAATGT		
Trypanosomatids	SEF	CTTTTGGTCGGTGGAGTGAT	406	Meeus et al., 2010
	SER	GGACGTAATCGGCACAGTTT		
VdMLV	VdMLV-F	ATCCCTTTTCAGTTCGCT	438	Gauthier et al., 2011
	VdMLV-R	AGAAGAGACTTCAAGGAC		

CHAPTER 5

WIDESPREAD OCCURRENCE OF HONEY BEE PATHOGENS IN SOLITARY BEES

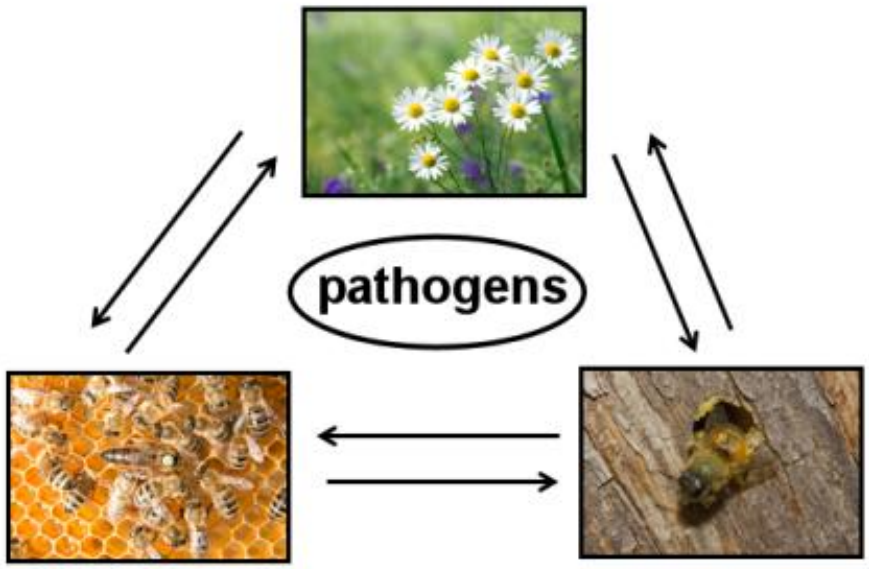
The work presented in Chapter 5 was adapted from the following manuscript:

J. Ravoet, L. De Smet, I. Meeus, G. Smagghe, T. Wenseleers, D.C. de Graaf. Widespread occurrence of honey bee pathogens in solitary bees. Journal of Invertebrate Pathology, 2014, 122: 55-58.
doi: 10.1016/j.jip.2014.08.007.

Contributions

D.C. de Graaf assisted with the study design. J. Ravoet executed all experiments, supported by L. De Smet. I. Meeus and G. Smagghe provided reagents. D.C. de Graaf and J. Ravoet wrote the manuscript, assisted by all co-authors. T. Wenseleers contributed by improving the language of the manuscript.

1. Graphical abstract



2. Abstract

Solitary bees and honey bees from a neighbouring apiary were screened for a broad set of putative pathogens including protists, fungi, spiroplasmas and viruses. Most sampled bees appeared to be infected with multiple parasites. Interestingly, viruses exclusively known from honey bees such as *Apis mellifera* Filamentous Virus and *Varroa destructor* Macula-like Virus were also discovered in solitary bees. A microsporidium found in *Andrena vaga* showed most resemblance to *Nosema thomsoni*. Our results suggest that bee hives represent a putative source of pathogens for other pollinators. Similarly, solitary bees may act as a reservoir of honey bee pathogens.

3. Introduction

There is a long tradition of studying the pathogens of the Western honey bee (*Apis mellifera*) and the list of honey bee pathogens has been expanded significantly from the sixties on. It became recently evident that common honey bee pathogens such as Deformed Wing Virus (DWV) can infect other bees as well (Furst et al., 2014; Levitt et al., 2013). Although several macroparasites of wild bees are well known (Westrich, 1990), reports on their microparasites are rather scarce. The few known

solitary bee specific parasites are fungi, including *Ascosphaera* spp. (Wynns et al., 2013) and *Antonospora scoticae* (Fries et al., 1999). The aim of this study was to investigate whether solitary bees sampled nearby an apiary harbour some of the known or recently discovered honey bee pathogens (Runckel et al., 2011). We screened for a broad set of parasitic micro-organisms, including fungi, protists, spiroplasmas and viruses.

4. Material and methods

4.1 Sample collection

For each species, three pooled samples of 10 bees were collected in 2012 at campus Sterre of Ghent University. *Osmia bicornis*, *Osmia cornuta* and *Heriades truncorum* were sampled at a bee hotel, close to an apiary. *Andrena vaga* and *Andrena ventralis* (only one sample of 10 bees) were caught nearby their nest aggregations. These species were identified using suitable keys (Scheuchl, 1996; Schmid-Egger and Scheuchl, 1997). Three different honey bee colonies were simultaneously (within one week) sampled at the neighbouring apiary.

4.2 RNA and DNA extraction

Ten bees were homogenised in 5 ml PBS in the presence of glass beads. Total RNA was extracted from 100 µl supernatant using the RNeasy Lipid Tissue Kit (Qiagen). Using random hexamer primers, 1 µg RNA was retro-transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). DNA was extracted from 120 µl supernatant using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions for animal tissues.

4.3 PCR and BeeDoctor analysis

All PCR reaction mixtures contained: 2 µM of each primer (Table A1); 1.0 mM $MgCl_2$; 0.2 mM dNTPs; 1.25 U Hotstar Taq DNA polymerase (Qiagen) and 1 µl cDNA or 3 µl DNA product (*Apis mellifera* Filamentous Virus (AmFV) and *Ascosphaera* spp. Detection) using described PCR cycles (Table A1). For our developed primers we used: 94°C for 15 min; [94°C for 30 s, 50°C (*Crithidia* spp. cytochrome *b*) or 55°C (LSV Orf1) for 30 s, 72°C for 1 min] 35 cycles, 72°C for 10 min. Positive and negative

controls were always included. Amplicons intended for Genbank submission were amplified with Hotstar High Fidelity Taq DNA polymerase (Qiagen). PCR products were electrophoresed using 1.4% agarose gels, stained with ethidium bromide and visualised under UV light. Honey bee samples (1 µl RNA) were screened using the BeeDoctor tool, capable of detecting actin (honey bee control gene), Acute Bee Paralysis Virus (ABPV), BQCV, Chronic Bee Paralysis Virus (CBPV), DWV, Sacbrood Virus (SBV) and Slow Bee Paralysis Virus (SBPV) (De Smet et al., 2012). ABPV probes amplify ABPV, Israeli Acute Paralysis Virus and Kashmir Bee Virus; DWV probes amplify DWV, Kakugo Virus and *Varroa destructor* Virus-1 (VDV-1). Solitary bees were analysed for these viruses using RT-PCR. Other viruses (Aphid Lethal Paralysis Virus strain Brookings (ALPV), AmFV, Big Sioux River Virus (BSRV), Lake Sinai Virus (LSV), Tobacco Ringspot Virus (TRSV) and *Varroa destructor* Macula-like Virus (VdMLV), bacteria (*Spiroplasma* spp.), fungi (*Ascosphaera* spp. and *Nosema* spp.) and protists (*Apicystis bomb*i and *Crithidia* spp.) were screened by PCR. The cytochrome *c* oxidase subunit I gene was used as control gene for the solitary bees.

4.4 Sequence analysis

Amplicons were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). Plasmids and PCR products were sequenced using gene-specific or M13 primers. Sequences are deposited as AB859946-AB859948 (DWV), AB860145-AB860146 (*Crithidia* spp.), AB859949-AB859952 (VdMLV), HG764796-HG764797 (BQCV), HG764798-HG764799 (SBV), KF768348-KF68351 (LSV), KJ685944 (AmFV), KJ685945-KJ685947 (*Ascosphaera* spp.). Phylogenetic trees were inferred via maximum likelihood (ML) using PhyML 3.0 (Guindon et al., 2010) with the Le-Gascuel (LG) amino acid substitution model (Le and Gascuel, 2008) and approximate likelihood ratio test non-parametric branch support based on a Shimodaira-Hasegawa-like (aLRT SH-like) procedure (Anisimova and Gascuel, 2006).

5. Results and discussion

We were able to demonstrate the presence of AmFV in all samples. Most other viruses (BQCV, DWV, SBV and VdMLV) were detected both in honey bees and in a smaller subset of solitary bees (see Table 1). ALPV was detected in honey bee samples only, but the viruses ABPV, BSRV, CBPV, SBPV and TRSV were not discovered at all. Protists and fungi appeared to be pervasive, whereas spiroplasmas were found only scarcely.

The discovered AmFV sequences were mutually identical and highly similar to Baculovirus sequences from Swiss bees (Genbank: JF304814) and *V. destructor* mites (Cornman et al., 2010) (Fig. A.1). Further, amplicons of the ribonucleotide reductase small subunit and thymidylate synthase were identical to those found in *V. destructor* (Genbank: GU980896-GU980897). The LSV strains found in honey bees, *A. vaga*, *O. bicornis* and *O. cornuta* were almost identical (Genbank: KF768348-KF68350). However, a deviating LSV strain (Genbank: KF768351) was detected both in *A. ventralis* and in some honey bees. Their Orf1 sequences showed only 76.7% amino acid similarity with each other. DWV was detected in all honey bee hives and in *O. bicornis*. Their 5' untranslated region (UTR) was equal to published DWV sequences. Although the 5' UTR amplicons appeared mutually identical, phylogenetic analysis of the DWV L protein resulted in two divergent strains (Figure 1). Strain e3 (Genbank: AB859948), only detected in honey bees, was part of the DWV clade. The other strains e5 and e10 (Genbank: AB859946, AB859947), detected in (different) honey bees and in *O. bicornis*, appeared to lie between DWV and VDV-1. The finding of VdMLV in several solitary bees was remarkable as they are not a known host of *V. destructor*, which is considered to be the primary vector (de Miranda et al., 2011). This indicates that transmission can occur by other means, like contaminated flowers (Singh et al., 2010).

Honey bee tryponasomatids were identified as *Crithidia mellificae* haplotype A (Morimoto et al., 2013) by their cytochrome *b* sequence (Genbank: AB860145). *O. bicornis* and *A. vaga* appeared to be infected with *Crithidia bombi* (Genbank: AB860146), hitherto only reported in bumble bees (Macfarlane et al., 1995).

The neogregarine *A. bombi*, found in the present study in all bee species, was previously only discovered in social bees (Lipa and Triggiani, 1996). Sequencing of its internal transcribed spacer (ITS) region from isolates of honey bees and *O. cornuta* assigned them as haplotype Uni (Maharramov et al., 2013).

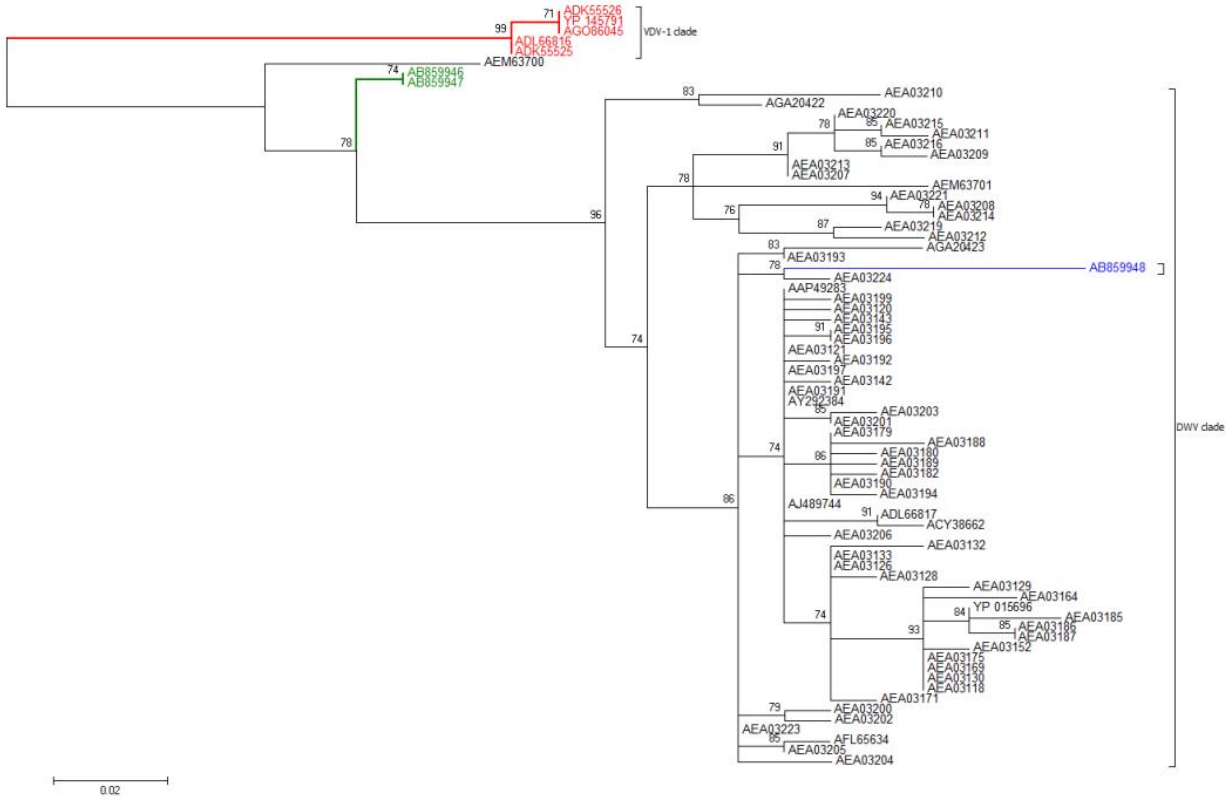


Figure 1. *Phylogeny of DWV L protein amino acid sequences of various isolates, including VDV-1 and Kakugo Virus. The phylogenetic tree was constructed using the maximum likelihood method under the LG parameter. Each sequence is indicated by its Genbank accession number. Strain e3 (Genbank: AB859948) is designated in blue, strains e5 and e10 (Genbank: AB859946, AB859947) in green. The VDV-1 clade is showed in red. Brach support for each node is indicated by aLRT values.*

Ascosphaera apis was detected in several honey bee samples. The other *Ascosphaera* spp. that we discovered in *O. cornuta* and *H. truncorum* (Genbank: KJ685945-KJ685947) matched (~98%) *Ascosphaera callicarpa* (Genbank: JX070046), previously reported from *Chelostoma florisomne* (Megachilidae) (Wynns et al., 2013). The related *Bettsia alvei* was also found in *O. cornuta*. Several sequences had no strong match (<93%) as reported before (Evison et al., 2012).

***Table 1**: Summary of pathogens detected in honey bees and solitary bees. Hive 3, 5 and 10: hive identification number*

Sample	Date	Virus	*Crithidia* spp.	*Neogregarinida*	*Nosema* spp.	*Ascosphaera* spp.	*Spiroplasma* spp.
Hive 3-1	4 April 2012	ALPV, BQCV, LSV, AmFV	-	*A. bombi*	*N. apis, N. ceranae*	-	-
Hive 5-1	4 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. apis, N. ceranae*	-	-
Hive 10-1	4 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	-
O. cornuta 1	4 April 2012	BQCV, LSV, AmFV, VdMLV	-	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	-
Hive 3-2	16 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. apis, N. ceranae*	*A. apis*	-
Hive 5-2	16 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*A. apis*	-
Hive 10-2	16 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*A. apis*	-
O. cornuta 2	16 April 2012	BQCV, LSV, AmFV, VdMLV	-	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	-
Hive 3-3	23 April 2012	ALPV, BQCV, LSV, AmFV	-	*A. bombi*	*N. apis, N. ceranae*	*Ascosphaera* spp.	-
Hive 5-3	23 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	-
Hive 10-3	23 April 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	-
O. cornuta 3	24 April 2012	BQCV, LSV, AmFV, VdMLV	-	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	-
A. vaga 1	24 April 2012	BQCV, LSV, AmFV	*C. bombi*	*A. bombi*	*N. thomsoni*	-	-
A. ventralis	24 April 2012	LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	-
Hive 3-4	15 May 2012	ALPV, BQCV, LSV,	-	*A. bombi*	*N. apis, N.*	*A. apis*	-

		AmFV			*ceranae*		
Hive 5-4	15 May 2012	ALPV, BQCV, LSV, AmFV	-	*A. bombi*	*N. ceranae*	-	-
Hive 10-4	15 May 2012	ALPV, BQCV, LSV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*A. apis*	-
A. vaga 2	8 May 2012	LSV, SBV, AmFV	*C. bombi*	*A. bombi*	*N. thomsoni*	-	-
A. vaga 3	8 May 2012	LSV, AmFV	*C. bombi*	*A. bombi*	*N. thomsoni*	-	
Hive 3-5	31 May 2012	ALPV, BQCV, DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	*S. melliferum*
Hive 5-5	31 May 2012	ALPV, BQCV, DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	-
Hive 10-5	31 May 2012	BQCV, DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	-
O. bicornis 1	30 May 2012	DWV, LSV, AmFV, VdMLV	*C. bombi*	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	*S. melliferum*
O. bicornis 2	30 May 2012	DWV, LSV, AmFV, VdMLV	*C. bombi*	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	*S. apis*
O. bicornis 3	30 May 2012	DWV, LSV, AmFV, VdMLV	*C. bombi*	*A. bombi*	*N. ceranae*	*Ascosphaera* spp.	*S. melliferum*
Hive 3-6	9 July 2012	ALPV, BQCV, DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	*S. melliferum*
Hive 5-6	9 July 2012	DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	*A. apis*	*S. melliferum*
Hive 10-6	9 July 2012	ALPV, BQCV, DWV, LSV, VdMLV, AmFV	*C. mellificae*	*A. bombi*	*N. ceranae*	-	*S. melliferum*
H. truncorum 1	5 July 2012	BQCV	-	-	*N. ceranae*	-	-
H. truncorum 2	5 July 2012	-	-	-	*N. ceranae*	-	-
H. truncorum 3	5 July 2012	BQCV	-	*A. bombi*	*N. ceranae*	-	-

The microsporidian parasite *N. ceranae* was detected in all species, except *A. vaga*. This gut parasite was previously also detected in bumble bees (Furst et al., 2014; Graystock et al., 2013; Li et al., 2012). Some honey bees were co-infected with *N. apis*. Surprisingly, the microsporidium detected in *A. vaga* (Genbank: KC596023) appeared to be highly related (99.8%) to *N. thomsoni* and other microsporidia from the same clade (Li et al., 2012). The spiroplasmas found in honey bees appeared to be *Spiroplasma melliferum*. *Spiroplasma apis* and *S. melliferum* were both discovered in *O. bicornis*, an unprecedented host (Clark et al., 1985; Mouches et al., 1983).

6. Conclusions

Our study identified several honey bee pathogens in solitary bees living in the proximity of an apiary. Our results suggest that bee hives represent a putative source of pathogens for other pollinators. Similarly, solitary bees may act as a reservoir of honey bee pathogens.

7. Acknowledgment

This work was supported by the Research Foundation-Flanders (FWO, research grant G.0628.11).

8. Corrigendum

As demonstrated by Schwarz and colleagues (Schwarz et al., 2015), two trypanosomatid species can infect honey bees, namely *C. mellificae* and *Lotmaria passim*. It appears that nearly all of the *C. mellificae* identified the Belgian samples should be redubbed *L. passim* (J. Ravoet, unpublished information; Chapter 8).

8. References

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55**,** 539-552.

Clark, T.B., Whitcomb, R.F., Tully, J.G., Mouches, C., Saillard, C. and others, 1985. Spiroplasma melliferum, a new species from the honeybee (Apis mellifera). Int J Syst Bacteriol 35**,** 296-308.

Cornman, R.S., Schatz, M.C., Johnston, S.J., Chen, Y.P., Pettis, J. and others, 2010. Genomic survey of the ectoparasitic mite Varroa destructor, a major pest of the honey bee Apis mellifera. Bmc Genomics 11.

de Miranda, J.R., Gauthier, L., Ribière, M., Chen, Y., 2011. Honey bee viruses and their effect on bee and colony health. In: Sammataro, D., Yoder, J.A. (Eds.), Honey Bee Colony Health: Challenges and Sustainable Solutions CRC Press, pp. 71-112.

De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y. and others, 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One 7**,** e47953.

Evison, S.E., Roberts, K.E., Laurenson, L., Pietravalle, S., Hui, J. and others, 2012. Pervasiveness of parasites in pollinators. PLoS One 7**,** e30641.

Fries, I., Paxton, R.J., Tengo, J., Slemenda, S.B., da Silva, A.J., Pieniazek, N.J., 1999. Morphological and molecular characterization of Antonospora scoticae n. gen., n. sp (Protozoa, Microsporidia) a parasite of the communal bee, Andrena scotica Perkins, 1916 (Hymenoptera, Andrenidae). Eur J Protistol 35**,** 183-193.

Furst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506**,** 364-366.

Graystock, P., Yates, K., Darvill, B., Goulson, D., Hughes, W.O., 2013. Emerging dangers: Deadly effects of an emergent parasite in a new pollinator host. J Invertebr Pathol 114**,** 114-119.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59**,** 307-321.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K. and others, 2013. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 176**,** 232-240.

Li, J.L., Chen, W.F., Wu, J., Peng, W.J., An, J.D. and others, 2012. Diversity of Nosema associated with bumblebees (Bombus spp.) from China. Int J Parasitol 42**,** 49-61.

Lipa, J.J., Triggiani, O., 1996. Apicystis gen. nov. and Apicystis bombi (Liu, Macfarlane & Pengelly) comb. nov. (Protozoa: Neogregarinida), a cosmopolitan parasite of Bombus and Apis (Hymenoptera: Apidae). Apidologie 27**,** 29-34.

Macfarlane, R.P., Lipa, J.J., Liu, H.J., 1995. Bumble bee pathogens and internal enemies. Bee World 76**,** 130-148.

Maharramov, J., Meeus, I., Maebe, K., Arbetman, M., Morales, C. and others, 2013. Genetic variability of the neogregarine Apicystis bombi, an etiological agent of an emergent bumblebee disease. PLoS One 8**,** e81475.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B. and others, 2013. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. Env Microbiol Rep 5**,** 74-77.

Mouches, C., Bove, J.M., Tully, J.G., Rose, D.L., Mccoy, R.E. and others, 1983. Spiroplasma apis, a new species from the honeybee Apis mellifera. Ann Inst Pasteur Mic A134**,** 383-397.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Scheuchl, E., 1996. Illustrierte Bestimmungstabellen der Wildbienen Deutschlands und Österreichs. Band II: Megachilidae - Melittidae. Eigenverlag, Velden.

Schmid-Egger, C., Scheuchl, E., 1997. Illustrierte Bestimmungstabellen der Wildbienen Deutschlands und Österreichs unter Berücksichtigung der Arten der Schweiz. Band III: Andreninae. Eigenverlag, Velden.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA viruses in hymenopteran pollinators: Evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5**,** e14357.

Schwarz, R.S., Bauchan, G., Murphy, C., Ravoet, J., de Graaf, D.C., Evans, J.D. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* n. gen., n. sp. J Eukaryot Microbiol. 2015, in press.

Westrich, P., 1990. Die Wildbienen Baden-Württembergs. Spezieller Teil. Eugen Ulmer Verlag, Stuttgart.

Wynns, A.A., Jensen, A.B., Eilenberg, J., 2013. Ascosphaera callicarpa, a new species of bee-loving fungus, with a key to the genus for Europe. PLoS One 8**,** e73419.

9. Supplementary information

Figure A.1: *Alignment of Baculovirus repeated open reading frame N-terminal domain (BroN) gene sequences. VDK00064516-1660_1, present in contig 154 of additional file 2 of (Cornman et al., 2010) was detected in Varroa mites in the USA. JF304814 originated from honey bees in Switzerland, KJ685944 was amplified in honey bees and several solitary bee species in Belgium.*

Table A.1: List of PCR primer used for each pathogen in this study.

Target	Primers	Sequence (5'-3')	Size (bp)	Reference
A. bombi	ApBF1	CGTACTGCCCTGAATACTCCAG	294	(Meeus et al., 2010)
	ApBR1	TGAAAGCGGCGTATACATGA		
AKI	AKI-F	CTTTCATGATGTGGAAACTCC	100	(Francis and Kryger, 2012)
	AKI-R	AAACTGAATAATACTGTGCGTA		
ALPV-Br	ALP-Br-F-2936	AACGTCGTATGCTACGATGAACTCG	464	(Runckel et al., 2011)
	ALP-Br-R-3400	GGGTTAAATTCAATTCCAGTACCACG G		
AmFV	AmFV-BroN-F *	CAGAGAATTCGGTTTTTGTGAGTG	551	(Hartmann et al., 2012)
	AmFV-BroN-R *	CATGGTGGCCAAGTCTTGCT		
	rrSSU-F	ACGAACGACTATCTAGCCATGAAC	591	(Cornman et al., 2010)
	rrSSU-R	GTCCGTTTCGGAGTGCATGAC		
	TS-F	CGCATGTACCAACAACTCGTAC	361	(Cornman et al., 2010)
	TS-R	CACAGTTGGTGTAGCGCAGT		
Ascosphaera spp	AscoAll1 *	GCACTCCCACCCTTGTCTA	550	(James and Skinner, 2005)
	AscoAll2 *	GAWCACGACGCCGTCACT		
	TW81	GTTTCCGTAGGTGAACCTGC	variable	(Curran et

	AB28	ATATGCTTAGTTCAGCGGGT		al., 1994)
BSRV	BSRV-4714F	RGTGCAGCTTTATGCGTTGCC	519	(Runckel et al., 2011)
	BSRV-37R	CCGCTGTTGAGAATAAGGATATCCAGG		
BQCV	BQCV-UTR-F	TGGTCAGCTCCCACTACCTTAAAC	700	(Singh et al., 2010)
	BQCV-UTR-R	GCAACAAGAAGAAACGTAAACCAC		
CBPV	CBPV 1-1	TCAGACACCGAATCTGATTA TG	570	(Blanchard et al., 2008)
	CBPV 1-2	ACTACTAGAAACTCGTCGCTTCG		
DWV L protein	DWV-F1425 *	CGTCGGCCTATCAAAG	417	(Forsgren et al., 2009)
	DWV-B1806 *	CTTTTCTAATTCAACTTCACC		
DWV UTR	DWV 20f	CGAATTACGGTGCAACTAAC	559	(Berenyi et al., 2007)
	DWV 578r	ACAATAGATGGTCGGTGACA		
LSV complex	LSVdeg-F *	GCCWCGRYTGTTGGTYCCCCC	578	(Ravoet et al., 2013)
	LSVdeg-R *	GAGGTGGCGGCGCSAGATAAAGT		
	770LSVorf-F	ACGATGTGCAGYYATGAGTA	770	This study
	770LSVorf-R	GAGGCCAACTGRTCAGG		
	866LSVorf-F	CGCCTGAYCAGTTGGCC	866	This study
	866LSVorf-R	CGWGGCCTCAGCACGA		
Nosema apis	NosaRNAPol-F2	AGCAAGAGACGTTTCTGGTACCTCA	297	(Gisder and Genersch, 2013)
	NosaRNAPol-R2	CCTTCACGACCACCCATGGCA		
Nosema ceranae	NoscRNAPol-F2	TGGGTTCCCTAAACCTGGTGGTTT	662	(Gisder et al., 2013)
	NoscRNAPol-R2	TCACATGACCTGGTGCTCCTTCT		
Nosema spp.	NOS-FOR *	TATGCCGACGATGTGATATG	~250	(Fernandez et al., 2012)
	NOS-REV *	CACAGCATCCATTGAAAACG		(Higes et al., 2006)
	SS18SF	GTTGATTCTGCCTGACGT	~1240	(Weiss and Vossbrinck, 1999)
	SS1537R	TTATGATCCTGCTAATGGTTC		
SBV	SBV-VP1b-F	GCACGTTTAATTGGGGATCA	693	(Singh et al., 2010)
	SBV-VP1b-R	CAGGTTGTCCCTTACCTCCA		
SBPV	F8156	GATTTGCGGAATCGTAATATTGTTTG	868	(de Miranda et al., 2010)
	B9023	ACCAGTTAGTACACTCCTGGTAACTT		

		CG		
Spiroplasma spp.	BS1-F	AAGTCGAACGGGGTGCTT	976	(Meeus et al., 2012)
	BS1-R	TGCACCACCTGTCTCAATGT		
Trypanosomatids	SEF *	CTTTTGGTCGGTGGAGTGAT	406	(Meeus et al., 2010)
	SER *	GGACGTAATCGGCACAGTTT		
	Tryp-cytb-F	TGTGGWGTKTGTTTAGC	490	This study
	Tryp-cytb-R	CRTCWGAACTCATAAAATAATG		
TRSV	TRSV-F2	GTGTGCTGTGACGGTTGTTCC	731	(Li et al., 2014)
	TRSV-R2	TGCCAGACCACCCAAGATTCC		
VdMLV	VdMLV-F	ATCCCTTTTCAGTTCGCT	438	(Gauthier et al., 2011)
	VdMLV-R	AGAAGAGACTTCAAGGAC		

A. bombi: *Apicystis bombi*, ABPV: Acute Bee Paralysis Virus complex, ALPV-Br: Aphid Lethal Paralysis Virus strain Brookings, AmFV: *Apis mellifera* Filamentous Virus, BSRV: Big Sioux River Virus, BQCV: Black Queen Cell Virus,CBPV: Chronic Bee Paralysis Virus, DWV: Deformed Wing Virus (DWV) complex, LSV: Lake Sinai Virus, SBV: Sacbrood Virus, SBPV: Slow Bee Paralysis Virus, TRSV: Tobacco Ringspot Virus, UTR: untranslated region, VdMLV: *Varroa destructor* Macula-like Virus

* PCR primers used for screening purposes

CHAPTER 6

VERTICAL TRANSMISSION OF HONEY BEE VIRUSES IN A BELGIAN QUEEN BREEDING PROGRAM

The work presented in Chapter 6 was adapted from the following manuscript:

J. Ravoet, L. De Smet, T. Wenseleers, D.C. de Graaf. Vertical transmission of honey bee viruses in a Belgian queen breeding program. BMC Veterinary Research, 2015, In Press.

Contributions

D.C. de Graaf designed the study. J. Ravoet performed all the experimental work, assisted by L. De Smet. T. Wenseleers carried out the statistical analysis. D.C. de Graaf and J. Ravoet wrote the manuscript, assisted by all co-authors.

1. Abstract

The Member States of European Union are encouraged to improve the general conditions for the production and marketing of apicultural products. In Belgium, programmes on the restocking of honey bee hives have run for many years. Overall, the success ratio of this queen breeding programme has been only around 50%. To tackle this low efficacy, we organized sanitary controls of the breeding queens in 2012 and 2014. We found a high quantity of viruses, with more than 75% of the egg samples being infected with at least one virus. The most abundant viruses were Deformed Wing Virus and Sacbrood Virus (≥ 40%), although Lake Sinai Virus and Acute Bee Paralysis Virus were also occasionally detected (between 10-30%). In addition, Aphid Lethal Paralysis Virus, Black Queen Cell Virus, Chronic Bee Paralysis Virus and *Varroa destructor* Macula-like Virus occurred at very low prevalences (≤ 5%). Remarkably, we found *Apis mellifera carnica* bees to be less infected with Deformed Wing Virus than Buckfast bees ($p < 0.01$), and also found them to have a lower average total number of infecting viruses ($p < 0.001$). This is a significant finding, given that Deformed Wing Virus has earlier been shown to be a contributory factor to winter mortality and Colony Collapse Disorder. Moreover, negative-strand detection of Sacbrood Virus in eggs was demonstrated for the first time. High pathogen loads were observed in this sanitary control program. We documented for the first time vertical transmission of some viruses, as well as significant differences between two honey bee races in being affected by Deformed Wing Virus. Nevertheless, we could not demonstrate a correlation between the presence of viruses and queen breeding efficacies.

2. Background

In view of the spread of varroasis – a mite infestation of the honey bee – over Europe and the problems which this disease has brought about in the beekeeping sector, the Member States of the European Union have been encouraged to set up national programmes aimed at improving the general conditions for the production and marketing of apicultural products. In Belgium, such apicultural programmes now exist for many years and particularly in the Flemish region, a lot of effort has been put in the restocking of hives. Within this programme, a limited number of recognized breeders are provided with the possibility to travel to a land mating yard in Belgium

(Kreverhille) and island mating yards in Germany (Spiekeroog, Norderney) and the Netherlands (Ameland, Marken) with selected virgin queens. When these fertilized queens perform well they become the new breeding queens two years later, and are distributed on a large scale among the other beekeepers. Overall, this programme enjoyed a high participation rate amongst the beekeepers, but failed to a certain extent in terms of the efficacy of the queen breeding programme. This is evident from the fact that in the past four years, between 5,948 and 6,195 larvae were grafted, but only 61.4-70.8% could be raised to newborn queens and from these only 75.0-79.9% became egg-laying. Thus overall, the success ratio of the queen breeding programme has been only 49.1-53.1%, a fairly low number (Buchler et al., 2013).

One of the measures that were taken to tackle this low breeding efficacy was the publication and distribution of a technical brochure describing the proper way to introduce a new queen into a bee colony. Since the problems persisted, we subsequently organized sanitary controls of the breeding queens in 2012 and 2014. This measure was taken given that honey bees can be exposed to several single stranded RNA viruses and transmission can occur both horizontally and vertically (reviewed by Chen et al. (Chen et al., 2006a; Chen and Siede, 2007)). In horizontal transmission, viruses are transmitted among individuals of the same generation. Vertical transmission occurs from mothers to their offspring and can have two main causes: (I) infected sperm originating from the drones and (II) contaminated eggs originating from infected spermatheca and/or ovaries of the queen. The reproducing individuals, the queen and the drones, have a protective status in the colony because they are fed by the nurse bees. Nevertheless, both castes are susceptible to parasites. Several viruses were already demonstrated in individual queens and drones (Chen et al., 2005a; Chen et al., 2006b; Chen et al., 2005b; Gregorc and Bakonyi, 2012; Retschnig et al., 2014; Shen et al., 2005). The presence of viruses in reproductive tissues of queens and drones were also investigated (Fievet et al., 2006; Francis et al., 2013; Gauthier et al., 2011; Yanez et al., 2012; Yue et al., 2006).

A non-destructive method to investigate whether vertical transmission occurs relies on examination of freshly laid eggs. In this study, we focused on a number of commonly occurring bee

viruses (Chen et al., 2007) e.g. Deformed Wing Virus (DWV), but also on a set of viruses that were recently discovered in the USA such as Lake Sinai Virus (LSV) (Runckel et al., 2011), and which we discovered to be present in Belgian apiaries as well (Ravoet et al., 2013). Moreover, using the BeeDoctor diagnostic tool (De Smet et al., 2012) which is based on the multiplex ligation-dependent probe amplification technology, we were also able to screen in parallel for the negative-strand intermediate.

Both *Apis mellifera carnica*-breeders and Buckfast-breeders participated in our study. *Apis mellifera carnica* or the carniolan honey bee is the subspecies of the European honey bee native to the Balkan Peninsula and represents the majority of Belgian bee populations due to massive import. This race is favoured for several reasons, e.g. non-aggressiveness and honey yield. The Buckfast bee is a combination race, a cross of various *Apis mellifera* subspecies and was developed in the United Kingdom during several decades.

3. Methods

Flemish honey bee queen breeders were instructed to collect 10 eggs from worker cells from the same honey bee colony, per sample. In the summer of 2012, 35 queen breeders collected a sample from one colony each. In 2014, a further 43 egg samples were obtained. This set originated from 11 queen breeders, who surveyed each several colonies, varying from one to nine. This resulted in a total of 78 egg samples used in this study. The eggs were preserved at -20°C, transported to the laboratory on dry ice and then stored at -80°C until the RNA was isolated, using the RNeasy Lipid Tissue (Qiagen). The eggs were homogenised in the presence of zirconium beads and 0.5 ml QIAzol lysis reagent (Qiagen). Using random hexamer primers, 200 ng RNA was retro-transcribed with the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific).

Although the BeeDoctor tool is capable to detect ten honey bee viruses (De Smet et al., 2012), we did not obtained good results due to the low virus concentrations in the eggs. Therefore, we examined the egg samples by RT PCR assays for the presence of viruses of the Acute Bee Paralysis

Virus (ABPV) complex (Francis and Kryger, 2012), Aphid Lethal Paralysis Virus strain Brookings (ALPV) (Ravoet et al., 2013), Black Queen Cell Virus (BQCV) (Singh et al., 2010), Chronic Bee Paralysis Virus (CBPV) (Blanchard et al., 2008),DWV (Forsgren et al., 2009), LSV (Ravoet et al., 2013), Sacbrood Virus (SBV) (Singh et al., 2010) and *Varroa destructor* Macula-like Virus (VdMLV) (Gauthier et al., 2011). Samples positive for the ABPV complex were re-analysed with specific primers for ABPV (Tentcheva et al., 2004), Israeli Acute Bee Paralysis Virus (Palacios et al., 2008) and Kashmir Bee Virus (Tentcheva et al., 2004). We used honey bee β-actin (Scharlaken et al., 2008) as a control gene to monitor the efficiency of the PCR reaction and its previous steps. All PCR reaction mixtures contained: 2 µM of each primer; 1 mM $MgCl_2$; 0.2 mM dNTPs; 1.2 U Hotstar Taq DNA polymerase (Qiagen) and 2 µl cDNA product.

Positive samples from the 2012 screening were analysed for the negative-strand of each detected virus, namely ABPV, ALPV, DWV, LSV and SBV. They were screened with the BeeDoctor tool (De Smet et al., 2012) in its uniplex modus, using 3 µl RNA.

PCR products were separated by electrophoresis using 1.4% agarose gels or 4% high resolution agarose gels for the MLPA PCR products, stained with ethidium bromide and visualised under UV light. Amplicons of each virus were sequenced on an ABI 3130XL platform with M13 primers after cloning with the TOPO TA Cloning Kit for sequencing (Invitrogen). DNA sequences were analysed using Geneious R7.

The incidence of the screened viruses (percentage infected) as well as the total virus load (total number of detected viruses) in *carnica* and Buckfast bees was compared using binomial and Poisson generalized linear mixed models with function glmer in package lme4 v. 1.1-7 in R v. 3.1.1. In these analyses, race and year were coded as fixed factors and breeder was coded as a random factor, and significance was assessed using Wald tests. Least square means on average infection percentages and total virus load and 95% Wald confidence limits were calculated using the effects package v. 3.0-3. Finally, a linear regression analysis was used to test the effect of virus load (total number of infecting viruses) on the percentage of queens that were born from grafted larvae, the

percentage of queens that went on to lay out of all larvae that were grafted and the percentage of all queens that were born that went on to lay. This analysis was performed in GraphPad Prism 6.

4. Results and discussion

In this study, we found a high prevalence of different honey bee viruses in eggs used in queen breeding operations (Table S1). Although we investigated representative samples consisting of ten eggs per sample, false negatives can be present. Over two sampling years, 75% (58/78) of the egg samples were infected with at least one virus whereof 32% (25/78) of the samples were infected with a single virus and 42% (33/78) were infected with multiple viruses (Figure 1).

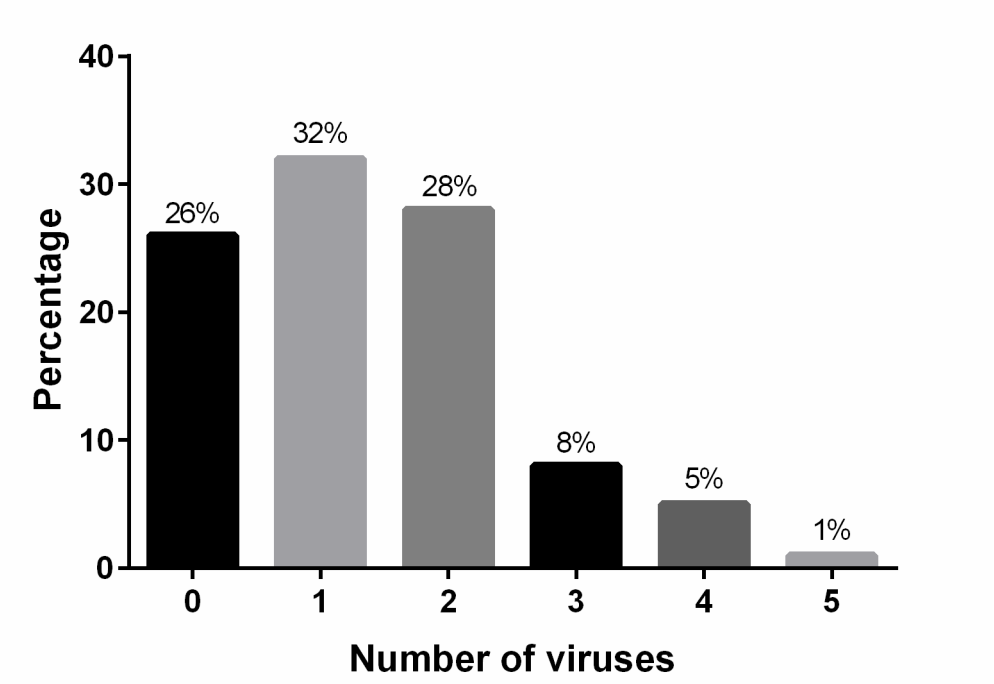


Figure 1*. Number of detected viruses and their prevalences. The samples used in our study were co-infected with a number of viruses, ranging from 1 to 5. In almost 26% of the samples were no viruses detected.*

The most abundantly detected viruses were DWV (40%, 31/78) and SBV (42%, 33/78). LSV and ABPV were moderately detected in 28% (22/78) and 14% (11/78) of the samples. The other viruses ALPV, BQCV, CBPV and VdMLV had only low prevalences, respectively 5% (4/78), 5% (4/78), 1% (1/78) and 3% (2/78). Remarkably, *carnica* had a significantly lower infection rate with DWV than

Buckfast [binomial GLMM, z=-3.048, p=0.002, 30% mean infection rate in *carnica* ([20%, 43%] 95% C.L.) vs. 73% mean infection rate in Buckfast ([49%, 88%] 95% C.L.)] (Figure 2) as well as a significantly lower total virus load (total number of detected viruses) per sample [Poisson GLMM, z=-3.911, p=9.10^{-5}, average of 1.1 infecting viruses in *carnica* ([0.8, 1.4] 95% C.L.) vs. an average of 2.3 infecting viruses in Buckfast ([1.7, 3.2] 95% C.L.)]. No significant differences were found in the incidence of the other viruses screened (binomial GLMM, $p > 0.05$).

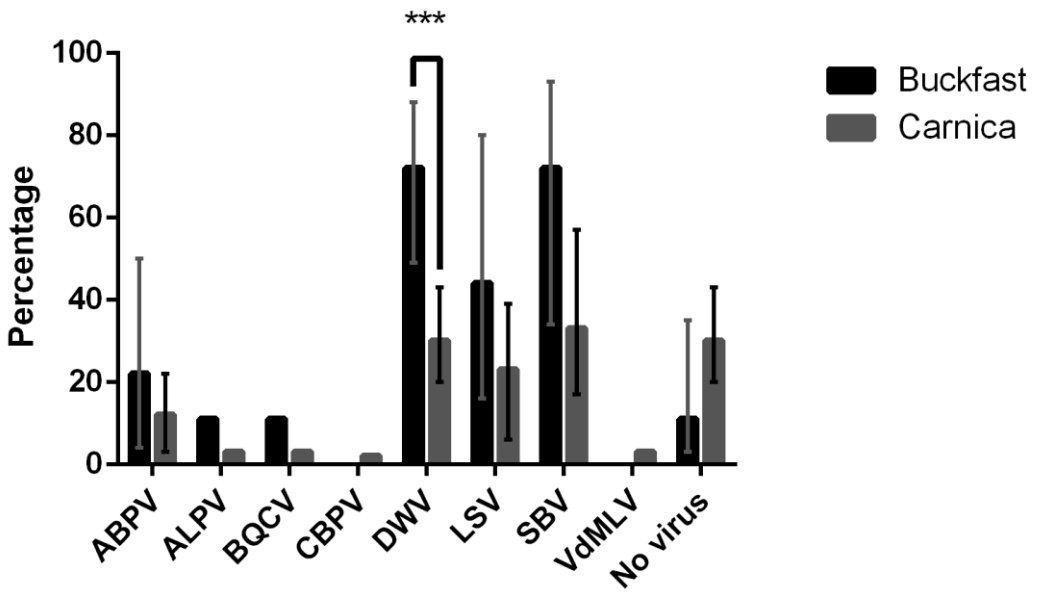


***Figure 2**. Comparison of the incidence of different viruses in* Apis mellifera carnica *and Buckfast bees, together with 95% confidence limits based on fitted binomial mixed models (incidence over the sampling years 2012 and 2014 was averaged and bee breeder was included as a random factor, n=78 samples). Accurate confidence limits could not be calculated for species with very low infection rates (≤5%), and are omitted from this graph. ***: significant difference with p < 0.01 (Wald tests, binomial GLMM).*

Our results, however, did not indicate a correlation between the virus burden (total number of infecting viruses) and queen breeding efficacy (Figure S1). It might be the case though that variation in beekeeping management skills required for successful queen breeding (Buchler et al., 2013) hides any effect of virus burden on queen breeding efficacy. Given the important effects that some of the viruses detected here have on honey bee health, including a large effect on winter

mortality (Berthoud et al., 2010; Genersch et al., 2010; Highfield et al., 2009; Nguyen et al., 2011), delayed negative effects on honey bee health are likely, particularly given the implied vertical transmission to offspring workers. Indeed, this study is the first to document vertical transmission for ALPV, LSV and VdMLV. This is another confirmation that these viruses can infect honey bees, especially given that the negative strand was previously detected (Ravoet et al., 2013; Runckel et al., 2011). Moreover, BQCV lethally affects developing queen larvae and pupae. After death of the pupae, the wall of the queen cell eventually colours dark (Chen et al., 2007). This virus is reported to be a common cause of queen larvae mortality (Anderson, 1993) and is correlated with the queenless condition of an apiary (Nguyen et al., 2011).

Furthermore, we have detected the negative-strand of SBV. Although this might indicate that SBV replicates in eggs, it is also possible that this originates from transovum transmission, such as surface contamination with sperm containing negative-strand RNAs. Replication of SBV was previously reported in adults and larvae of European (*A. mellifera*) and Asian honey bees (*Apis cerana*) (Bailey, 1968; Bailey, 1969; Mussen and Furgala, 1977). This virus is frequently found in adult bees that are covertly infected. A Belgian screening of adult forager bees revealed a prevalence of 19% (De Smet et al., 2012), but this varies greatly in other European countries (Antunez et al., 2012; Forgach et al., 2008; Tentcheva et al., 2004). Larvae can be overtly infested, which then results in a failure to pupate and eventually death (Chen et al., 2007). Nonetheless, problems with this virus are seldom reported by beekeepers, in contrast to the Asian serotypes that infect *A. cerana* (Liu et al., 2010; Roberts and Anderson, 2014). Although SBV is mainly horizontally transmitted, its detection in eggs demonstrated that vertical transmission also occurs. It can be expected that a replicating virus in honey bee eggs can have consequences for the development into a queen, resulting in a clinical relevance for queen breeding, and can also have knock-on effects after being transmitted to the offspring workers or drones (Chen et al., 2007).

A broad virus screening of honey bee eggs was not yet performed. Nevertheless, few studies reported the presence of viruses (Chen et al., 2005a; Chen et al., 2006b; Shen et al., 2005; Singh et

al., 2010; Yue et al., 2007) but only limited numbers of colonies were screened. However, our study of fertilised eggs does not allow us to pinpoint the infection source, queen or drone, which could be important for eventual remedial actions. Because surface-sterilisation was not applicable in our study design, we could not distinguish between viruses on the surface of the eggs (transovum transmission) or within the eggs (transovarian transmission). Because of the possible transovum transmission, the emerging larvae will not necessarily be infected with viruses as previously demonstrated (Chen et al., 2006b). Nevertheless, these larvae are exposed to horizontal transmission via feeding (reviewed by Chen et al. (Chen et al., 2006a)).

5. Conclusions

A survey of viruses in honey bee eggs in the context of a queen breeding program demonstrated high incidences of two viruses (DWV and SBV) and moderate to low incidences of a further six viruses (ABPV, ALPV, BQCV, CBPV, LSV and VdMLV). Transmission (transovum or transovarian) of some viruses (ALPV, LSV, VdMLV) was demonstrated for the first time as well as negative-strand detection of SBV. We could not demonstrate a correlation between the presence of viruses and the low queen breeding efficacies. Remarkably, we found *Apis mellifera carnica* bees to be less infected with Deformed Wing Virus ($p < 0.01$) than Buckfast bees, and also found them to have a lower average total number of infecting viruses ($p < 0.001$). This is a significant finding, given that Deformed Wing Virus has earlier been shown to be a contributory factor to winter mortality, and offers interesting perspectives for breeding virus-resistant bees. However, we cannot make general conclusions about the virus-resistant state of *carnica* race compared to Buckfast race solely based on our data. We can only state that this was observed in our limited dataset. Concluding, further sanitary screenings in the context of queen breeding seems advisory, especially because BQCV infection is a common cause of queen larval death (Anderson, 1993).

6. Acknowledgements

This study was supported by the Research Foundation of Flanders (FWO-Vlaanderen G.0628.11). We would like to the participating beekeepers for supplying the honey bee egg samples.

7. Ethics statement

The study involved the European honey bee (*Apis mellifera*), which is neither an endangered nor a protected species.

8. Competing interests

All authors have declared that no financial or commercial interests exist.

9. References

Anderson, D.L., 1993. Pathogens and queen bees. Australasian Beekeeper 94**,** 292-296.

Antunez, K., Anido, M., Garrido-Bailon, E., Botias, C., Zunino, P. and others, 2012. Low prevalence of honeybee viruses in Spain during 2006 and 2007. Res Vet Sci 93**,** 1441-1445.

Bailey, L., 1968. Multiplication of Sacbrood Virus in the adult honeybee. Virology 36**,** 312-&.

Bailey, L., 1969. The multiplication and spread of sacbrood virus of bees. Ann Appl Biol 63**,** 483-491.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., Neumann, P., 2010. Virus infections and winter losses of honey bee colonies (Apis mellifera). J Apicult Res 49**,** 60-65.

Blanchard, P., Olivier, V., Iscache, A.L., Celle, O., Schurr, F. and others, 2008. Improvement of RT-PCR detection of chronic bee paralysis virus (CBPV) required by the description of genomic variability in French CBPV isolates. J Invertebr Pathol 97**,** 182-185.

Buchler, R., Andonov, S., Bienefeld, K., Costa, C., Hatjina, F. and others, 2013. Standard methods for rearing and selection of Apis mellifera queens. J Apicult Res 52.

Chen, Y.P., Evans, J.D., Feldlaufer, M., 2006a. Horizontal and vertical transmission of viruses in the honeybee, Apis mellifera. J Invertebr Pathol 92**,** 152-159.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F., 2005a. Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (Apis mellifera L.). Appl Environ Microbiol 71**,** 436-441.

Chen, Y.P., Pettis, J.S., Collins, A., Feldlaufer, M.F., 2006b. Prevalence and transmission of honeybee viruses. Appl Environ Microbiol 72**,** 606-611.

Chen, Y.P., Pettis, J.S., Feldlaufer, M.F., 2005b. Detection of multiple viruses in queens of the honey bee Apis mellifera L. J Invertebr Pathol 90**,** 118-121.

Chen, Y.P., Siede, R., 2007. Honey bee viruses. Adv Virus Res 70**,** 33-80.

De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y. and others, 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One 7**,** e47953.

Fievet, J., Tentcheva, D., Gauthier, L., de Miranda, J., Cousserans, F. and others, 2006. Localization of deformed wing virus infection in queen and drone Apis mellifera L. Virol J 3.

Forgach, P., Bakonyi, T., Tapaszti, Z., Nowotny, N., Rusvai, M., 2008. Prevalence of pathogenic bee viruses in Hungarian apiaries: situation before joining the European Union. J Invertebr Pathol 98**,** 235-238.

Forsgren, E., de Miranda, J.R., Isaksson, M., Wei, S., Fries, I., 2009. Deformed wing virus associated with Tropilaelaps mercedesae infesting European honey bees (Apis mellifera). Exp Appl Acarol 47**,** 87-97.

Francis, R.M., Kryger, P., 2012. Single assay detection of Acute Bee Paralysis Virus, Kashmir Bee Virus and Israeli Acute Paralysis Virus. J Apic Sci 56**,** 137-146.

Francis, R.M., Nielsen, S.L., Kryger, P., 2013. Patterns of viral infection in honey bee queens. J Gen Virol 94**,** 668-676.

Gauthier, L., Ravallec, M., Tournaire, M., Cousserans, F., Bergoin, M. and others, 2011. Viruses associated with ovarian degeneration in Apis mellifera L. queens. PLoS One 6**,** e16217.

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C. and others, 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41**,** 332-352.

Gregorc, A., Bakonyi, T., 2012. Viral infections in queen bees (Apis mellifera carnica) from rearing apiaries. Acta Vet Brno 81**,** 15-19.

Highfield, A.C., El, N.A., Mackinder, L.C., Noel, L.M., Hall, M.J. and others, 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Appl Environ Microbiol 75**,** 7212-7220.

Liu, X., Zhang, Y., Yan, X., Han, R., 2010. Prevention of Chinese sacbrood virus infection in Apis cerana using RNA interference. Curr Microbiol 61**,** 422-428.

Mussen, E.C., Furgala, B., 1977. Replication of Sacbrood Virus in larval and adult honeybees, Apis mellifera. J Invertebr Pathol 30**,** 20-34.

Nguyen, B.K., Ribiere, M., Vanengelsdorp, D., Snoeck, C., Saegerman, C. and others, 2011. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res 50**,** 195-202.

Palacios, G., Hui, J., Quan, P.L., Kalkstein, A., Honkavuori, K.S. and others, 2008. Genetic analysis of Israel acute paralysis virus: Distinct clusters are circulating in the United States. J Virol 82**,** 6209-6217.

Ravoet, J., Maharramov, J., Meeus, I., De, S.L., Wenseleers, T. and others, 2013. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS One 8**,** e72443.

Retschnig, G., Williams, G.R., Mehmann, M.M., Yanez, O., de Miranda, J.R., Neumann, P., 2014. Sex-Specific Differences in Pathogen Susceptibility in Honey Bees (Apis mellifera). PLoS One 9.

Roberts, J.M., Anderson, D.L., 2014. A novel strain of sacbrood virus of interest to world apiculture. J Invertebr Pathol 118**,** 71-74.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Scharlaken, B., de Graaf, D.C., Goossens, K., Peelman, L.J., Jacobs, F.J., 2008. Differential gene expression in the honeybee head after a bacterial challenge. Dev Comp Immunol 32**,** 883-889.

Shen, M.Q., Cui, L.W., Ostiguy, N., Cox-Foster, D., 2005. Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. J Gen Virol 86**,** 2281-2289.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA viruses in hymenopteran pollinators: evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5**,** e14357.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F. and others, 2004. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl Environ Microbiol 70**,** 7185-7191.

Yanez, O., Jaffe, R., Jarosch, A., Fries, I., Moritz, R.F.A. and others, 2012. Deformed wing virus and drone mating flights in the honey bee (Apis mellifera): implications for sexual transmission of a major honey bee virus. Apidologie 43**,** 17-30.

Yue, C., Schroder, M., Bienefeld, K., Genersch, E., 2006. Detection of viral sequences in semen of honeybees (Apis mellifera): Evidence for vertical transmission of viruses through drones. J Invertebr Pathol 92**,** 105-108.

Yue, C., Schroder, M., Gisder, S., Genersch, E., 2007. Vertical-transmission routes for deformed wing virus of honeybees (Apis mellifera). J Gen Virol 88**,** 2329-2336.

10. Supplementary information

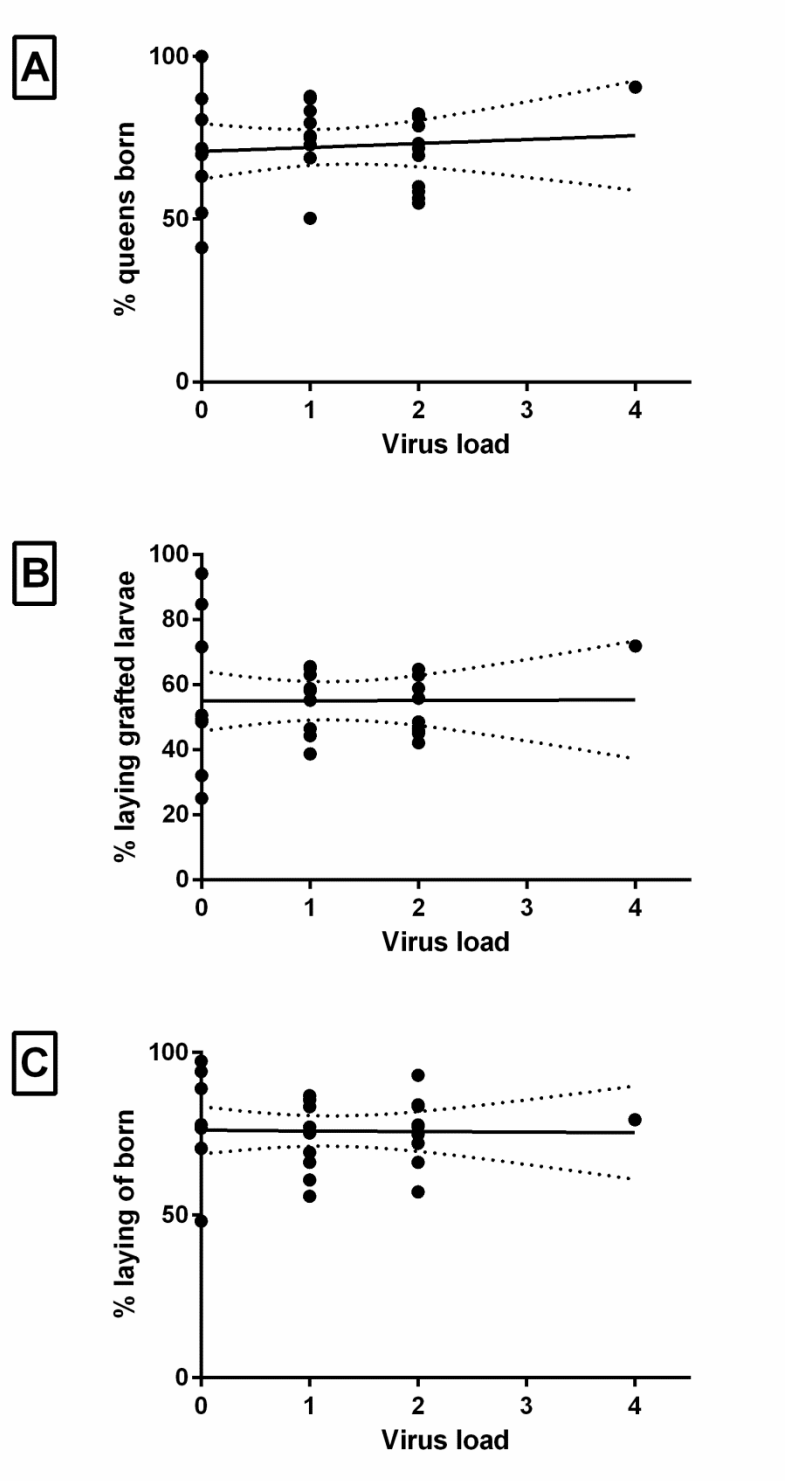


Figure S1. *Linear regression analysis of the effect of virus loads (total number of detected viruses) on queen breeding efficacies: effect on (A) the percentage of queens that were born from grafted larvae, (B) the percentage of queens that went on to lay out of all larvae that were grafted and (C) the percentage of all queens that were born that went on to lay.*

***Table S1**: Overview of the detected viruses in honeybee egg samples, subdivided per year. For each sample is the corresponding apiary, bee race and total virus burden shown. The virus prevalence per sampling year and the overall occurrence are indicated.*

ABPV: Acute Bee Paralysis Virus, ALPV: Aphid Lethal Paralysis Virus strain Brookings, BQCV: Black Queen Cell Virus, CBPV: Chronic Bee Paralysis Virus, DWV: Deformed Wing Virus, LSV: Lake Sinai Virus, SBV: Sacbrood Virus and VdMLV: Varroa destructor Macula-like Virus

SAMPLING IN 2012

Apiary	Race	ABPV	ALPV	BQCV	CBPV	DWV	LSV	SBV	VdMLV	Negative	Virus load
1	Buckfast	0	0	0	0	1	0	1	0	0	2
2	Buckfast	0	0	0	0	1	0	1	0	0	2
3	Buckfast	0	0	0	0	1	0	1	0	0	2
4	Buckfast	0	0	0	0	0	0	0	0	1	0
5	Buckfast	0	0	0	0	0	0	0	0	1	0
6	Buckfast	0	0	0	0	1	0	0	0	0	1
7	Buckfast	0	0	0	0	1	1	0	0	0	2
8	Buckfast	0	1	0	0	1	1	1	0	0	4
9	Buckfast	0	0	0	0	0	0	1	0	0	1
10	Carnica	0	0	0	0	0	0	1	0	0	1
11	Carnica	0	0	0	0	0	0	1	0	0	1
12	Carnica	0	0	0	0	0	0	1	0	0	1
13	Carnica	0	0	0	0	0	0	1	0	0	1
14	Carnica	0	0	0	0	1	0	1	0	0	2
15	Carnica	0	0	0	0	0	0	0	0	1	0
16	Carnica	0	0	0	0	0	0	0	0	1	0
17	Carnica	0	0	0	0	0	1	1	0	0	2
18	Carnica	0	0	0	0	0	0	0	0	1	0
19	Carnica	0	0	0	0	0	1	1	0	0	2
20	Carnica	0	0	0	0	0	0	0	0	1	0
21	Carnica	0	0	0	0	1	0	0	0	0	1
22	Carnica	0	0	0	0	0	0	0	0	1	0
23	Carnica	0	0	0	0	0	0	0	0	1	0

24	Carnica	1	0	0	0	0	0	1	0	0	2
25	Carnica	0	0	0	0	1	0	1	0	0	2
26	Carnica	0	0	0	0	0	0	1	0	0	1
27	Carnica	0	0	0	0	1	0	1	0	0	2
28	Carnica	0	1	0	0	1	0	0	0	0	2
29	Carnica	0	0	0	0	1	0	0	0	0	1
30	Carnica	0	0	0	0	0	1	0	0	0	1
31	Carnica	1	0	0	0	0	0	0	0	0	1
32	Carnica	0	0	0	0	0	0	0	0	1	0
33	Carnica	0	0	0	0	1	0	1	0	0	2
34	Carnica	0	0	0	0	0	0	1	0	0	1
35	Carnica	0	0	0	0	0	0	1	0	0	1
Total		2	2	0	0	13	5	19	0	9	41
Percentage		6%	6%	0,0%	0,0%	37%	14%	54%	0,0%	26%	N.A.

SAMPLING IN 2014

Apiary	Race	ABPV	ALPV	BQCV	CBPV	DWV	LSV	SBV	VdMLV	Negative	Virus load
1	Buckfast	1	0	0	0	1	1	1	0	0	4
1	Buckfast	0	0	0	0	1	1	1	0	0	3
1	Buckfast	1	0	1	0	1	1	1	0	0	5
1	Buckfast	1	0	0	0	1	0	1	0	0	3
1	Buckfast	0	0	0	0	1	0	1	0	0	2
2	Buckfast	1	0	0	0	0	1	0	0	0	2
2	Buckfast	0	1	0	0	0	0	1	0	0	2
2	Buckfast	0	0	1	0	1	1	1	0	0	4
3	Buckfast	0	0	0	0	1	1	1	0	0	3
4	Carnica	0	0	0	0	0	1	1	0	0	2
4	Carnica	0	0	0	0	0	1	1	0	0	2
5	Carnica	0	0	0	0	0	0	1	0	0	1
5	Carnica	1	0	0	0	1	0	1	0	0	3
6	Carnica	0	0	0	0	1	1	0	0	0	2
6	Carnica	1	0	0	0	0	1	0	0	0	2

6	Carnica	0	0	1	0	1	1	0	1	0	4
6	Carnica	0	0	0	0	0	0	0	0	1	0
6	Carnica	0	1	0	0	1	0	0	0	0	2
6	Carnica	0	0	0	0	0	0	0	0	1	0
7	Carnica	0	0	0	0	0	1	1	0	0	2
7	Carnica	0	0	0	0	1	1	0	1	0	3
8	Carnica	0	0	0	0	0	1	0	0	0	1
8	Carnica	0	0	0	0	0	0	0	0	1	0
8	Carnica	1	0	0	0	0	1	0	0	0	2
8	Carnica	0	0	0	0	0	1	0	0	0	1
9	Carnica	0	0	0	0	0	1	0	0	0	1
9	Carnica	0	0	0	0	1	0	0	0	0	1
9	Carnica	0	0	0	0	0	0	0	0	1	0
9	Carnica	1	0	0	0	0	0	0	0	0	1
9	Carnica	1	0	0	0	0	0	0	0	0	1
10	Carnica	0	0	0	0	1	0	0	0	0	1
10	Carnica	0	0	0	0	0	0	0	0	1	0
10	Carnica	0	0	1	0	1	0	1	0	0	3
10	Carnica	0	0	0	0	0	0	0	0	1	0
11	Carnica	0	0	0	1	0	0	0	0	0	1
11	Carnica	0	0	0	0	0	0	0	0	1	0
11	Carnica	0	0	0	0	0	0	0	0	1	0
11	Carnica	0	0	0	0	0	0	0	0	1	0
11	Carnica	0	0	0	0	1	0	0	0	0	1
11	Carnica	0	0	0	0	0	0	0	0	1	0
11	Carnica	0	0	0	0	1	0	0	0	0	1
11	Carnica	0	0	0	0	1	0	0	0	0	1
11	Carnica	0	0	0	0	0	0	0	0	1	0
Total		9	2	4	1	18	17	14	2	11	67
Percentage		21%	5%	9%	2%	42%	40%	33%	5%	26%	N.A.
Overall prevalence		14%	5%	5%	1%	40%	28%	42%	3%	26%	N.A.

PART III

IN-DEPTH CHARACTERIZATION OF SELECTED HONEY BEE PARASITES

CHAPTER 7

CHARACTERIZATION OF TWO SPECIES OF TRYPANOSOMATIDAE FROM THE HONEY BEE APIS MELLIFERA: *CRITHIDIA MELLIFICAE* LANGRIDGE AND MCGHEE, 1967 AND *LOTMARIA PASSIM* N. GEN., N. SP.

The work presented in Chapter 7 was adapted from the following manuscript:

R.S. Schwarz, G. Bauchan, C. Murphy, J. Ravoet, D.C. de Graaf, J.D. Evans. Characterization of Two Species of Trypanosomatidae from the Honey Bee Apis mellifera: *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* n. gen., n. sp., Journal of Eukaryote Microbiology. In Press. doi: 10.1111/jeu.12209.

Contributions

J.D. Evans and R. Schwarz designed the study. R. Schwarz performed the experiments concerning the cell line cultures, *in vivo* inoculations and the phylogenetic analyses. The DNA purification, gene cloning and sequencing were executed by R. Schwarz for the cell lines and by J. Ravoet, supported by D.C. de Graaf, for the Belgian samples. G. Bauchan and C. Murphy performed the electron microscopy and Confocal Laser-scanning microscopy experiments. R. Schwarz wrote the manuscript, assisted by all co-authors.

1. Abstract

Trypanosomatids are increasingly recognized as prevalent in European honey bees (*Apis mellifera*) and by default are attributed to one recognized species, *Crithidia mellificae* Langridge and McGhee, 1967. We provide genetic and ultrastructural data for type isolates of *C. mellificae* (ATCC 30254 and 30862) in comparison with two recent isolates from *A. mellifera* (BRL and SF). Phylogenetics unambiguously identify strains BRL/SF as a novel taxonomic unit distinct from *C. mellificae* strains 30254/30862 and assign all four strains as lineages of a novel clade within the subfamily Leishmaniinae. *In vivo* analyses show strains BRL/SF preferably colonize the hindgut, lining the lumen as adherent spheroids in a manner identical to previous descriptions from *C. mellificae*. Microscopy images show motile forms of *C. mellificae* are distinct from strains BRL/SF. We propose the binomial *Lotmaria passim* n. gen., n. sp. for this previously undescribed taxa. Analyses of new and previously accessioned genetic data show *C. mellificae* is still extant in bee populations, however *L. passim* n. gen., n. sp. is currently the predominant trypanosomatid in *A. mellifera* globally. Our findings require that previous reports of *C. mellificae* be reconsidered and that subsequent trypanosomatid species designations from Hymenoptera provide genetic support.

2. Introduction

Due to their worldwide agricultural significance, eusocial colony behavior and semi-domestication, European honey bees (*Apis mellifera*) are model organisms for a variety of applied and pure research endeavors (Dietemann et al. 2013). Correspondingly, the suite of pathogens and symbionts they host are of heightened interest (e.g. Cox-Foster et al. 2007; Evans and Schwarz 2011; Forsgren and Fries 2010; Genersch et al. 2005; Higes et al. 2013; Klee et al. 2007; Moran et al. 2012; Vásquez et al. 2012). Currently recognized enteric unicellular parasites of *A. mellifera* represent four suprakingdom-level groups of the eukaryotes (sensu Adl et al. 2005; Adl et al. 2012): Amoebozoa, Chromalveolata, Excavata, and Opisthokonta. Of these, trypanosomatids belonging to Excavata [Euglenozoa: Kinetoplastea: Trypanosomatida: Trypanosomatidae] have been known to infect *A. mellifera* since at

least 1912 (Fantham and Porter 1912) with a peppering of reports in the following century supporting their generally common and global distribution including Europe (Fantham and Porter 1912; Fyg 1954; Lom 1962; Lotmar 1946; Orantes-Bermejo 1999; Ravoet et al. 2013), Africa (Porter 1945), Australia (Langridge 1966; Langridge and McGhee 1967), North America (e.g. Cox-Foster et al. 2007; Runckel et al. 2011; van Engelsdorp et al. 2009), South America (Teixeira et al. 2008) and Asia (Morimoto et al. 2012; Yang et al. 2013). Reports of their taxonomic diversity have been in conflict, however, with some researchers reporting multiple species (Fantham and Porter 1912; Fyg 1954) while others believed a single, polymorphic species was present (Langridge and McGhee 1967; Lotmar 1946). This confusion was not surprising since taxonomy of trypanosomatids at the time relied on morphological characteristics (e.g. Hoare and Wallace 1966; Vickerman 1976; Wallace 1966), which genetic analyses have since shown are often unreliable and misleading.

Trypanosomatidae are obligate parasites and include in part genera (i.e. Leishmania, Phytomonas, Trypanosoma) comprised of dixenous species, requiring two hosts to complete their lifecycle (invertebrate and vertebrate or plant). Although comparatively less studied, many invertebrates are parasitized by lineages of monoxenous trypanosomatid species, requiring only one host to complete their lifecycle, and are important to consider for a clear understanding of the biology and evolutionary history of this entire family. Classically, trypanosomatids have been categorized according to six major morphotypes based on the flagellated stage of development (reviewed in Wheeler et al. 2013), yet multiple genera can be assigned to individual morphotypes. This is most pronounced with the promastigote morphotype, which has been described from the following genera: *Crithidia*, *Leishmania*, *Leptomonas*, *Herpetomonas*, *Phytomonas*, and *Wallaceina* (recently argued to be renamed as *Wallacemonas* by Kostygov et al. 2014). Thus, phylogenetics are essential for accurate taxonomic classification to circumvent homoplasies among unrelated lineages of trypanosomatids (e.g. Vickerman 1994; Votýpka et al. 2012; Wheeler et al. 2013; Yurchenko et al. 2008) and to identify cryptic species within morphologically indistinguishable populations (Schmid-Hempel and Tognazzo 2010). For this reason, current trypanosomatid research efforts rely on

phylogenetics when characterizing new (e.g. Jirku et al. 2012; Maslov et al. 2010; Votýpka et al. 2013; Yurchenko et al. 2006a,b) and previously described taxa (e.g. Teixeira et al. 2011; Yurchenko et al. 2014). Nonetheless, most of these new taxa continue to be arbitrarily assigned to previously accepted genera based on morphotypes despite conflicting phylogenetic placement. This approach has artificially created polyphyletic genera within the Trypanosomatida (e.g. Merzlyak et al. 2001; Yurchenko et al. 2008) that are only now beginning to be revised based entirely on phylogenetics (Teixeira et al. 2011; Borghesan et al. 2013; Kostygov et al. 2014).

Phylogenetic analyses of trypanosomatids typically involve two nuclear DNA loci for which sequences across a large diversity of taxa are available: glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) and the 18S small subunit ribosomal RNA (SSU). These two loci have recently been used to establish the newly recognized trypanosomatid subfamily Leishmaniinae (Jirku et al. 2012), previously referred to as the slowly evolving (SE) clade (Merzylak et al. 2001). This subfamily includes a robust lineage comprised of *Leishmania* species (the namesake of the subfamily) as well as two polyphyletic genera, *Crithidia* and *Leptomonas*. However, taxonomic research of this subfamily has focused on species derived from Hemiptera (suborder Heteroptera) or Diptera hosts, highlighting the need for additional taxa from a broader host range to clarify lineages within this subfamily (Maslov et al. 2013). The class to which trypanosomatids belong, Kinetoplastea, refers to the unique mass of kinetoplast DNA (kDNA) within the elongated mitochondria of these cells. The kDNA provides an alternative source of genetic material to which traditional nuclear phylogenetics can be compared, and its value has yet to be broadly applied by systematists. The cytochrome *b* gene encoded on kDNA, *Cytb*, is an important locus for distinguishing genotypes within species of *Trypanosoma* (Spotorno et al. 2008) and *Leishmania* (Asato et al. 2009) and has recently been used (in part) to characterize two closely related taxonomic lineages of trypanosomatids infecting bumblebees, *Crithidia bombi* and *Crithidia expoeki* (Schmid-Hempel and Tognazzo 2010).

Early research interests on honey bee trypanosomatids produced two key reports from *A. mellifera*. First (Lotmar 1946) was a detailed account of adult bees with a unique gut scarring

pathology, termed “Schorfbienen” (scab bees), with which trypanosomatids were intimately associated and suggested to cause. Both motile, flagellated forms and non-motile round forms (described here as ‘spheroids’) were described as adherent to the lumenal epithelium surrounding melanized ‘scab’ regions in a specific area of the gut called the pylorus, a highly infolded region that regulates the transition of gut contents between the ventriculus (midgut) and ileum (small intestine). Although no physical specimens were archived, detailed illustrations and photomicrographs showed these flagellates typically had acutely pointed anteriors from which the flagellum extended and enlarged rounded posteriors as well as some thin, highly elongated forms consistent with promastigote and choanomastigote morphotypes. Additional emphasis was placed on their highly specific niche within the pylorus, hence the association with melanized tissue here, which rarely extending anteriorly into the midgut or distally into the small intestine. The population was attributed to a new species with the provisional name *Leptomonas apis*, with genus assignment based on morphological standards at the time.

Two decades later another trypanosomatid from *A. mellifera* was characterized and denominated *Crithidia mellificae* (Langridge 1966; Langridge and McGhee 1967), thus becoming the first formally and widely accepted species in honey bees. While the description of *L. apis* was noted by Langridge and McGhee, their isolate was ascribed to an entirely different genus and species. This was justified by differences in cell morphology and site of tissue colonization described for each. *Crithidia mellificae* showed occasional slightly elongated promastigotes but primarily choanomastigotes, with truncated anteriors and rounded to acute posteriors. Within the host gut, *C. mellificae* primarily colonized the rectum in large numbers via spheroids attached to the lumenal surface that formed a compact, single layer of parasites. An earlier report of trypanosomatids (uncharacterized) in *A. mellifera* also recognized the rectum as a primary site of colonization (Fyg 1954). Axenic cultures of *C. mellificae* were successfully established for its characterization and thereafter, two archived type cultures from Georgia, U.S.A. were deposited by one of the authors, R.

B. McGhee, to the American Type Culture Collection (ATCC): strain ATCC 30254 isolated from *A. mellifera* and strain ATCC 30862 isolated from the wasp *Vespula squamosa* (eastern yellow jacket).

These two key descriptions provided an excellent foundation for understanding honey bee trypanosomatids. However, interest in them waned and gave way to the common practice of lumping honey bee trypanosomatids into a single taxon, with *C. mellificae* the largely accepted species. Recent applications of molecular methods used in diagnostic bee pathogen surveys (e.g. Ravoet et al. 2013; Runckel et al. 2011; van Engelsdorp et al. 2009) have confirmed the general abundance of trypanosomatids, yet the lack of clarity surrounding their taxonomy is an impediment. Studies that aimed to understand the role of microbes in diseased colonies (Cox- Foster et al. 2007; vanEngelsdorp et al. 2009) used vague reference of trypanosomatid genetic signals to family (Trypanosomatidae) only, ignoring any species level insight. Alternatively, despite the historical disparity over taxa, publicly accessioned trypanosomatid genetic data have been arbitrarily assigned to *C. mellificae* when isolated from *A. mellifera* (Morimoto et al. 2012; Runckel et al. 2011; Runckel et al. 2014; Schmid-Hempel and Tognazzo 2010) and from Asian honey bee *Apis cerana* (Yang et al. 2013), leading others to speciously designate homologous sequence data (Cornman et al. 2012; Ravoet et al. 2013). However, one study (Cornman et al. 2012) specifically used reference genetic material from a type strain of *C. mellificae* (ATCC 30254) to recognize that a divergent clade of trypanosomatids existed in honey bee colonies from the USA. This type strain of *C. mellificae* has also been used in controlled studies where they were found to stimulate complex honey bee immune responses (Schwarz and Evans 2013), identifying important implications these parasites may have on overall honey bee health.

Toward improved understanding of trypanosomatids in bees and insect trypanosomatid taxonomy, we examined the two archived type strains of *C. mellificae* (30254 and 30862) and provide the first nuclear and kDNA sequence data for this species. We also provide nuclear and kDNA sequence data from two recent trypanosomatid axenic cultures established from *A. mellifera* in the USA (strains BRL and SF) that are genetically and morphologically distinct from *C. mellificae*.

Phylogenetic analyses of concatenated gGAPDH and SSU clarify 3 distinct clades within the Leishmaniinae subfamily: the *Leishmania* (Clade 1), the *Crithidia* (Clade 2) and a novel clade (Clade 3) that includes all trypanosomatids isolated from Hymenoptera (e.g. bees, bumblebees and wasps) and comprised of species classically assigned to either *Crithidia* or *Leptomonas* based on morphology. We propose that strains BRL and SF are type specimens (hapantotype BRL strain; parahapantotype SF strain) for a new genus and species of Trypanosomatidae within Clade 3.

3. Material and methods

3.1 Cell line cultures

All cell lines were maintained at 25°C axenically in "supplemented DS2" medium: Insectagro DS2 (Cellgro, Manassas, U.S.A.), 5% (v/v) fetal bovine serum (Cellgro) and 100 IU/mL penicillin – 100 µg/mL streptomycin (Cellgro). Two axenic cell lines of *C. mellificae* were obtained from ATCC (Manassas, U.S.A.): 1) *C. mellificae* Langridge and McGhee (ATCC 30254) and 2) *C. mellificae* Langridge and McGhee (ATCC 30862). An axenic trypanosomatid culture isolated from the dissected ileum of an adult female *A. mellifera* at the Bee Research Lab (BRL strain ATCC 00359) in Beltsville, Maryland, U.S.A. was established in September 2012. The ileum was removed with sterile tools, submerged in 1 mL of supplemented DS2 medium in a 1.7 mL microtube and gently macerated with a sterile pestle. After 48 hours incubation at 25°C, an active culture was expanded in supplemented DS2 with added amphotericin B (2.5 µg/mL) until bacterial and fungal contaminants were no longer observed at which point cultures were cryopreserved. A fourth trypanosomatid axenic cell line (SF strain ATCC PRA-403) was isolated from *A. mellifera* in San Francisco, California, U.S.A. as described previously (Runckel et al. 2011).

3.2 *In vivo* Inoculations with Strain BRL

Promastigotes of strain BRL were cultured in supplemented DS2 media and prepared for *per os* inoculation as described previously (Schwarz and Evan 2013) in 20% sucrose solution (1:5 sucrose to 1x phosphate buffered saline (PBS)) at the time of inoculation. A brood frame was removed from a

colony maintained at the U.S.D.A. BRL and incubated in a frame cage to recover recently emerged workers (< 2 d) that were then divided into two treatment groups and hand fed either 5 µl of 1:1 sugar water or 5 µl of a strain BRL suspension (10,000 cells / µl) then maintained separately according to treatment group in bee cups (Evans et al. 2009) at 34 °C + 50% relative humidity with *ad libidum* sterile sucrose solution until dissection of foregut and hindgut tissues at 10 to 11 days post inoculation (p.i.) (n = 20 per treatment group) and light microscopy examination at 400x to 1,000x magnification.

3.3 DNA purification, gene cloning, and sequencing

DNA was extracted from cultures of axenic trypanosomatid cell lines by homogenizing cells with 1mm glass beads in 2% (w/v) hexadecyltrimethylammonium bromide (CTAB) buffered with 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 0.2% 2-mercaptoethanol, 50 µg proteinase K (Promega; Madison, U.S.A.) and 5% (v/v) RNase cocktail (Life Technologies, Carlsbad, U.S.A.) using a FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, U.S.A.). DNA was purified with phenol:chloroform:isoamyl alcohol (25:24:1) phase separation followed by alcohol precipitation and then resuspended in nuclease-free water.

DNA purified from each cell line was used as template for polymerase chain reaction (PCR) to clone 3 nuclear genome loci: 1) *gGAPDH*, 2) SSU rRNA and 3) the internal transcribed spacer (*ITS*) regions from *18S* to *28S* rRNA (partial *18S*, entire *ITS-1*, *5.8S* rRNA, *ITS-2*, partial *28S* rRNA) and one mitochondrial locus, *Cytb*. Primers targeting these 4 loci were as follows: gGAPDH forward 5'-ATG GCT CCG (A/C)TC AAG GTT GGC-3' and reverse 5'-TTA CAT CTT CGA GCT CGC G(C/G)(C/G) GTC-3' with a 55°C annealing step (modified from Yurchenko et al. 2006); SSU forward 5'-GGC GTC TTT TGA CGA ACA AC-3' and reverse 5'- TAC GTT CTC CCC CGA ACT AC-3' with a 60°C annealing step (designed using Primer 3 in this study); ITS region forward 5'-GTC GTT GTT TCC GAT GAT GGT G-3' and reverse 5'- CCT GCC AAC TTG ACA CTG C-3' with a 57°C annealing step (forward modified from Teixeira et al. 2008 and reverse designed in this study), Cytb forward 5'-TCG TGT AAA GCG GAG AAA GAA GA-3' and

reverse 5'-ACA CAA ACG TTC ACA ATA AAA AGC A-3' with a 60°C annealing step (designed in this study using Primer3).

Several honey bee samples from a Belgian trypanosomatid screening (Ravoet et al. 2013) were selected for this study. Five µl of RNA (variable concentration) were reverse transcribed using random hexamer primers with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania). Four specimens of *O. bicornis* were also collected in May 2012 at campus Sterre, Ghent, Belgium. Temporary trypanosomatid cultures (with contaminating bacteria) were established using a described protocol (Popp and Latorff 2011), from which DNA was extracted with Dneasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol for cell cultures. To amplify trypanosomatid specific genes from infected bees (*A. mellifera*, *O. bicornis*), previously published primers were used for *gGAPDH* (Yang et al. 2013), *Cytb* was amplified using the primer Tryp-cytb-F 5'-TGT GGW GTK TGT TTA GC-3' and Tryp-cytb-R 5'-CRT CWG AAC TCA TAA AAT AAT G-3' with a 50°C annealing step, and *SSU* was amplified using the above primers designed for this study. PCR reactions contained 2 µM of forward and reverse primer; 1 mM $MgCl_2$; 1.25 U Hotstar HiFidelity DNA polymerase (Qiagen) and 1 µl cDNA (*A. mellifera*) or 100 ng DNA (*O. bicornis*).

Column purified (QIAprep, Qiagen) recombinant plasmid amplicons were bidirectionally sequenced with T7 and SP6 priming sites using BigDyeR Terminator on a ABI3730XL capillary sequencer (Macrogen, Rockville, U.S.A.). Sequencher 5.2.3 (Gene Codes Corporation, Ann Arbor, U.S.A.) software was used to assess sequence quality, unambiguously determine DNA sequence, and build contigs for each insert. All non-redundant sequences isolated were accessioned to Genbank and are presented in Table S1-S5.

3.4 Phylogenetic analyses

Previously accessioned trypanosomatid sequences were retrieved from Genbank and aligned with sequences we recovered from each locus using MUSCLE (Edgar 2004) with minor hand correction to minimize gaps. Single locus alignments were analyzed using both maximum likelihood (ML) discrete-character method and Neighbor-Joining (NJ) distance method in MEGA 6.06 for MacOS (Tamura et al.

2013). Best-fit nucleotide substitution testing of 24 models based on lowest Bayesian information criterion (BIC) inferred by Nearest-Neighbor Interchange heuristic search determined the General Time Reversible (GTR) + discrete Gamma Distribution (G) + evolutionarily invariable sites (I) was optimal for ML analysis of gGAPDH and SSU while Tamura-Nei (TN93) + G was optimal for *Cytb*. All NJ method analyses used Tamura-Nei model including transitions and transversions, uniform rates among sites and pairwise deletion of gaps/missing data. All sites in aligned regions were used and areas of no coverage in shorter sequences were ignored. Since both gGAPDH and SSU alignments had BIC support for the same optimal model, we generated a concatenated alignment for analysis using the most commonly recovered sequence variants from each locus to create a single, representative sequence for each trypanosomatid cell line. Tree topologies were tested with 1,000 bootstraps using *Bodo* (SSU and gGAPDH) or *Trypanosoma* (*Cytb*) species as outgroups. Optimal phylograms were imported to TreeGraph 2 (Stover and Muller 2010) for editing. The trees shown in this manuscript (Fig. 1-2) are available in TreeBASE ID 16436.

3.5 Confocal Laser-scanning microscopy

Live cells were pelleted from axenic cultures for 5 min at 425x *g*, fixed in 4% paraformaldehyde in 1x PBS (w/v) for 30 min at 4 °C, pelleted at 239x *g* and resuspended in 1x PBS then stained using 4',6-diamidino-2-phenylindole (DAPI) (NucBlueR Fixed Cell Stain, Life Technologies) for 5 min. Stained cells were placed in cover glass bottom petri dishes (MatTeck Corp., Ashland, U.S.A.) and viewed with differential interference contrast (DIC) on a ZeissTM LSM710 confocal laser scanning microscopy (CLSM) system as described previously (Macarisin et al. 2010 and 2012). Briefly, the images were observed using a Zeiss Axio ObserverTM inverted microscope with 100x 1.4 NA Plan-Apochromat objectives and a 405 nm diode laser with a pin hole of 63 µm passing through a MBS 405 beam splitter filter with limits set between 410-485 nm. Zeiss ZenTM 2012 software was used to obtain 15-20 z-stack images to produce the 3D renderings which were used to develop 2D maximum intensity projections.

3.6 Electron microscopy

Scanning electron microscopy (SEM). Live cells were pelleted for 5 min at 425x *g* and fixed with 3% glutaraldehyde in 0.05M PBS for 2 h at room temperature then overnight at 4 °C. Fixed cells were washed 6 times in PBS then dehydrated in a graded series of ethanol. Samples were critical point dried in a Tousimis Samdri-780A (Tousimis Research Corporation, Rockville, U.S.A.), placed onto ultra smooth (12mm diameter) carbon adhesive discs (Electron Microscopy Sciences, Inc., Hatfield, U.S.A.) attached to 15 cm x 30 cm copper plates and sputter coated using a magnetron sputter head equipped with a platinum target. Samples were observed in an S-4700 field emission SEM (Hitachi High Technologies America, Inc., Dallas, U.S.A.) equipped with a Quorum CryoPrep PP2000 (Quorum Technologies Ltd., East Sussex, U.K.) cryotransfer system using accelerating voltage of 5 kV. Images were captured using a 4pi Analysis System (Agilent Technologies, Durham, U.S.A.).

Transmission electron microscopy (TEM). Live cells were fixed in 2.5% glutaraldehyde (v/v) in 0.1M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature then overnight at 4 °C. Samples were then rinsed 6 times in 0.1M sodium cacodylate buffer, post-fixed in 2% (w/v) osmium tetroxide for 2 h, dehydrated in ethanol series then embedded in acrylic resin (LR White Resin System, London, U.K.) and cured at 55 °C for 24 h prior to sectioning using a Reichert- Jung/AO ultra-microtome fitted with a Diatome diamond knife. Sections were stained with 4% uranyl acetate and 3% lead citrate, viewed in an HT-7700 microscope (Hitachi High Technologies America) at 80 kV and imaged using an AMT High-Res CCD camera.

4. Results

4.1 Genetic characterization of four axenic trypanosomatid strains

DNA templates from each of the *C. mellificae* type strains (30254 and 30862) as well as two recent axenic isolates from *A. mellifera*, strain BRL and strain SF, were PCR amplified at three nuclear loci (gGAPDH, SSU, and *ITS1-5.8S-ITS2*) and one kDNA (*Cytb*) locus. From 226 total recombinant plasmid clone inserts, we obtained 99 unique sequences in total that were accessioned to Genbank as

Table 1. *Inter- and intraspecific identity among unique DNA sequences from* Lotmaria passim *n.gen., n. sp. (strains BRL and SF) and* Crithidia mellificae *(strains 30254 and 30862) trypanosomatid isolates at four genetic loci.*

	Cytb	**gGAPDH**	**SSU**	**ITS1-5.8S-ITS2**
	Identity (%) between *L. passim* and *C. mellificae*			
BRL vs. 30254	88.46 – 88.62	92.32 – 92.80	94.84 – 95.48	64.69 – 65.69
SF vs. 30254	88.29 – 88.46	93.18 – 93.66	94.84 – 95.48	65.57 – 66.50
BRL vs. 30862	88.12 – 88.29	91.07 – 92.89	95.35 – 95.48	65.57 – 65.69
SF vs. 30862	87.95 – 88.12	91.93 – 93.76	95.23 – 95.48	66.44 – 66.50
Alignment length	589 sites	1,041 sites	775 sites	1,606 sites
	Identity (%) within *L. passim*			
BRL vs. SF	99.32 – 99.49	98.66 – 99.04	99.74 – 100	98.35 – 99.14
BRL only	99.66 – 99.83	99.62 – 99.71	99.87	98.48 – 99.87
SF only	99.66 – 99.83	98.85 – 99.81	99.74 – 99.87	98.09 – 99.93
Alignment length	589 sites	1,041 sites	773 sites	1,519 sites
	Identity (%) within *C. mellificae*			
30254 vs. 30862	99.49 – 99.66	97.12 – 99.23	99.87 – 100	98.46 – 98.77
30254 only	99.66 – 99.83	98.94 – 99.42	99.34 – 99.87	98.53 – 99.38
30862 only	99.66 – 99.83	97.69 – 99.90	99.87	99.54 – 99.92
Alignment length	589 sites	1,041 sites	763 sites	1,296 sites

reference material for these 4 strains (Table S1). Nucleotide sequence identity among the axenic strains at these four loci revealed two consistent and distinct taxonomic groups: strains BRL and SF each shared higher identity with one another than with strains 30254 or 30862 (Table 1). Nucleotide identity among sequences from strains BRL and SF ranged from 98.09% (*ITS1-5.8S-ITS2*) to 100% (SSU) while identity from strains 30254 and 30862 ranged from 97.12% (gGAPDH) to 100% (SSU). When sequences from strains BRL and SF were both compared to those of strains 30254 and 30862, identity ranged from 64.69% (*ITS1-5.8S-ITS2*) to 95.48% (SSU) between these two taxonomic groups. Sequence identity between taxonomic group BRL/SF compared to group 30254/30862 was

consistently lower at all four loci than sequence identity among strains BRL/SF or among 30254/30862.

Variation among sequences at the gGAPDH and *Cytb* locus were due to single nucleotide polymorphisms (SNPs) while the *SSU* and *ITS1-5.8S-ITS2* loci included variation due to SNPs and insertions/deletions (indels). Homologous regions of *SSU* were 10 bp shorter in strains 30254 and 30862 (763 bp) compared to strains BRL and SF (773 bp). Cloned fragments spanning the full *ITS1-5.8S-ITS2* region in strains BRL and SF ranged from 1,492 to 1,511 bp (1,519 aligned sites) and ranged from 1,279 to 1,294 bp (1,296 aligned sites) in strains 30254 and 30862.

Table 2. *Predicted protein identity among axenic isolates of* Lotmaria passim *n. gen., n. sp. (strains BRL and SF) and* Crithidia mellificae *(strains 30254 and 30862).*

Isolate	Identity (%) to BRL consensus	Identity (%) to 30254 consensus
	[a]gGAPDH	
BRL	99.1 to 100	93.6 to 94.2
SF	97.6 to 99.4	93.6 to 95.9
30254	93.3 to 94.5	99.1 to 100
30862	92.7 to 94.5	97.9 to 100
	[b,c]Cytb	
BRL	99.4 to 100	97.9 to 98.4
SF	99.4 to 100	97.9 to 98.4
30254	97.9 to 98.4	99.4 to 100
30862	97.9 to 98.4	99.4 to 100

[a] Based on 28 unique sequences 347 aa's in length.
[b] Based on 13 unique sequences 195 aa's in length.
[c] Translated using genetic code for protozoan mitochondrial DNA.

To assess potential functional variation, we contrasted predicted protein sequences of gGAPDH and *Cytb* (Table 2). The range of identities at both loci formed two, non-overlapping groups: one was comprised of strains 30254/30862 and the other of strains BRL/SF. Identity across 347 predicted amino acids (aa's) of gGAPDH ranged from 97.6% to 100% 323 within strains BRL/SF and 97.9% to 100% within strains 30254/30862. Between these two taxonomic groups, identity ranged from 92.7% to 95.9%. Similarly, the 195 predicted aa's of Cytb showed strains BRL/SF were more similar to each other than to any 30254/30862 sequences and vice versa (both were 99.4% to 100%

identical within group). Contrasting the two groups to each other, identity ranged from 97.9% to 98.4%. Predicted aa alignments are available in Fig. S1 (gGAPDH) and Fig. S2 (Cytb).

4.2 Trypanosomatid sequences isolated *in vivo* from bees

Fragments of trypanosomatid nucleic acids (*gGAPDH*, 492 bp; *SSU*, 763 to 775 bp; *Cytb*, 452 bp) amplified directly out of *A. mellifera* and *Osmia bicornis* (red mason bee) collected in Belgium recovered 39 (*A. mellifera*) and 2 (*O. bicornis*) unique sequences that shared high sequence identity to either taxonomic group BRL/SF or 30254/30862. Nearly all sequences isolated from *A. mellifera* (gGAPDH, KM066212-KM066224; *SSU*, KM066227-KM066239; *Cytb*, KM066240-KM066250) had high identity to sequences of group BRL/SF (99.34% to 100%). However, two SSU sequences recovered from *A. mellifera* (KM066225, KM066226) were 99.87% and 100% identical to taxonomic group 30254/30862. The two trypanosomatid sequences recovered from *O. bicornis* also belonged to the 30254/30862 group (gGAPDH, KM066211, 99.56%; *Cytb*, KM066251, 100%).

4.3 Phylogenetic analyses

Unambiguous and unique gGAPDH, SSU and *Cytb* DNA sequences isolated from axenic strains 30254, 30862, BRL and SF along with sequences isolated in vivo from infected honey bees (*A. mellifera*) and red mason bees (*O. bicornis*) were aligned to homologous sequences extracted from Genbank that represented a broad range of available Trypanosomatidae taxa. Both gGAPDH and SSU were the best represented loci in Genbank with which we produced alignments containing 160 and 126 sequences in total, respectively, while *Cytb* was poorly represented for which we aligned 51 sequences in total. Concatenated gGAPDH-SSU analyses (Fig. 1) placed all of our Trypanosomatidae strains (30254, 30862, BRL, SF) into a novel clade within the Leishmaniinae subfamily (*sensu stricto* Maslov and Lukeš in Jirku et al. 2012) adjacent to two additional clades with strong ML and NJ bootstrap support, respectively: Clade 1 (*Leishmania*; 98, 99), Clade 2 (*Crithidia*; 72, 67) and novel Clade 3 (87, 86).

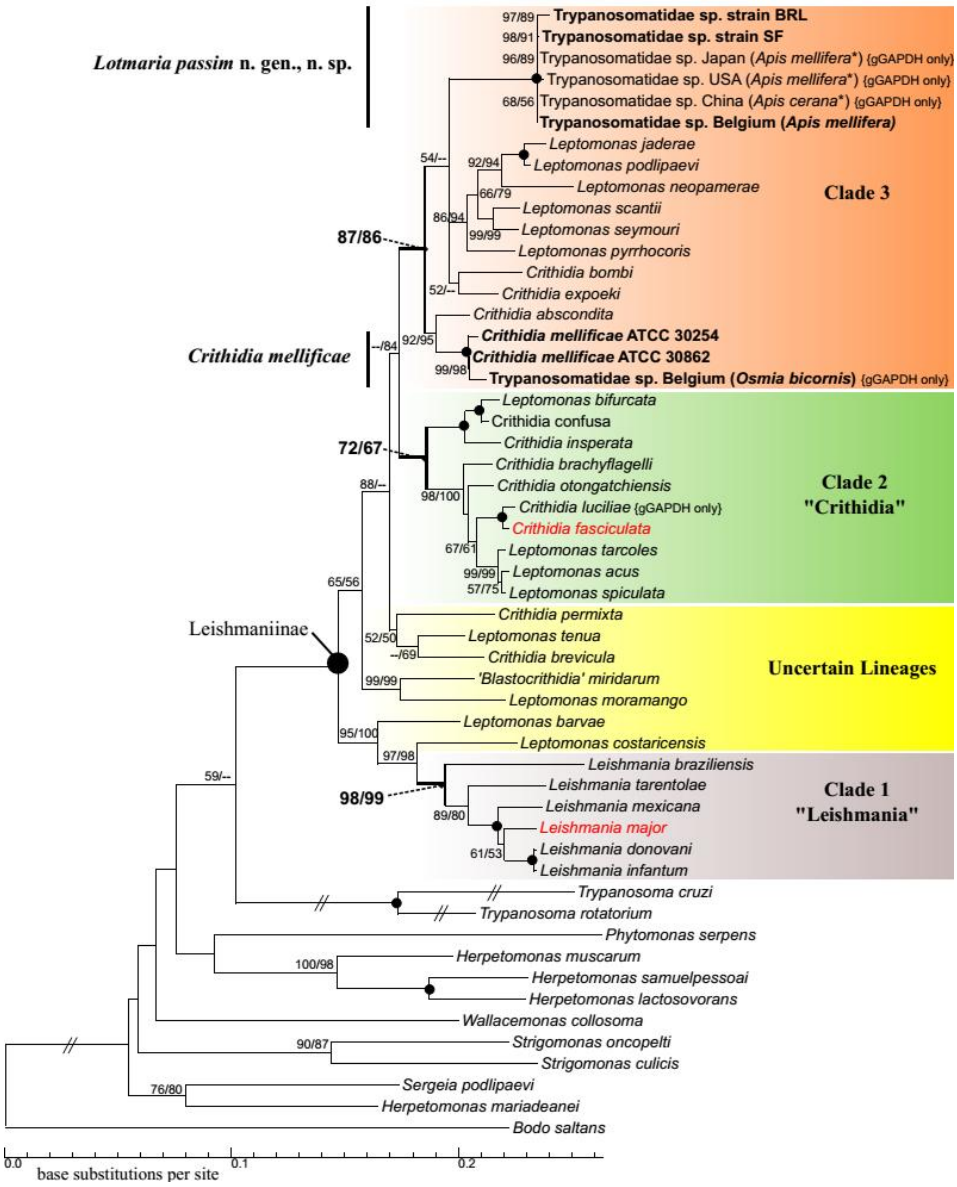


Figure 1. *Phylogeny reconstruction by ML method using concatenated gGAPDH and SSU sequences from four axenic strains (30254/30862* Crithidia mellificae*; BRL/SF* Lotmaria passim *n. gen., n. sp.) and from in vivo isolated sequences. Alignments were made with previously accessioned Kinetoplastea (accession numbers available in TreeBASE ID 16436), totaling 53 sequences and 1,978 sites. Both loci were independently best modeled using GTR+G+I based on lowest BIC, justifying concatenation and analysis with the same model. Original sequences are in bold font, represented by the most prevalent sequence variants from each strain. Node support values for ML and NJ methods are shown, respectively, with support <50% (1,000 bootstraps) not shown and 100% support using both methods indicated with a black circle. Some taxa for which only the gGAPDH locus was available are noted. The base of the Leishmaniinae subfamily is indicated and robust clades within are highlighted (Clade 1, 2 and 3). Country of origin and host species are given for non-cultured isolates. *Originally accessioned as* C. mellificae*. Cross-hatched branches are half their original length.*

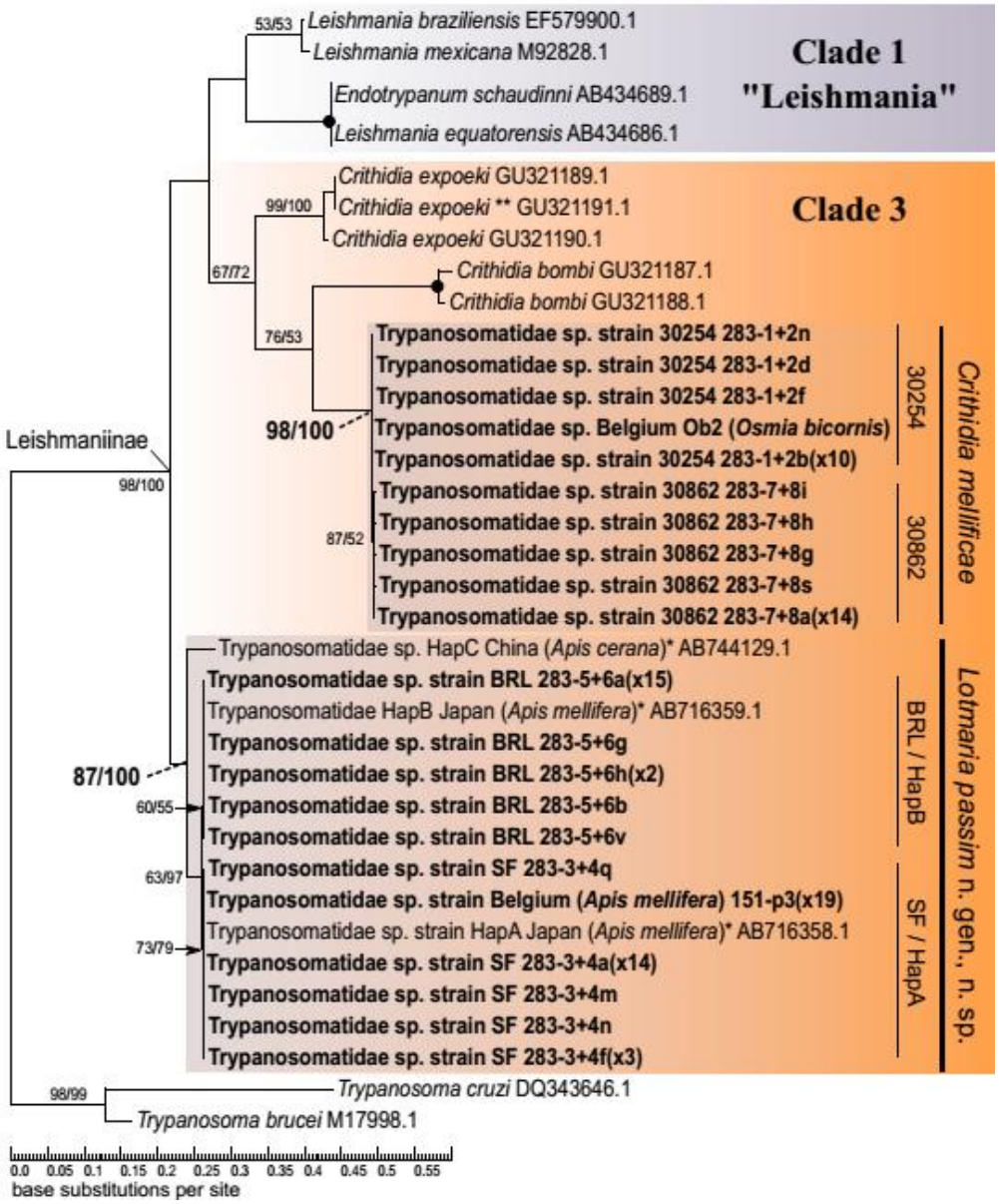


Figure 2. *Trypanosomatid phylogeny reconstruction using Cytb sequences provides resolution of genotype lineages within Clade 3. Sequence alignments (593 sites) were analyzed by ML and NJ methods and best-fit model TN93+G (G = 0.2521; lnL = -3726.7857 from lowest BIC = 8728.709), with brach support >50% (1,000 bootstraps) shown, respectively. Nodes with black circles have 100% support. All original sequences are shown in bold font with the multiple of identical sequences recovered in parentheses where applicable. Country of origin and host species are given for non-cultured isolates. For brevity, select leaves are shown from the original tree (Fig. S9-S10). *These sequences were originally accessioned as* Crithidia mellificae*. **Accessioned as* C. mellificae *(Schmid-Hempel and Tognazzo 2010) but identified here as* Crithidia expoeki *based on sequence identity to this species. Tree files with accession numbers available via TreeBASE ID 16436.*

Within Clade 3, our sequences consistently resolved into two clearly distinct taxonomic units with strong support (bootstrap % for ML and NJ methods): strains 30254/30862 (99, 98) and strains BRL/SF (100, 100). Single gene trees for gGAPDH, SSU and *Cytb* robustly resolved all sequence variants according to these two taxonomic groups as well, among a broader range of previously accessioned Trypanosomatidae sequences (Fig. S3-S10).

First, all sequences isolated from strains 30254 and 30862 (*C. mellificae*) in addition to two SSU sequences isolated in vivo from *A. mellifera* (Fig. S4) and two sequences isolated in vivo from *O. bicornis*, gGAPDH (Fig. S3) and Cytb (Fig. 2) formed a novel taxonomic unit (ML and NJ bootstrap support %, respectively): gGAPDH (98, 100), SSU (93, 99), Cytb (98, 100). A second clade consistently resolved was comprised of all sequence variants from strains BRL and SF as well as all but two sequences isolated in vivo from *A. mellifera* in Belgium (ML and NJ bootstrap support %, respectively): gGAPDH (98, 100), SSU (99, 96), Cytb (87, 100).

All trypanosomatid sequences previously accessioned as *C. mellificae* belong to the BRL/SF clade and not to the 30254/30862 *C. mellificae* clade (Fig. 1-2, S3-S10). gGAPDH sequences isolated from *A. mellifera* in the U.S.A. (JF423199) and Japan (AB716357) and a gGAPDH sequence isolated from *Apis cerana* (Asian honey bee) in China (AB745489) are all members of the BRL/SF clade (Fig. 1 indicated by *). An SSU sequence (GU321196) isolated from *A. mellifera* in Switzerland (Fig. S4 indicated by *) and three *Cytb* sequences (AB716358, AB716359 and AB744129) originally ascribed to *C. mellificae* as “haplotype” variants A, B and C, respectively (Fig. 2 indicated by *), all belong to the BRL/SF clade as well. BLAST heuristic algorithm searches using promiscuous to strict parameters did not identify additional sequences belonging to the BRL/SF or 30254/30862 clades not already included in our alignments.

Despite flawed species designations, “haplotype” distinctions made previously using *Cytb* (Morimoto et al. 2012) are corroborated by our data and show strain BRL forms a distinct subclade (ML = 60, NJ = 55) with “haplotype B” sequence and strain SF forms a distinct subclade (ML = 73, NJ = 79) with “haplotype A” sequence (Fig. 2, S9-10). None of the sequences we isolated were within the

"haplotype C" subclade, represented solely by a sequence from *A*. *cerana*, yet our analyses support that this sequence is a lineage of the BRL/SF clade. Similarly, distinct subclades within the 30254/30862 clade were resolved using *Cytb*, such that the 5 unique sequences obtained from strain 30862 formed a distinct subclade from strain 30254 by ML (87) and NJ (52) analyses. The *Cytb* sequence we isolated from *O. bicornis* clustered with the 30254 subclade. Finally, one *Cytb* sequence (GU321191) originally accessioned as *C. mellificae* clearly belongs to the *Crithidia expoeki* clade (Fig. 2 indicated by **). Tables S2-S5 correlate sequence names used in the phylogenies to their Genbank accession numbers for novel sequences obtained from this study. Concatenated gGAPDH-SSU and *Cytb* alignment files are available in Table S6-S7 and contain accession numbers for each sequence.

4.4 Morphology and ultrastructure

Cultures of *C. mellificae* 30254 and 30862 had noticeably different predominant cell morphology when compared to those of strain BRL and SF. Representative confocal microscopy images show the predominant morphology of *C. mellificae* 30254 (Fig. 3A) and strain BRL (Fig. 3D) in culture, both of which had a single, long, free flagellum inserted at the apical end of the cell. We regularly observed cells with the choanomastigote morphology in cultures of both *C. mellificae* isolates (30254 and 30862) and cell polymorphism consistent with what has already been described (Langridge and McGhee 1967). In contrast, BRL and SF strains predominantly exhibited more elongated, tear-drop shaped cells typical of a promastigote morphotype that narrowed posteriorly to a short caudate (tail-like) extension. Brightly fluorescent kDNA and slightly more diffusely fluorescent nuclear DNA were localized with DAPI fluorescence and CLSM. The kDNA was located anterior (closer to the flagellum insertion point) to the nucleus in both *C. mellificae* 30254 (Fig. 3B-C) and strain BRL (Fig. 3E-F) cells.

Measurements from microscopy images of *C. mellificae* 30254 choanomastigotes showed cells (n = 50) averaged 6.62 µm in length (S.D. ± 1.23 µm, range 4.61 – 8.88 µm) by 3.32 µm widest width (S.D. ± 0.43 µm, range 2.47 – 4.38 µm). Average promastigote length of strain BRL cells (n = 50) were 7.44 µm in length (S.D. ± 1.59 µm, range 4.66 – 11.40 µm) by 3.15 µm widest width (S.D. ± 0.76

µm, range 1.50 – 4.65 µm). These measurements were made on the dominant morphotype from axenic cultures and do not include transitional variants that were approaching or at the spheroid stage. SEM (Fig. 4A-C) and TEM (Fig. 4D-K) imaging of *C. mellificae* 30254 revealed the choanomastigotes had deep, narrow lateral grooves (Fig. 4A-C) formed by the plasma membrane that were most likely structurally supported by a network of subpellicular microtubules (Fig. 4D,K). A single flagellum inserted into a narrow, anterior pocket that extended 415 into approximately half the length of the cell (Fig. 4D, H) with no apparent spicules or extensions at the point of insertion (Fig. 4B arrowhead). The flagellum was comprised of an axoneme (Fig. 4D-E) with typical 9x2 +2 microtubule structure (Fig. 4F) and a cryptic paraflagellar rod (PFR; Fig. 4H-J). kDNA was adjacent to the basal bodies at the base of the flagellum (Fig. 4E) within an elongated mitochondrion (Fig. 4E-G). TEM imaging corroborated confocal microscopy results that showed kDNA lies just anterior to the adjacent nucleus (Fig. 4D, I), which showed an electron dense nucleolus (Fig. 4G). Additional typical trypanosomatid cell structures included glycosomes, acidocalcisomes and spongiome (Fig. 4D). The unique promastigote cell morphology of strain BRL was more clearly discerned via SEM (Fig. 5A-C, E) and TEM (Fig. 5D, F-L) from that of *C. mellificae* 30254. In addition to the caudate, posterior extension (Fig. 5A-C, K) BRL promastigotes had a broad, deep lateral groove (Fig. 5A-C) in contrast to the narrow grooves of *C. mellificae* (Fig. 4A-C). Distinguishing these strains further was the common presence of a short spicule that extended from the flagellar pocket (Fig. 5C-D arrowheads) of strain BRL promastigotes that appeared to arise from the flagellum at its insertion point into the flagellar pocket, and thus did not appear to be emergent flagella from early cell division since each flagellum originated deep within the flagellar pocket and was comprised of an axoneme independently surrounded by plasma membrane (Fig. 5I arrowheads). 5H) was localized anterior and adjacent to the nucleus (Fig. 5K, L), as determined with DAPI staining (Fig. 3D-F), at the base of a flagellum that had an axoneme with 9x2 +2 microtubule structure and a proximal cryptic PFR (Fig. 5G, J). Additional ultrastructural features included a large nucleus and nucleolus, acidocalcisomes, glycosomes, basal bodies and a contractile vacuole (Fig. 5D, F, J-L).

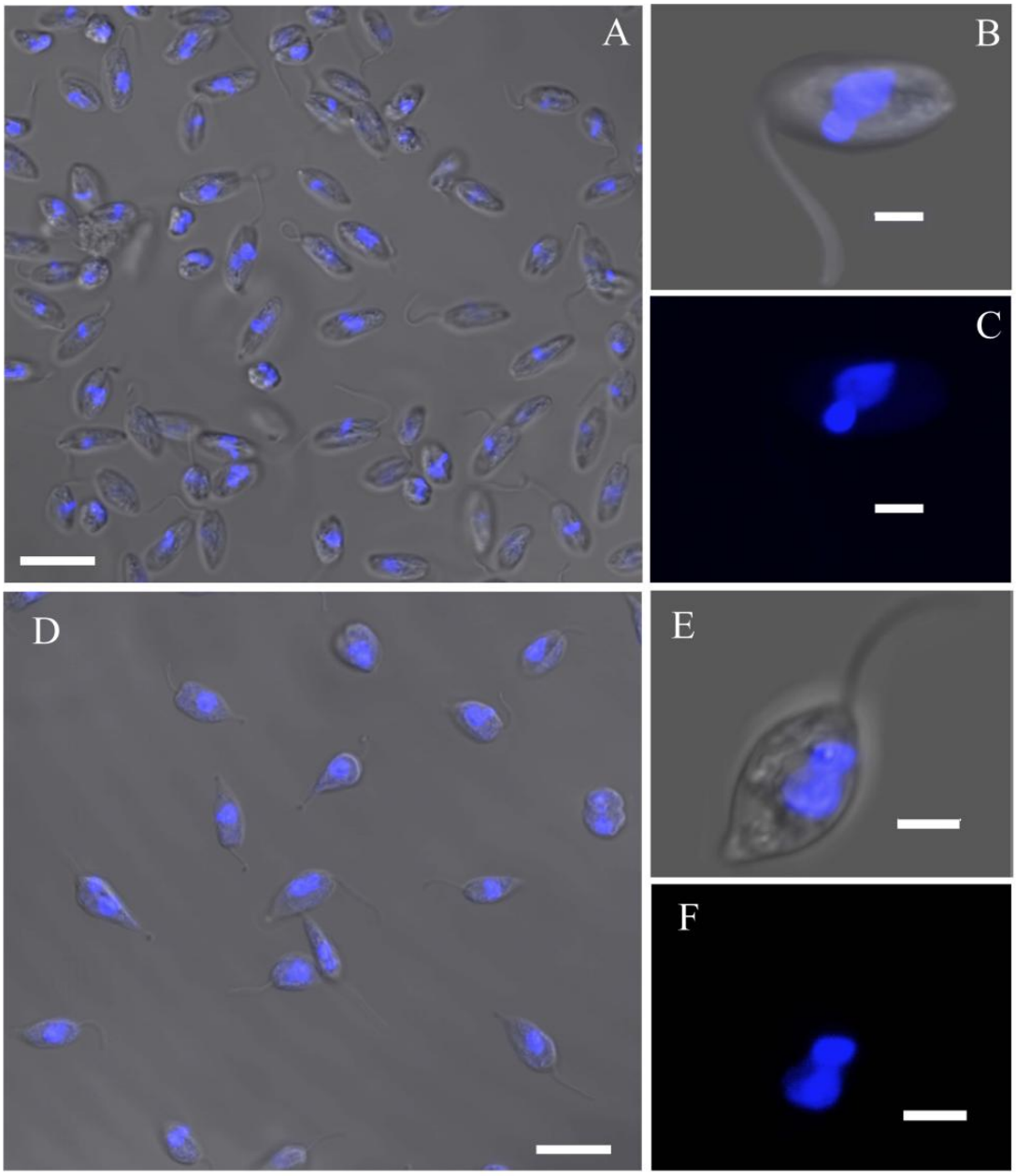


Figure 3. *Confocal microscopy of* Crithidia mellificae *strain 30254 (**A**-**C**) and* Lotmaria passim *n. gen., n. sp. strain BRL (**D**-**F**) showing the smaller and overall brighter kinetoplast in relation to the larger and less bright nucleus. Typical cell morphology of fixed axenic cell culture with DAPI fluorescence and DIC (A, D). Single cell view of fixed and DAPI-stained* C. mellificae *with DIC and fluorescence (B) and with fluorescence only (C). DAPI-stained* L. passim *with fluorescence and DIC (E) and with fluorescence only (F). Arrows point to the anterior flagellum insertion point. Scale bars: 10 µm (A, D) and 2 µm (B-C, E-F).*

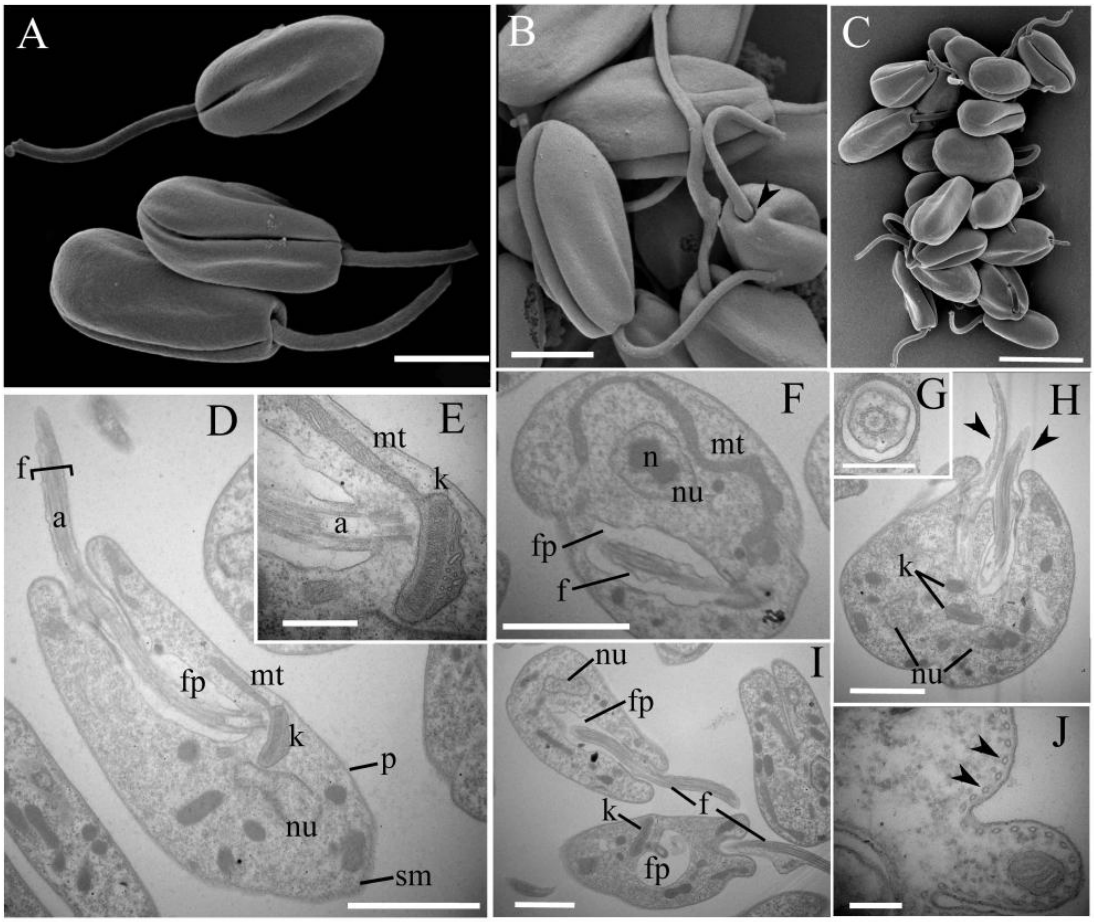


Figure 4. *SEM (A-C) and TEM (D-K) images of* Crithidia mellificae *strain 30254.* ***A-C****. Views of typical choanomastigotes with multiple deep, narrow lateral grooves and anterior flagellum insertion point. Anterior view into insertion point of flagellum (B, arrowhead).* ***D, H.*** *Longitudinal sections of choanomastigotes showing typical cell and organelle features. The paraflagellar rod (PFR) is indicated (H, white arrowheads).* ***E****. Higher magnification of (D) showing kinetoplast in extended mitochondrion, microtubules of the axoneme and basal bodies.* ***F.*** *Cross section of flagellar pocket (seen as the plasma membrane ring encircling the flagellum) showing 9x2 + 2 microtubule architecture of the axoneme.* ***G****. Cross section of a choanomastigote showing extended mitochondrion.* ***I****. Cytokinesis in progress along the longitudinal axis with two flagella (black arrowheads), kinetoplasts and nuclei visible. PFR is indicated (white arrowheads).* ***J.*** *Cross-section of a free flagellum with PFR (white arrowheads).* ***K****. Subpellicular microtubule network (black arrowheads) visible beneath the plasma membrane shown in cross section at a lateral groove. Abbreviations: acidocalcisome ("ac"), axoneme ("a"), basal bodies ("b"), flagellum ("f"), flagellar pocket ("fp"), glycosome ("g"), kinetoplast ("k"), mitochondrion ("mt"), nucleus ("nu"), nucleolus ("n"), subpellicular microtubules ("sm"), spongiome ("sp"). Scale bars: 5 µm (C), 2 µm (A-B, D, G-I), 500 nm (E-F, J), 200 nm (K).*

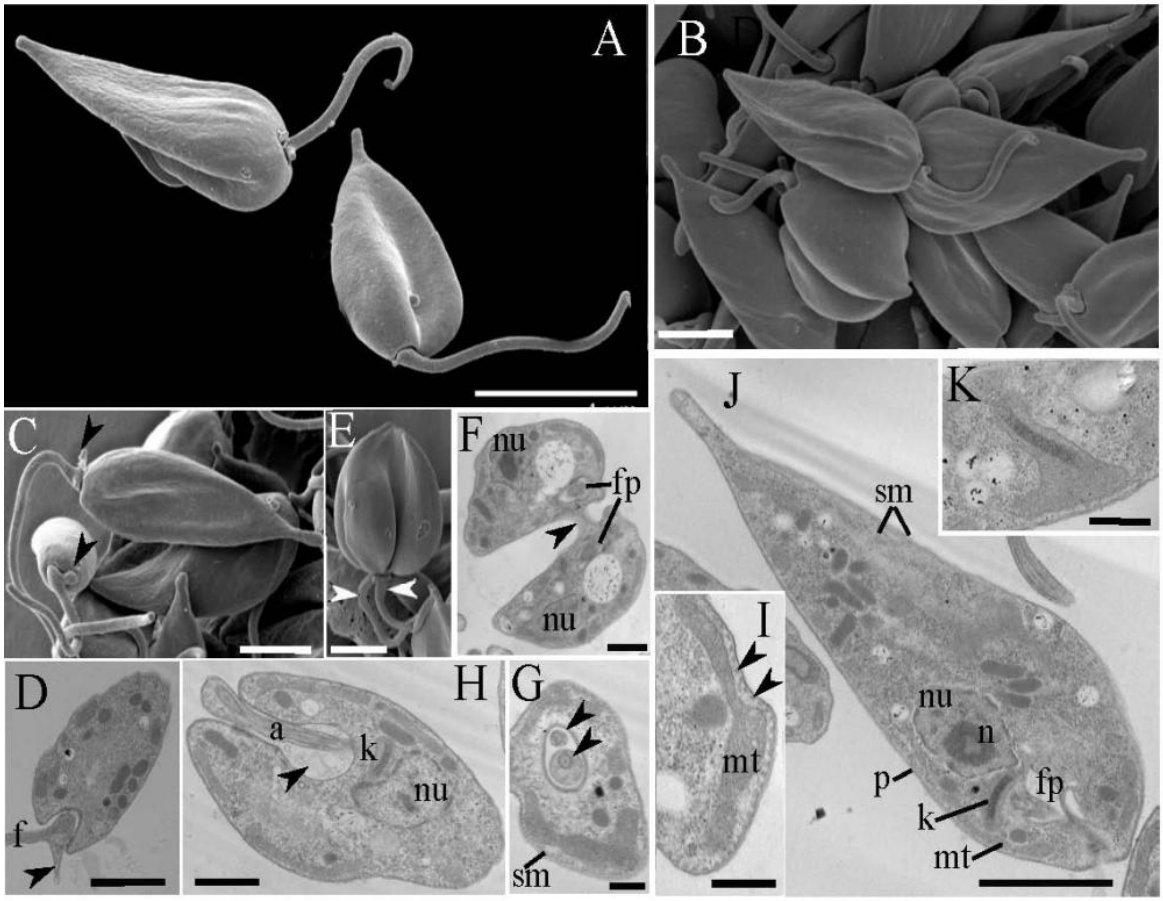


Figure 5. *SEM (A-C, E) and TEM (D, F-L) of* Lotmaria passim *n. gen., n. sp. strain BRL.* ***A***-***B****. Typical lanceolate promastigote cell morphology with broad, deep lateral groove, anterior flagellum insertion point and caudate posterior extension visible.* ***C-D****. A commonly present spicule (black arrowheads) at the insertion point of the flagellum is an extension of the plasma membrane from the flagellum (D).* ***E-F****. Cytokinesis along the longitudinal axis seen using SEM (E) with two developed flagella (white arrowheads) and in TEM cross section (F) where only a small section of plasma membrane (black arrowhead) remains holding the two cells together.* ***G.*** *Longitudinal section of flagellum proximal to the cell body with paraflagellar rod (PFR, white arrowheads) alongside the axoneme 146uminal146ules.* ***H-I.*** *Cross sections of promastigotes showing subpellicular microtubule network (H, black arrowheads) beneath the plasma membrane and early stage cell division (I) showing two flagella developed within the flagellar pocket (black arrowheads).* ***J****. Cross section of free flagellum showing cryptic PFR (white arrowhead).* ***K-L.*** *Longitudinal sections of promastigotes showing typical cell and organelle ultrastructure. A portion of the extending mitochondrion from the kinetoplast is shown in detail (L). Abbreviations: acidocalcisome ("ac"), axoneme ("a"), basal bodies ("b"), contractile vacuole ("cv"), flagellum ("f"), flagellar pocket ("fp"), glycosome ("g"), kinetoplast ("k"),mitochondrion ("mt"), nucleus ("nu"), nucleolus ("n"). Scale bars: 4 µm (A), 1008 2 µm (B-E, K), 1 µm (F-G), 500 nm (H-J, L).*

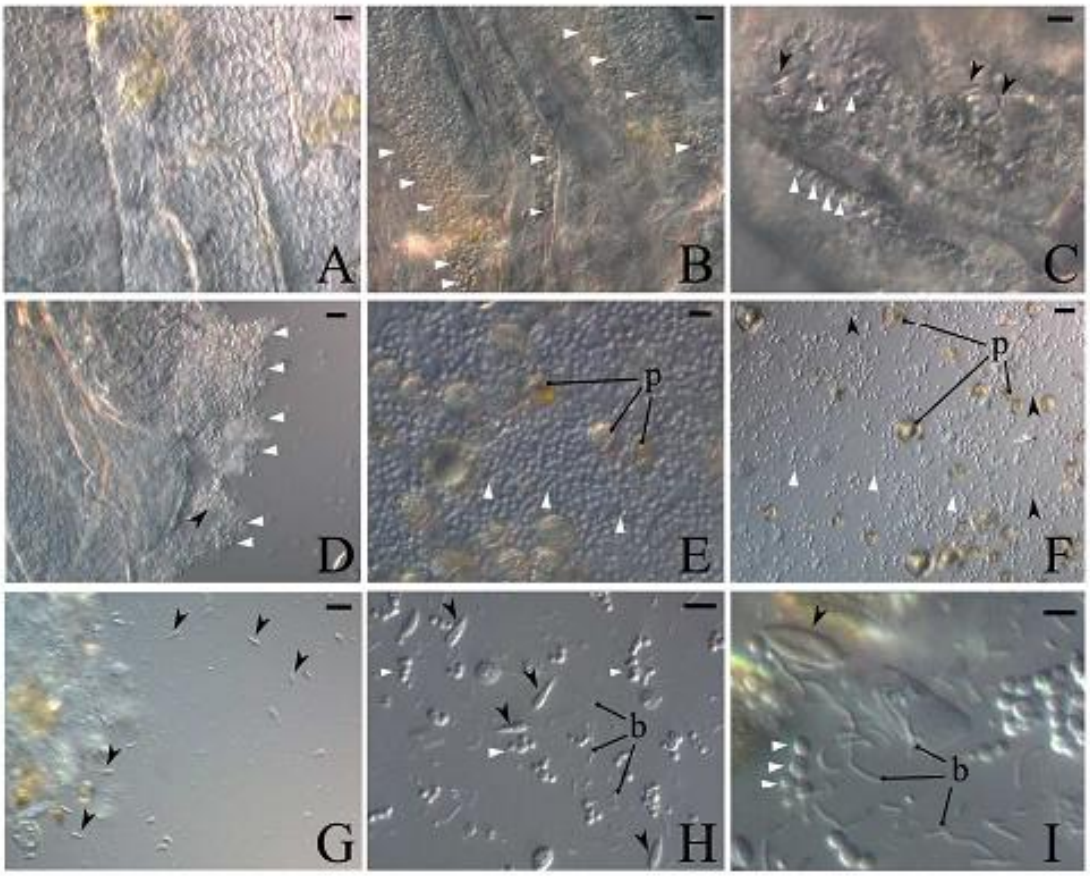


Figure 6. *Light microscopy of* Lotmaria passim *n. gen., n. sp. strain BRL in vivo followingexperimental inoculation of* Apis mellifera *showing both spheroid (white arrowheads) andpromastigote (black arrowheads) morphology.* ***A****. Tissue from lower ileum of uninfected controlbee.* ***B****-****C****. Spheroids form a single, dense layer along the 147uminal surface of the lower ileumwith several promastigotes among them.* ***D****. Spheroid layer peeling away from the lumenalsurface of the ileum where it was cut just anterior to the rectum.* ***E-F****. Rectal tissue macerateswith typical dense colonization by spheroids among pollen grains ("p") and severalpromastigotes (F).* ***G-I****. Free swimming promastigotes from dissected hindgut tissues amongdislodged spheroids and unidentified bacteria ("b") from the rectum (H,I). Promastigotesshowed identical morphology to those from axenic culture, including the broad, deep lateralgroove (I). Scale bars: 10 µm (B-C, E, H-I), 20 µm (A, D, F-G).*

Further, flagellated cells from both *C. mellificae* 30254 (Fig. 4I) and BRL (Fig. 5E-F) cultures were regularly seen undergoing cytokinesis yet only BRL strain cells were observed to have these spicules. The flagellar pocket of strain BRL did not extend as deeply into the cell (Fig. 5K) as *C. mellificae*. kDNA within a large extended mitochondrion lying just beneath the subpellicular microtubule network (Fig.

5H) was localized anterior and adjacent to the nucleus (Fig. 5K, L), as determined with DAPI staining (Fig. 3D-F), at the base of a flagellum that had an axoneme with 9x2 +2 microtubule structure and a proximal cryptic PFR (Fig. 5G, J). Additional ultrastructural features included a large nucleus and nucleolus, acidocalcisomes, glycosomes, basal bodies and a contractile vacuole (Fig. 5D, F, J-L).

In addition to typical cytokinesis, two additional states of intercellular adherence were regularly observed in our trypanosomatid cell cultures (not shown). First, both *C. mellificae* 30254/30862 and BRL/SF strain cells would occasionally form aggregates of up to ~30 cells by entwining their flagella, forming what are known as “rosettes” (Dwyer et al. 1974). Second, adherent pairs of BRL/SF strain promastigotes occasionally formed when two cells conjoined via the caudate extensions, in a posterior-posterior fashion, forming a “doublet” (Wheeler et al. 2011). This form of intercellular adherence was not observed in *C. mellificae* cultures. Both of these examples of intercellular adherence may have been artifacts of cell culture since they were not observed in vivo.

4.5 Tissue tropism of strain BRL

Primary site of colonization in experimentally infected bees (n = 20) was the anterior rectum, particularly surrounding the rectal papillae, often extending into the distal end of the ileum just proximal to the rectum. The lumenal surfaces of these sites in uninfected bees (Fig. 6A) were noticeably different from infected bees, which were largely covered by a dense layer of spheroids (Fig. 6B-C) approximately 3-4 µm in diameter. When disrupted from their *in vivo* location (Fig. 6D-I), these cells often separated from one another. Spheroids were only observed in the hindgut and were common in the rectum (n = 17) and occasional in the ileum (n = 8). Within the ileum, spheroids were almost exclusively observed attached at the distal end proximal to the rectum, except for one observation in the pylorus of the ileum (n = 1). No spheroids were observed in the crop or midgut.

Less numerous but consistently present were promastigotes distributed among the spheroids on the hindgut lumenal surface (Fig. 6C-D). In vivo morphology of promastigotes was identical to those from axenic culture and showed typical acute posteriors (Fig. 6G-H) and a deep lateral groove (Fig. 6I). Rarely, more rounded (blunt posterior) promastigotes were observed in the ileum and

rectum (not shown). Promastigotes also displayed the spiraling motility as observed from axenic cultures. Promastigotes were common in the rectum (n = 17), occasional in the ileum (n = 10), and rare in the midgut (n = 1) and crop (n = 2). Three bees from the experimentally infected group did not have detectable trypanosomatids at the time of observation, apparently having cleared the infection. No scarring nor specific colonization of the pylorus as described by Lotmar (1946) was observed from any of these experimental inoculations. None of the sugar water control group bees had visually detectable trypanosomatids (n = 20).

5. Discussion

This study was spurred by a recognized need to contrast recent trypanosomatid isolates from honey bees to type strain species of *C. mellificae* (Evans and Schwarz 2011), with building evidence provided by a study that contrasted healthy versus diseased colonies and identified trypanosomatid sequences as highly divergent from *C. mellificae* strain 30254 (Cornman et al. 2012). This study further fueled interest with the finding that these were the most prevalent non-viral parasites in honey bee colonies. The establishment of two recent honey bee Trypanosomatidae strains in axenic culture, SF (Runckel et al. 2011) and BRL (this study), allowed us to reliably obtain genetic data and contrast it with the putative axenic type strains of *C. mellificae*. Our analyses of gGAPDH, SSU, *Cytb* and ITS1-5.8S-ITS2 loci from these four strains consistently resolved stains BRL/SF and strains 30254/30862 into two non-overlapping taxonomic groups, both of which were nested within the Leishmaniinae subfamily. Trypanosomatidae strains BRL and SF represent a novel taxonomic unit distinct from any previously published characterizations and Trypanosomatidae strains 30254 and 30862 are conspecifics of the previously designated taxa *C. mellificae*. For these reasons, strains BRL and SF are denominated and described as type specimens for a novel taxon, *Lotmaria passim* n. gen., n. sp. in accordance with ICZN guidelines (ICZN 1999).

Phylogenetics using *gGAPDH-SSU* placed all Hymenoptera trypanosomatids within a novel clade (Clade 3) of the Leishmaniinae subfamily that includes the following lineages: 1) *L. passim* from *A. mellifera* (including strains BRL/haplotype B, SF/haplotype A), 2) *L. passim* from *A. cerana* (China

haplotype C), 3) *C. mellificae* from *A. mellifera* (strain 30254) 4) *C. mellificae* from *V. squamosa* (strain 30862) 5) *C. bombi* from *Bombus lucorum* and 6) *C. expoeki* from *B. lucorum*. Importantly, this shows that these species shared a common ancestor that was different from the common ancestor of species within the *Leishmania* clade (Clade 1) and the *Crithidia* clade (Clade 2). The phylogenetic placement of hymenopteran trypanosomatids to the same clade within the Leishmaniinae, referred to here as Clade 3, has been a consistent finding (Morimoto et al. 2012; Runckel et al. 2014; Schmid-Hempel and Tognazzo 2010). In addition to the hymenopteran trypanosomatids, we show that Clade 3 also contains species from Hemiptera hosts (*C. abscondita*, *L. jaderae*, *L. neopamerae*, *L. podlipaevi*, *L. pyrrhocoris*, *L. scantii*, and *L. seymouri*). Review of studies that have included only the hemipteran trypanosomatids from Clade 3 confirm the common ancestry of these seven species (e.g. Kostygov et al. 2014; Yurchenko et al. 2014) and the validity of Clade 3 as a distinct lineage of this subfamily. Single gene analyses of gGAPDH sequences corroborated this result, but SSU data alone did not resolve any phylogenetic positions of taxa within the Leishmaniinae. Too few *Cytb* accessions are available at present to decipher broader relationships among the Leishmaniinae taxa, but this was the most valuable locus tested to phylogenetically differentiate honey bee Trypanosomatidae strains from one another and interspecific relationships to *C. mellificae*, *C. bombi* and *C. expoekii*, as found in previous work (Morimoto et al. 2012; Schmid-Hempel and Tognazzo 2010). These findings confirm the value of kDNA for resolving strain differences within monoxenous trypanosomatids (Wallace et al. 1983), similar to what has been found in dixenous species (e.g. Asato et al. 2009; Spotorno et al. 2008).

Trypanosomatid systematics originally used morphological features to define monoxenous taxa to genera but it soon became apparent that this was creating immense confusion since many were indistinguishable due to intraspecific polymorphism and homoplasies across lineages. This was particularly true for taxa assigned to the genus *Leptomonas* or *Crithidia* based on two morphotypic homoplasies: promastigotes and choanomatigotes, respectively. As a first attempt to reduce the confusion and refine trypanosomatid taxonomy, recommendations were put forth for new species

descriptions to use not only host type and cell morphology but also the establishment of axenic cell cultures from which kDNA fingerprinting, nutritional requirements and growth parameters could be assessed (Wallace et al. 1983). Although most of these recommendations were rarely applied, establishment of axenic cell cultures did gain support and resulted in an invaluable resource of monoxenous trypanosomatid cell lines that were archived, primarily at ATCC. Because of such archived specimens (ATCC 30254 and ATCC 30862), we were able to determine that the honey bee trypanosomatid *C. mellificae* is distinct from the novel honey bee isolates here defined as *L. passim*.

Current standards for protist descriptions and taxonomy require genetic data, which by default, becomes the sole determinant for taxa that cannot be reliably distinguished from other genera and/or species by morphological features. Among the Trypanosomatidae, the current broadly standard loci are *gGAPDH* and *SSU*, with *Cytb* a standard locus for some taxa. Phylogenetics using primarily *gGAPDH* and *SSU* either individually or concatenated have confirmed that classical taxonomic assignments based on morphology do not accurately reflect the evolutionary history of some lineages. For a time, *Crithidia* and *Leptomonas* were accepted as polyphyletic but it is now easy to explain that this is a result of traditional genus assignment misled by morphotypic homoplasies, which probably reflect convergent evolution rather than evolution from a common ancestor. Despite this recognition and self admonishment for doing so, authors have continued to assign new Trypanosomatidae taxa based on cell morphology to the limited, classically defined genera that are deeply entrenched in the literature instead of defining novel genera in accordance with the current standards of phylogenetics. The massive undertaking of restructuring monoxenous trypanosomtid taxa based on phylogenetics is underway, by validation and new assignments to existing genera (Borghesan et al. 2012; Teixeira et al. 2011) and by erecting novel genera (Kostygov et al. 2014; Votypka et al. 2013; Votypka et al. 2014), to which our report contributes.

5.1 Novel genus assignment of strains BRL and SF

As a member of the novel Clade 3, strains BRL and SF are shown to be evolutionarily associated with other species classically assigned to *Crithidia* and *Leptomonas* based on morphology. *Crithidia*

fasciculate Leger, 1904 is the type species for the genus and is part of a distinct clade ("Crithidia" Clade 2) within Leishmaniinae shown here and similarly in prior work (e.g. "C" clade in Yurchenko et al. 2009; "Cf" clade in Jirku et al. 2012). Since phylogenetics did not place strains BRL and SF within the accepted type species clade, we could not justify assigning it to this genus.

Validity of the genus *Leptomonas* Kent, 1880 was brought into question at its inception by Kent himself (Kent 1880) when he described the type species *L. bütschlii* Kent, 1880 from an aquatic nematode (Nematoda; Adenophorea; Enoplida; Tobrilidae; *Tobrilus gracilis (*syn. *Trilobus gracilis*)). Nonetheless, a parasitic flagellate discovered in the hindgut of a water scorpion (Hemiptera, Nepidae, *Nepa*) shared the same general cell features described from the *Leptomonas* type species and was assigned to this genus as *L. jaculum* Leger, 1902. This paved the way for subsequent genus denominations of insect flagellates with a promastigote morphotype, the validity of which has been in question for decades (Maslov et al. 2013; Wallace 1966). Although no type material for *L. bütschlii* or *L. jaculum* are available to our knowledge, a trypanosomatid was recently isolated from the same host genus from which *L. jaculum* was originally described (*Nepa* sp.) and established as a 'neotype' (Kostygov et al. 2007). If this neotype is accepted, it represents the type material for the first insect Trypanosomatidae assigned to the genus *Leptomonas*. Phylogenetics using type DNA from this neotype (EF184218) and other Trypanosomatidae sequences established the phylogenetic clade for *L. jaculum* (Clade 5 in Maslov et al. 2013), which is not a member of the Leishmaniinae subfamily and is clearly a sister clade to the *Blastocrithidia* as shown repeatedly in numerous studies (e.g. Maslov et al. 2010; Votypka et al. 2012; Yurchenko et al. 2009). This invalidates *Leptomonas* as a candidate genus for taxa within the Leishmaniiane subfamily. Since genus assignment of strains BRL and SF within our Clade 3 to *Crithidia* or *Leptomonas* could not be justified, we were required to denominate the new genus *Lotmaria* Evans and Schwarz, 2014.

5.2 Comparison of *L. passim* n. gen., n. sp. to prior descriptions of honey bee trypanosomatids

The characterization of a honey bee trypanosomatid by R. Lotmar (1946) provided thorough written and diagrammatic descriptions for the species proposed as *L. apis*, however no archived type specimens nor genetic characterizations were made. Although we now know morphology cannot be the determining factor for putative species identification, it is valuable and required to address this point since it is the only information with which to make comparisons to historical characterizations. The description of *L. apis* is distinct from the BRL/SF *L. passim* taxon we propose here in two important ways. First, several careful drawings and descriptions of *L. apis* characterized the flagellated stages as having a narrow anterior end from which the flagellum originated with the cell widening distally to culminate in a large, rounded posterior end that typified this species. This morphology is more consistent with *C. mellificae* than with *L. passim*. No promastigotes with acute posteriors like those characteristic of *L. passim* were described for *L. apis*. Interestingly, a later report on trypanosomatids found in honey bees (Lom 1962) described flagellated stages as having "…the posterior end tapering and often drawn out into a sharp point", which is consistent with *L. passim* and as we show is a feature helpful to differentiate *L. passim* from *C. mellificae*. However, these trypanosomatids were attributed by the author to the genus *Crithidia* and presumed to be opportunistic infections, thus no species designation was proposed. Rarely thin, elongated forms of *L. apis* were also observed by Lotmar, which is a morphology reported from other insect trypanosomatids (e.g. *Blechomonas maslovi*, *Blechomonas wendygibsoni*, *Leptomonas spiculata*) but never for *C. mellificae* (Langridge 1966, Langridge and McGhee 1967, this report) and we have not observed such morphotypes from *L. passim*.

A second distinction we identified from Lotmar's characterization of *L. apis* was her careful description that *L. apis* specifically settled on and colonized the epithelium of the pyloric region of the gut, occasionally slightly anterior or posterior into the midgut and upper ileum. She occasionally observed flagellated stages throughout various regions of the gut, but never noted a case of

colonized rectum tissue, which we show appears to be the preferred niche colonized by *L. passim* and has previously been shown as the preferred niche for *C. mellificae* as well (Langridge and McGhee 1967). These key distinctions support to the best possible ability that *L. passim*, represented by strains BRL and SF, is not the proposed *L. apis* taxa Lotmar observed in honey bees from several regions of Europe. The description of *L. apis* also does not fully support the features of *C. mellificae* as understood by Langridge and McGhee (1967) who noted Lotmar's report of *L. apis* but did not contrast their novel species description of *C. mellificae* to *L. apis*.

We also note that the spheroid morphotype we describe for *L. passim* (non-motile, round adherent cell) is identical to descriptions and images of *C. mellificae* in honey bees as "large numbers of the rounded parasites attached to the wall of the rectum…" (Langridge and McGhee 1967). Although these cells have the same morphology as amastigotes described from *Leishmania* spp., this term is associated with the intracellular stage within mammalian hosts. Spheroids are a diagnostic feature of both *C. mellificae* and *L. passim* (Clade 3) in honey bee hosts, but given that limited experimental in vivo work has been performed with monxenous insect trypanosomatids, it remains to be seen if and how prevalent this morphotype is in other clades.

5.3 Identifying trypanosomatids from Hymenoptera and host specificity

Our data support that *L. passim* is currently more prevalent in honey bees than *C. mellificae*, is globally distributed, and has been present in *A. mellifera* since at least 2010 based on the earliest accessioned sequence we were able to confirm (GU321196). The majority of original trypanosomatid sequences amplified from *A. mellifera* in Belgium belonged to *L. passim* except for two *SSU* sequences from *C. mellificae*. Thus, current populations of *A. mellifera* host both species but *L. passim* predominates. It is unclear at this time why *C. mellificae* has been infrequently detected in *A. mellifera* recently, yet must have been relatively prevalent nearly 50 years ago when it was repeatedly isolated and described in Australia (Langridge 1966, Langridge and McGhee 1967) and from the U.S.A. (type specimen accessions). We have determined that all previously accessioned sequence data designated as *C. mellificae* are incorrectly attributed and actually belong to the new

species *L. passim*. Given that 1) both axenic isolates (BRL and SF) of *L. passim* were established from the U.S.A. between 2010 and 2012, 2) the vast majority of original sequence data obtained from *A. mellifera* in Belgium are from *L. passim* and 3) all previously accessioned data from honey bees in China, Japan, Switzerland and the U.S.A. are actually lineages of *L. passim* and not *C. mellificae* as previously presumed, we conclude that *L. passim* is global and commonly infects honey bees (*A. mellifera*) while *C. mellificae* is currently comparatively infrequent. Further, *L. passim* is not strictly host-specific as sequence data obtained from *A. cerana* (AB744129) also belongs to this new species.

As trypanosomatids progress through different stages of their cell cycle, variation in promastigote morphologies are well documented from *Leishmania* (Clark 1959; Wheeler, Gluenz and Gull 2011). The flagellated stage of *C. mellificae* and *L. passim* as we have shown here can be generally useful to help discern these two species within honey bees, however, putative species designation requires genetic confirmation. Previously published light microscopy images of *C. bombi* and *C. expoeki* from bumblebees are indistinguishable from the images of *C. mellificae* we present here and cross-infections of bumblebee and honey bee eukaryotic endoparasites do occur (Plischuk et al. 2011; Ruiz-Gonzalez and Brown 2006). Further, cryptic species may be present that cannot be distinguished from one another by cell morphology, further supporting the need for genetic confirmation.

Our analyses clarify that *C. mellificae* promiscuously utilizes a wide variety of hymenopteran hosts, including not only *A. mellifera* but also *V. squamosa*, *O. bicornis* (shown here) and *Osmia cornuta* (unpublished results) and provides a contradictory example to the paradigm that insect trypanosomatids are host-specific. By contrast, *L. passim* has so far only been isolated from *A. mellifera* and in one instance *A. cerana*, supportive of the paradigm that trypanosomatids have limits to the range of hosts they can infect. Certainly, behavioral and metabolic differences may provide barriers that drive evolutionary divergence and host specificity. Previous discussions have speculated that parasites (Evans and Schwarz 2011) and trypanosomatids specifically (McGhee and Cosgrove 1980) may be delimited among honey bees due to unique social behaviors such as adult-to-larva

food provision (royal jelly) and adult-to660adult food sharing (trophallaxis). Metabolic limits to cross-species susceptibility have been empirically demonstrated specifically in *A. mellifera* via challenges with trypanosomatids isolated from flies (Diptera), including *Crithidia luciliae*, *Crithidia fasciculata*, *Crithidia culicidarum*, and *Stigomonas* (*Crithidia) oncopelti*, none of which established successful infections within *A. mellifera* (Lom 1962). Now, this improved characterization of inter- and intragenetic diversity among *C. mellificae* and *L. passim* provides the foundation for future work on host diversity and distribution that will elaborate on the life history of these key representatives of monoxenous trypanosomatids.

6. Taxonomic summary

Super-group Excavata (Cavalier-Smith, 2002) emend. Simpson, 2003

Phylum Euglenozoa (Cavalier-Smith, 1981) emend. Simpson, 1997

Class Kinetoplastea Honigberg, 1963

Subclass Metakinetoplastina Vickerman, 2004

Order Trypanosomatida (Kent, 1880) emend. Vickerman, 2004

Family Trypanosomatidae Doflein, 1901

Subfamily Leishmaniinae Maslov and Lukeš, 2012

***Lotmaria* n. gen. Evans and Schwarz, 2014**

Diagnosis: Monoxenous parasites strictly of the Leishmaniinae subfamily and part of a distinct phylogenetic clade, referred to here as Clade 3, that currently includes taxa assigned to two other genera based on classical morphology, which is now recognized as unacceptable for taxonomic characterization: *Crithidia abscondita*, *Crithidia bombi*, *Crithidia expoeki*, *Crithidia mellificae*, *Leptomonas jaderae*, *Leptomonas neopamerae*, *Leptomonas podlipaevi*, *Leptomonas pyrrhocoris*, *Leptomonas scantii*, and *Leptomonas seymouri*.

Remarks: Phylogenetics invalidates *Leptomonas* Kent, 1880 as a genus assignment for taxa within the Leishmaniianae subfamily. Phylogenetics also show that species assigned to the genus *Crithidia*

Leger, 1904 must fall within the lineage associated with the type species for this genus, *C. fasciculata*, referred to here as Clade 2.

Type species: *Lotmaria passim* n. sp.

Etymology: The genus is named in honor of microbiologist and honey bee expert Ruth Lotmar, who produced extremely detailed reports of trypanosomatids from Hymenoptera in the middle of the 20th century. Ruth Lotmar was chosen as a representative for the many Hymenoptera trypanosomatid researchers whose combined work has pioneered an understanding of the host parasite biology of monoxenous insect trypanosomes and to underscore the value Hymenoptera trypanosomatids have made toward improved understanding of Trypanosomatidae evolutionary history. The genus suffix "-ia" was chosen to show phylogenetic affiliation with the two other prominent clades of the Leishmaniinae subfamily, *Leishmania* and *Crithidia*.

***Lotmaria passim* n. sp. Schwarz, 2014**

Diagnosis: Promastigotes are lanceolate to tear-drop shaped, have a single free flagellum lacking a membrane that inserts into a flagellar pocket opening at the broad, rounded anterior end of the cell. Length 7.44 µm (4.66 - 11.40 µm), width 3.15 µm (1.50 - 4.65 µm). Promastigotes are horizontally compressed with a deep groove oblique to the anterior-posterior axis, creating a wide axis and a narrow axis tapering to a typically caudate posterior extension. The kinetoplast is anterior to the nucleus. A short spicule (finger-like projection) often occurs at the opening of the flagellar pocket adjacent to the flagellum, visible only using electron microscopy. Spheroids (diameter = 3 - 4 µm) adhere to the gut wall in a single layer and often in dense aggregates, particularly among the rectal papillae (anterior rectum) and into the lower ileum. Polymorphic cell stages that range between the described promastigote to the spheroid form may be seen.

Type taxon: Hapantotype strain BRL (ATCC 00359).

Type host: In hind gut (ileum) of adult female *Apis mellifera ligustica* (Hymenoptera, Apidae).

Other hosts: *Apis cerana*.

Type locality: U.S. Department of Agriculture Bee Research Lab apiary in Beltsville, Maryland, U.S.A. (39° 2'26.09"N, 76°51'42.25"W).

Type material: Giemsa-stained hapantotype specimen mounts have been deposited to the U.S. National Parasite Collection (USNPC) in Beltsville, Maryland, U.S.A. for *L. passim* n. gen., n. sp. strain BRL (USNPC No. 108271.00) as well as parahapantotype mounts of *L. passim* n. gen., n. sp. strain SF (USNPC No. 108272.00). Cultures of the hapantotype strain BRL have been accessioned and are available (ATCC 00359, Protistology Collection in Manassas, Virginia, U.S.A.)

Etymology: The species name is derived from the Latin word *passim* meaning "everywhere" in reference to its global and pervasive distribution within honey bees. The species name also contains the anagram "apis m" for its type host species *Apis mellifera*.

Gene sequences: The species is identified by the following unique DNA sequences obtained from the *L. passim* n. gen., n. sp. hapantotype strain BRL and deposited to GenBank: gGAPDH (KJ713353 to KJ713356), SSU *rRNA* (KJ713377 and KJ713378), *Cytb* (KJ684960 to KJ684964), and ITS1, 5.8S, and ITS2 region (KJ722737 to KJ722744). Unique genetic sequences from the *L. passim* n. gen., n. sp. parahapantotype strain SF deposited to Genbank may also be used: gGAPDH (KJ713346 to KJ713352), SSU rRNA (KJ713371 and KJ713376), *Cytb* (KJ684955 to KJ684959), and ITS1, 5.8S, and ITS2 region (KJ722728 to KJ722736).

7. Acknowledgements

Christopher Pooley of the USDA Soybean Genomics and Improvement Lab prepared digital photographs and strain SF was kindly provided by Dr. Michelle Flenniken of Montana State University. R.S.S. is grateful to Dr. Allen Smith of the USDA Diet, Genomics and Immunology Lab for cell culture knowledge and material support, Dawn Lopez and Margaret Smith of the USDA Bee Research Lab for helpful discussions regarding the manuscript, Dr. Eric Hoberg and Patricia Pilitt of the USNPC for clarification of type specimen designations and accessioning assistance, Juliane Birke who provided an English translation of the R. Lotmar manuscript. The thorough efforts of three

anonymous reviewers greatly improved this manuscript. R.S.S. was supported in part by U.S. National Science Foundation Dimensions in Biodiversity grant 1046153. J.R. and D.C.G. acknowledge funding by the Research Foundation-Flanders (FWO, research grant G.0628.11).

8. References

Adl, S.M., Simpson, A.G.B., Farmer, M.A., Andersen, R.A., Anderson, O.R., et al., 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol, 52:399-451.

Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., et al., 2012. The revised classification of eukaryotes. J Eukaryot Microbiol, 59:429-493.

Asato, Y., Oshiro, M., Myint, C.K., Yamamoto, Y., Kato, H., Marco, J.D., Mimori, T., Gomez, E.A.L., Hashiguchi, Y., Uezato, H., 2009. Phylogenetic analysis of the genus Leishmania by cytochrome b gene sequencing. Exp Parasitol, 121:352-361.

Borghesan, T. C., Ferreira, R. C., Takata, C. S. A., Campaner, M., Borda, C. C., Paiva, F.,Milder, R. V., Teixeira, M. M. G., Camargo, E. P. 2013. Molecular phylogenetic redefinition of Herpetomonas (Kinetoplastea, Trypanosomatidae), a genus of insect parasites associated with flies. Protist, 164:129-152.

Clark, T. B. 1959. Comparative morphology of four genera of Trypanosomatidae. J. Protozool.,6:227-232.Cornman, R. S., Tarpy, D. R., Chen, Y., Jeffreys, L., Lopez, D., Pettis, J. S., vanEngelsdorp, D., Evans, J. D., 2012. Pathogen webs in collapsing honey bee colonies. PLoS ONE,7:e43562.

Cox-Foster, D. L., Conlan, S., Holmes, E. C., Palacios, G., Evand, J. D., Moran, N. A., Quan, P.L., Briese, T., Hornig, M., Geiser, D. M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S. K., Simons, J. F., Egholm, M.,Pettis, J. S., Lipkin, W. I,. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science, 318:283-287.

Dietemann, V., Ellis, J. D., Neumann, P., 2013. COLOSS BEEBOOK: Standard Methods for Apis mellifera research. International Bee Research Association, Treforest, United Kingdom.p. 1-614.

Dwyer, D. M., Langreth, S. G., Dwyer, N. K., 1974. Evidence for a polysaccharide 781 surface coatin the developmental stages of Leishmania donovani: a fine structure-cytochemical study. Z Parasitenk, 43:227-249.

Edgar, R. C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 32:1792–1797.

Evans, J. D., Chen, Y. P., di Prisco, G., Pettis, J. S., Williams, V. 2009. Bee cups: single-use cages for honey bee experiments. J Apicult Res, 48:300–302.

Evans, J. D. and Schwarz, R. S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol, 19:614-620.

Fanthan, H. B. and Porter, A., 1912. Note on certain protozoa found in bees. Suppl J Board Agricul, 19:138.

Forsgren, E. and Fries, I., 2010. Comparative virulence of Nosema ceranae and Nosema apis in individual European honey bees. Vet Parasitol, 170:212-217.

Fyg, W., 1954. Uber das Vorkommen von Flagellaten im Rectum der Honigbiene (Apis mellifica L.). Mitteilungen der Schweizerischen Entomologischen Gesellschaft, 27:423-428.

Genersch, E., Ashiralieva, A., Fries, I., 2005. Strain- and genotype-specific differences in virulence of Paenibacillus larvae subsp. larvae, a bacterial pathogen causing American foulbrood disease in honeybees. Appl Env Microbio., 71:7551-7555.

Higes, M., Meana, A., Bartolome, C., Botias, C., Martin-Hernandez, R., 2013. Nosema ceranae (Microsoporidia), a controversial 21st century honey bee pathogen. Env Microbiol Rep 5:17-29.

Hoare, C. A. and Wallace, F. G., 1966. Developmental stages of trypanosomatid flagellates: a new terminology. Nature, 212:1385-1386.

ICZN, 1999. International Code of Zoological Nomenclature. 4th ed. The International Trust for Zoological Nomenclature, London.

Jirku, M., Yurchenko, V. Y., Lukeš, J., Maslov, D. A., 2012. New species of insect trypanosomatids from Costa Rica and the proposal for a new subfamily within the Trypanosomatidae. J Eukaryot Microbiol, 59:537-547.

Kent, W. S. 1880. A manual of the infusoria, volume 1. David Bogue, London, United Kingdom. p. 1-472.

Klee, J., Besana, A. M., Genersch, E., Gisder, S., Nanetti, A., Tam, D. Q., Chinh, T. X., Puerta, F., Ruz, J. M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I., Paxton, R. J., 2007. Widespread dispersal of the microsporidian Nosema ceranae, an emergent pathogen of the western honey bee, Apis mellifera. J Invert Pathol, 96:1-10.

Kostygov, A. Y., Frolov, A. 2007. Leptomonas jaculum (Leger, 1902) Woodcock 1914: a leptomonas or a blastocrithidia? Parazitologiia, 41:126-136.

Kostygov, A. Y., Grybchuk-Ieremenko, A., Malysheva, M. N., Frolov, A. O., Yurchenko, V., 2014. Molecular revision of the genus Wallaceina. Protist, 165:594-604.

Langridge, D. F., 1966. Flagellated protozoa (Trypanosomidae) in the honey bee, Apis mellifera, in Australia. J Invert Pathol, 8:124-126.

Langridge, D. F., McGhee, R. B., 1967. Crithidia mellificae n. sp. an acidophilic trypanosomatid of the honey bee Apis mellifera. J Protozool, 14:485-487.

Lom, J., 1962. The occurence of a Crithidia-species within the gut of the honey-bee, Apis mellifica L. Entomophaga Mémoire hors Série, 2:91-93.

Lotmar, R., 1946. Uuber flagellaten und bakterien im duundarm der honigbiene (Apis mellifica). Beih. Schweiz. Bienen-Zeit., 2:49-76.

Macarisin, D., Bauchan, G., Fayer, R., 2010. Spinacia oleracea L. leaf 827 stomata harboring Cryptosporidium parvum oocysts: a potential threat to food safety. Appl Env Microbiol, 76:555-559.

Macarisin, D., O'Brien, C., Fayer, R., Bauchan, G., Jenkins, M., 2012. Immunolocalization of β- and δ-giardin within the ventral disk in trophozoites of Giardia duodenalis using multiplex laser scanning confocal microscopy. Parasitol Res, 111:241-248.

Maslov, D. A., Yurchenko, V. Y., Jirku, M., Lukeš, J., 2010. Two new species of trypanosomatid parasites isolated from Heteroptera in Costa Rica. J Eukaryot Microbiol, 57:177-188.

Maslov, D. A., Votypka, J., Yurchenko, V., Lukeš, J., 2013. Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol, 29:43-52.

McGhee, R. B., Cosgrove, W. B., 1980. Biology and physiology of the lower Trypanosomatidae. Microbiol Rev, 44:140-173.

Merzlyak, E., Yurchenko, V., Kolesnikov, A. A., Alexandrov, K., Podlipaev, S. A., Maslov, D. A., 2001. Diversity and phylogeny of insect trypanosomatids based on small subunit rRNA genes: polyphyly of Leptomonas and Blastocrithidia. J Eukaryot Microbiol, 48:161-169.

Moran, N. A., Hansen, A. K., Powell, J. E., Sabree, Z. L., 2012. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. PLoS ONE, 7(4): e36393.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B., Peng, G., Kadowaki, T., 2012. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. Environ Microbiol Rep, 5:74-77.

Orantes-Bermejo, F. J., 1999. A scientific note on the prevalence of trypanosomatid parasites of honey bees (Apis mellifera L.) in southern Spain. Apidologie, 30:77-78.

Plischuk, S., Meeus, I., Smagghe, G., Lange, C, E., 2011. Apicystis bombi (Apicomplexa: Neogregarinorida) parasitizing Apis mellifera and Bombus terrestris (Hympenoptera: Apidae) in Argentina. Environ Microbiol Rep, 3:565–568.

Popp, M. and Latorff, H. M. G., 2011. A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of Crithidia bombi (Trypanosomatidae), a parasite of bumblebees. J Eukaryot Microbiol, 58:7-10.

Porter, A,. 1945. Some animal parasites of bees observed in three continents. Brit Beekeepers Assoc In Lotmar R. 1946.

Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G., de Graaf, D. C., 2013. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS ONE, 8:e72443.

Ruiz-Gonzalez, M. X. and Brown, M. J. F., 2006. Honey bee and bumblebee trypanosomatids: specificity and potential for transmission. Ecol Entomol, 31:616-622.

Runckel, C., Flenniken, M. L., Engel, J. C., Ruby, J. G., Ganem, D., Andino, R., DeRisi, J. L., 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS ONE, 6:e20656.

Runckel, C., DeRisi, J., Flenniken, M. L., 2014. A draft genome of the honey bee trypanosomatid parasite Crithidia mellificae. PLoS ONE, 9:e95057.

Schmid-Hempel, R. and Tognazzo, M., 2010. Molecular divergence defines two distinct lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. J Eukaryot Microbiol, 57:337-345.

Schwarz, R. S. and Evans, J. D., 2013. Single and mixed-species trypanosome 872 and microsporidian infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. Dev Comp Immunol, 40:300–310.

Spotorno, A. E., Cordova, L., Solari, A., 2008. Differentiation of Trypanosoma cruzi I subgroups through characterization of cytochrome b gene sequences. Inf Gen Evol, 8:898-900.

Stover, B. C. and Muller, K. F., 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics, 11:7.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol, 30:2725-2729.

Teixeira, E. W., Message, D., Chen, Y., Pettis, J., Evans, J. D., 2008. First metagenomic analysis of microorganisms in honey bees from Brazil. B Indústr Anim, 65:355-361.

Teixeira, M. M. G., Borghesan, T. C., Ferreira, R. C., Santos, M. A., Takata, C. S. A., Campaner, M., Nunes, V. L. B., Milder, R. V., de Souza, W., Camargo, E. P., 2011. Phylogenetic validation of the genera Angomonas and Strigomonas of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. Protist, 162:503-524.

vanEngelsdorp, D., Evans, J. D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B. K., Frazier, M., Frazier, J., Cox-Foster, D., Chen. Y., Underwood, R., Tarpy, D. R., Pettis, J. S., 2009. Colony collapse disorder: a descriptive study. PLoS ONE, 4:e6481.

Vasquez, A., Forsgren, E., Fries, I., Paxton, R. J., Flaberg, E., Szekely, L., Olofsson, T. C., 2012. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. PLoS ONE, 7(3):e33188.

Vickerman, K. 1976. The diversity of the kinetoplastid flagellates. In: Lumsden, W. H. R., Evans, D. A. (ed.), Biology of the Kinetoplastida. Academic Press, London. p. 1-34.

Vickerman, K. 1994. The evolutionary expansion fo the trypanosomatid flagellates. Int J Parasitol, 24:1317-1331.

Votypka, J., Klepetkova, H., Jirku, M., Kment, P., Lukeš, J. ,2012. Phylogenetic relationships of trypanosomatids parasitising true bugs (Insecta: Heteroptera) in sub-Saharan Africa. Int J Parasitol, 42:489-500.

Votypka, J., Sukova, E., Kraeva, N., Ishemgulova, A., Duži, I., Lukeš, J., Yurchenko, V., 2013. Diversity of trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus Blechomonas gen. n. Protist, 164:763-781.

Votypka, J., Kostygov, A. Y., Kraeva, N., Grybchuk-Ieremenko, A., Tesařova, M., Grybchuk, D., Lukeš, J., Yurchenko, V., 2014 Kentomonas gen. n., a new genus of endosymbiont containing Trypanosomatids of Strigomonadinae subfam. n. Protist, ahead of print doi.org/10.1016.

Wallace, F. G., 1966. The trypanosomatid parasites of insects and arachnids. Exp. Parasitol., 18:124-193.

Wallace, F. G., Camargo, E. P., McGhee, R. B., Roitman, I., 1983. Guidelines for the description of new species of lower trypanosomatids. J Protozool, 30:308-313.

Wheeler, R. J., Gluenz, E., Gull, K., 2011. The cell cycle of Leishmania: morphogenetic events and their implications for parasite biology. Molec Microbiol, 79:647-662.

Wheeler, R. J., Gluenz, E., Gull, K., 2013. The limits on trypanosomatid morphological diversity. PLoS ONE, 8:e79581.

Yang, B., Peng, G., Li, T., Kadowaki, T., 2013. Molecular and phylogenetic characterization of honey bee viruses, Nosema microsporidia, protozoan parasites, and parasitic mites in China. Ecol Evol, 3:298-311.

Yurchenko, V., Lukeš, J., Xu, X., Maslov, D. A., 2006a. An integrated morphological and molecular approach to a new species description in the Trypanosomatidae: the case of Leptomonas podlipaevi n. sp., a parasite of Boisea rubrolineata (Hemiptera: Rhopalidae). J Eukaryot Microbiol, 53:103-111.

Yurchenko, V., Lukeš, J., Jirku, M., Zeledón, R., Maslov, D. A., 2006b. Leptomonas costaricensis sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus Leishmania. Parasitology, 133:537-546.

Yurchenko, V. Y., Lukeš, J., Tesařová, M., Jirku, M., Maslov, D. A., 2008. Morphological discordance of the new trypanosomatid species phylogenetically associated with the genus Crithidia. Protist, 159:99-114.

Yurchenko, V. Y., Lukeš, J., Jirku, M., Maslov, D. A., 2009. Selective recovery of the cultivation-prone components from mixed trypanosomatid infections: a case of several novel species isolated from neotropical Heteroptera. Int J Syst Evol Microbiol, 59:893-909.

Yurchenko, V. Y., Votýpka, J., Tesařová, M., Klepetková, H., Kraeva, N., Jirku, M., Lukeš, J., 2014. Ultrastructure and molecular phylogeny of four new species of monoxenous trypanosomatids from flies (Diptera: Brachycera) with redefinition of the genus Wallaceina. Folia Parasitologica, 61:97-112

CHAPTER 8

DIFFERENTIAL DIAGNOSIS OF THE HONEY BEE TRYPANOSOMATIDS *CRITHIDIA MELLIFICAE* AND *LOTMARIA PASSIM*

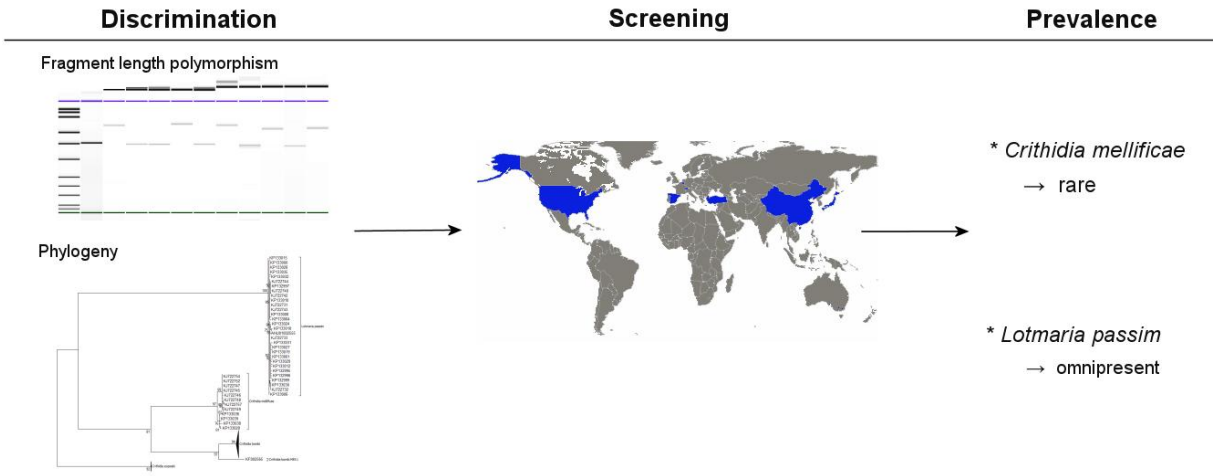
The work presented in Chapter 8 was adapted from the following manuscript:

J. Ravoet, R. S. Schwarz, T. Descamp, O. Yanez, O. Tozkar, R. Martin-Hernandez, L. De Smet, J. DeRisi, M. Higes, T. Wenseleers, R. Schmid-Hempel, P. Neumann, T. Kadowaki, J.D. Evans, D.C. de Graaf, Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. Unpublished work, submitted to Journal of Invertebrate Pathology.

Contributions

D.C. de Graaf and J. Ravoet designed the study. J. Ravoet performed the experimental work, assisted by T. Descamp for the cell cultivation. J.D. Evans, M. Higes, T. Kadowaki, R. Martin-Hernandez, P. Neumann, R. Schmid-Hempel, R. Schwarz, O. Tozkar and O. Yanez provided DNA or cDNA samples. The manuscript was drafted by J. Ravoet, assisted by L. De Smet. The language was edited by J.D. Evans, R. Schwarz and T. Wenseleers, supported by the co-authors through the writing phase.

1. Graphical abstract



2. Abstract

Trypanosomatids infecting honey bees have been poorly studied with molecular methods until recently. After the description of *Crithidia mellificae* Langridge and McGhee, 1967 it took about forty years until molecular data for honey bee trypanosomatids became available and were used to identify and describe a new trypanosomatid species from honey bees, *Lotmaria passim* Evans and Schwarz, 2014. However, an easy method to distinguish them without sequencing is not yet available. Research on the related bumble bee parasites *Crithidia bombi* and *Crithidia expoeki* revealed a fragment length polymorphism in the internal transcribed spacer 1 (ITS1), which enabled species discrimination. In search of fragment length polymorphisms for differential diagnostics in honey bee trypanosomatids, we studied honey bee trypanosomatid cell cultures of *C. mellificae* and *L. passim*. This research resulted in the identification of fragment length polymorphisms in ITS1 and ITS1-2 markers, which enabled us to develop a diagnostic method to differentiate both honey bee trypanosomatid species without the need for sequencing. Further investigation confirmed that *L. passim* is the dominant species in Belgium, Japan and Switzerland. We found *C. mellificae* only rarely in Belgian honey bee samples, but not in honey bee samples from other countries. *C. mellificae* was also detected in mason bees (*Osmia bicornis* and *Osmia cornuta*) besides in honey bees. Further, the characterization and comparison of additional markers from *L. passim* strain SF (published as *C. mellificae* strain SF) and a Belgian honey bee sample revealed very low divergence in the 18S rRNA,

ITS1-2, 28S rRNA and cytochrome *b* sequences. Nevertheless, a variable stretch was observed in the gp63 virulence factor.

3. Introduction

Trypanosomatids can infect a diverse range of organisms like insects, plants and vertebrates (Simpson et al., 2006). Human parasites with an insect-mediated transmission (dixenous trypanosomatids), such as some *Trypanosoma* spp. and *Leishmania* spp., received a lot of attention due to their medical importance. In contrast, trypanosomatids solely infecting insects (monoxenous trypanosomatids) are comparatively neglected.

The early descriptions of these monoxenous parasites were based on their morphology and host (Wallace, 1966). It was believed that a trypanosomatid could infect only one host species, the so called 'one host – one parasite' paradigm. The rise of molecular methods like PCR led to the awareness that the previously used criteria of morphology and host species had only limited significance in taxonomy, thus invalidating this paradigm (Maslov et al., 2013). Numerous molecular markers, like the 18S ribosomal RNA (rRNA), the spliced leader RNA and the glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), are commonly used in current genotyping studies of insect trypanosomatids (Cepero et al., 2014; Hamilton et al., 2004; Maslov et al., 1996; Schwarz et al., 2015; Westenberger et al., 2004).

Several insect groups like true bugs (Heteroptera), flies (Diptera) and fleas (Siphonaptera) were recently studied for their trypanosomatid diversity (Maslov et al., 2007; Tyc et al., 2013; Votypka et al., 2013; Westenberger et al., 2004), but many other insect groups remain inadequately studied. For instance, most currently known hymenopteran trypanosomatids were described decades ago, based on the outdated morphology and host species criteria. For example, *Herpetomonas swainei* (Smirnoff and Lipa, 1970) and *Crithidia cimbexi* (Lipa and Smirnoff, 1971) were reported from sawflies (Hymenoptera, suborder Symphyta) yet they remained neglected after the initial research and no molecular markers are currently available. In contrast, trypanosomatids

from social bees (bumble bees and honey bees) were poorly studied for a long time but interest in them revived since they were shown to have negative effects on these economically important and interesting pollinators (Brown et al., 2000; Brown et al., 2003; Gegear et al., 2005; Ravoet et al., 2013; Schwarz and Evans, 2013).

Nowadays, *Crithidia bombi* (Lipa and Triggiani, 1988) is a well studied parasite from bumble bee species (*Bombus* spp.) that occurs worldwide. Microsatellite data showed that many clones are circulating (Schmid-Hempel and Reber, 2004) and by investigating several molecular markers it became clear that a second species can also infect bumble bees, namely *Crithidia expoeki* (Schmid-Hempel and Tognazzo, 2010). It was also suggested that *C. bombi* can be vectored by honey bees (Ruiz-Gonzalez and Brown, 2006).

Contrary to bumble bees, trypanosomatids infecting honey bees (*Apis mellifera*) were poorly studied until some years ago despite the fact that *Crithidia mellificae* was already described in 1967 (Langridge and Mcghee, 1967). A trypanosomatid cell culture from American honey bees was later on deposited as *C. mellificae* ATCC 30254 in 1974, followed by *C. mellificae* ATCC 30862 from *Vespula squamosa* in 1978. When sequence data of the 18S rRNA, gGAPDH and cytochrome *b* markers became recently available for the bumble bee trypanosomatids, sequences from a honey bee trypanosomatid were presumed to be derived from *C. mellificae* (Schmid-Hempel et al., 2010). At the same period, trypanosomatids were readily detected in the USA from multiple studies (Cox-Foster et al., 2007; Runckel et al., 2011; vanEngelsdorp et al., 2009). The honey bee trypanosomatid cell culture strain SF (ATCC PRA 403) (Runckel et al., 2011) and subsequent draft genome assembly (Runckel et al., 2014) were arbitrarily assigned as *C. mellificae* yet current honey bee trypanosomatid sequences from colonies in the U.S. were noted to be divergent from *C. mellificae* ATCC 30254 (Cornman et al., 2012). The high genetic distance between this strain and recently obtained sequences published as *C. mellificae* was later on demonstrated by analysing the gGAPDH gene and recognized that the latter group belonged to another taxon which should be renamed accordingly (Cepero et al., 2014). Concurrently, a third honey bee trypanosomatid cell culture isolated in 2012

from the USA, strain BRL (ATCC 000359), was in use along with strain SF to formally contrast *C. mellificae* with the newly recognized taxon now denominated as *Lotmaria passim* (Schwarz et al. 2015), represented by hapantotype strain BRL and parahapantotype strain SF.

Nowadays, *L. passim* appears to be the predominant parasite in honey bees worldwide (Schwarz et al., 2015), in contrast to the 'true' (=taxonomically valid) *C. mellificae*. Since the prevalence of *C. mellificae* might be underestimated, we searched for a convenient diagnosis method. Several loci, including cytochrome *b* and gGAPDH, showed enough interspecific identity to discriminate between both species, but sequencing remains necessary. Techniques like fragment length polymorphism and high resolution melting (HRM) were previously used to identify trypanosomatids in other host species (Higuera et al., 2013; Schmid-Hempel et al., 2010; Zackay et al., 2013). We investigated both methods using infected honey bee samples from different geographic locations and trypanosomatid cell types from honey bees and bumble bees. This can provide an easy diagnostic method to differentiate both honey bee trypanosomatids, without the need for sequencing.

4. Material and methods

4.1 Sample selection

For this study we used honey bee (*A. mellifera*) samples from Japan (10 cDNA samples), Spain (bee samples from 10 colonies), Switzerland (10 DNA samples) and Turkey (30 cDNA samples), infected with trypanosomatids as confirmed by previous 18S rRNA detection. In addition, we used 8 Belgian honey bee samples (cDNA) from which the partial 18S rRNA, GAPDH and cytochrome *b* genes of trypanosomatids were previously determined (Schwarz et al., 2015). Single bee specimens from one of these Belgian samples were further investigated. DNA samples of *C. bombi* types A1 (BJ08.079) and A2 (AK08.053), *C. expoeki* types B1 (BJ08.078) and B2 (AK08.231), *C. mellificae* (ATCC 30862, ATCC 30254) and *L. passim* strain SF (ATCC PRA-403; deposited as *C. mellificae* strain SF (Runckel et

al., 2014; Runckel et al., 2011)) and *L. passim* strain BRL (ATCC 00359 (Schwarz et al., 2015)) were also included.

4.2 Cell culture

Crithidia mellificae ATCC 30254 was first cultivated in ATCC medium 355 as recommended. Further sub-cultivation was performed in Brain Heart Infusion (BHI) medium, supplemented with 3.5 µg/ml hemin and 2% (v/v) anti-contamination cocktail (Maser et al., 2002). The other cell lines were cultivated as described previously (Salathe et al., 2012; Schwarz et al., 2015). Cultivation of trypanosomatids from *Osmia bicornis* and *Osmia cornuta* were attempted as described earlier (Popp and Lattorff, 2011).

4.3 DNA/RNA extraction and cDNA synthesis

The nucleic acids from the infected Belgian (Ravoet et al., 2013), Japanese (Morimoto et al., 2013), Swiss (unpublished results) and Turkish honey bee samples (unpublished results) were extracted earlier. For the Spanish samples, ten bees were homogenized in 5 ml PBS by mechanical agitation in a BulletBlender for 5 minutes. After centrifugation at 13,300 g for 5 minutes, extracellular RNA was isolated from 140 µl supernatant using the QiaAmp Viral RNA mini kit (Qiagen). Total RNA was extracted from five individual honey bees from one Belgian sample, which appeared to be infected with the two trypanosomatids. The individual bees were only used to investigate the trypanosomatid diversity. They were directly homogenized in 1 ml QIAzol by mechanical agitation in the presence of glass beads (2 mm). RNA was further extracted using the RNeasy Lipid Tissue mini kit (Qiagen).For the cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), 5 µl extracellular RNA or 2 µg total RNA were reverse transcribed using random hexamer primers.

DNA was extracted from 4 ml liquid culture (supplemented BHI medium) of *Crithidia mellificae* ATCC 30254 and trypanosomatids from *Osmia bicornis* and *Osmia cornuta* using the protocol for cell cultures of the DNeasy Blood & Tissue Kit (Qiagen). DNA from *C. bombi*, *C. expoeki*, *C. mellificae* ATCC 30862 and *L. passim* strain SF (ATCC PRA-403; deposited as *C. mellificae* strain SF

(Runckel et al., 2014; Runckel et al., 2011)) and *L. passim* strain BRL (ATCC 00359 (Schwarz et al., 2015)) was extracted as previously described (Salathe et al., 2012; Schwarz et al., 2015).

4.4 PCR amplification

All PCR reaction mixtures contained: 2 µM of each primer (Table A1); 1.0 mM $MgSO_4$; 1.25 U Hotstar Taq HiFidelity DNA polymerase (Qiagen) and 1 µl cDNA or DNA. Published PCR assays were performed (see Table A1 for references), but for the newly designed primers we used the following cycling conditions: 95°C for 5 min; 94°C for 30 s, 55°C or 50°C (the latter only for the cytochrome *b* amplification) for 30 s, 72°C for 1 min, 35 cycles; final elongation 72°C for 10 min, hold at 4°C. Several new primer pairs for the amplification of 18S rRNA, cytochrome *b*, gp63 (glycoprotein of 63 kDa) and ITS1-2 (partial 18S rRNA – complete ITS1 – complete 5.8S rRNA – complete ITS 2 – partial 28S rRNA) were designed based on published sequences of *C. bombi*, *Crithidia fasciculata*, *C. mellificae* and/or *L. passim* (Table A1).

All PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualised under UV light. Amplicons were purified or gel-extracted, and subsequently cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen). Four plasmids of each amplicon were purified and sequenced using M13 primers on an ABI3730XL platform (Applied Biosystems). Different internal primer pairs (Table A1) were used to sequence the 18S rRNA amplicons.

Cloned plasmids were processed for 30 min with FastDigest EcoRI (Thermo Scientific) and analysed using 1.5% agarose gel electrophoresis or capillary electrophoresis on a Bioanalyzer (Agilent Technologies). For the latter, the fragments were separated using a Agilent DNA 1000 kit (Agilent Technologies). The different wells were aligned at 15 bp and 15,000 bp.

4.5 High resolution melting analysis

Using the Type-it HRM PCR kit (Qiagen) with 10 µM primer pairs (HRM-cytb-F – HRM-cytb-R or IR1 - 5.8R; Table A1) and 1 µl cDNA or DNA, we performed the high resolution melting (HRM) analyses. PCR products were analyzed with a LightScanner machine (BioFire Diagnostics).

4.6 Sequence analysis

All obtained sequences were assembled, BLAST-searched and aligned with MUSCLE in Geneious R7 to confirm their identity. The 18S rRNA sequences of the *C. bombi*, *C. expoeki*, *C. mellificae* and *L. passim* strains were assembled *de novo*. Afterwards, they were mapped onto the genome sequence of *L. passim* strain SF; ATCC PRA-403 (published as *C. mellificae* strain SF (Runckel et al., 2014; Runckel et al., 2011)), together with 18S rRNA sequences assigned to *C. mellificae* (Genbank: AB738082, AB745487, AB745488, GU321196, KF607064, KJ704242-KJ704251). The draft genome of *L. passim* strain SF; ATCC PRA-403 was BLAST-searched to identify homologous gp63, 18S rRNA, 28S rRNA and ITS1-2 sequences. In addition, ITS1 sequences from *C. bombi* (Genbank: GU321121-GU321168, KF002565), *C. expoeki* (Genbank: GU321169-GU321186) were downloaded from Genbank. Additional sequences were made available for this study by R. Schwarz: *C. mellificae* ATCC 30254 (Genbank: KJ722745- KJ722753), *C. mellificae* ATCC 30862 (Genbank: KJ722754-KJ722757), *L. passim* strain BRL; ATCC 00359 (Genbank: KJ722738- KJ722744) (Schwarz et al., 2015) and *L. passim* strain SF; ATCC PRA-403 (Genbank: KJ722728-KJ722737). Gp63 proteins from *Crithidia* spp. were retrieved by a BLASTP-search and investigated for conserved domains (Marchler-Bauer et al., 2011).

Phylogenetic analysis of the ITS1 locus was performed to assign the trypanosomatid sequences. Selection of the best fitted maximum likelihood models was based on the Bayesian information criterion (BIC), as implemented in MEGA6. The analysis was performed using the Kimura 2-parameter model with gamma distributed rate variation among sites (K80+G) using PhyML 3.0 (Guindon et al., 2010). The reliability was assessed by approximate likelihood ratio test non-parametric branch support based on a Shimodaira-Hasegawa-like (aLRT SH-like) procedure (Anisimova and Gascuel, 2006).

All unique sequences obtained in this study were deposited in Genbank under accession numbers KM980179 (*L. passim* gp63 fragment from honey bees), KM980180-81 (*L. passim* cytochrome *b* sequences from honey bees), KM980182-KM980188 (*C. bombi*, *C. expoeki*, *C. mellificae* and *L. passim* 18S rRNA sequences from bumble bees and honey bees), KM980189-90 (*L. passim* 28S

rRNA fragments from honey bees), KM980191-98 (*C. bombi*, *C. expoeki*, *C. mellificae* and *L. passim* ITS1-2 sequences from bumble bees and honey bees), KP133036-KP133039, KP133020,KP133022, KP133023 (*C. mellificae* ITS1 sequences from honey bees), KP115801-KP115804 (*C. mellificae* ITS1 sequences from mason bees) and KP132992-KP133019, KP133021, KP133023-KP133035 (*L. passim* ITS1 sequences from honey bees).

5. Results

Using the hapantotype (ATCC 30254 and ATCC 00359) and parahapantotype (ATCC 30862 and PRA 403) strains of *C. mellificae* and *L. passim*, respectively (hereafter 'type strains'), we were able to distinguish them in initial HRM analyses. Nevertheless, a broader screening with infected honey bee samples from a widespread origin failed to differentiate them (data not shown). Therefore we investigated several loci in search for a PCR based fragment length polymorphism.

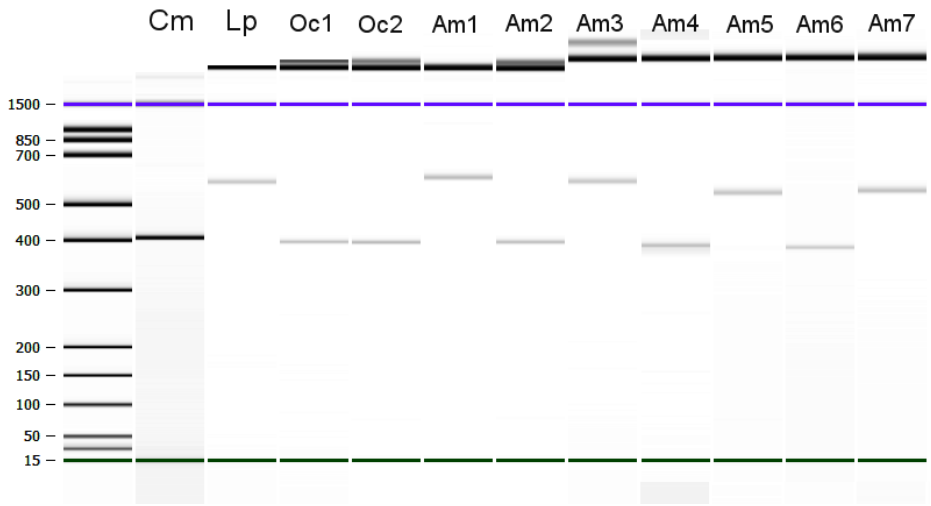


***Figure 1**: Example of ITS1 fragment length polymorphism visualized by capillary electrophoresis. L: 15-15.000 bp molecular weight ladder (Agilent Technologies). Clones derived from* C. mellificae *ATCC 30254 (Cm),* L. passim *strain BRL (Lp),* O. cornuta *trypanosomatid culture (Oc1 and Oc2) and a pooled honey bee sample (Am1-Am7) are included in this analysis. The fragments of* C. mellificae *are situated around 410 bp and those of* L. passim *around 550 bp. The fragments above 1.500 bp are derived from the pCR4 cloning vector.*

First the 18S rRNA (Genbank: KM980182-7), ITS1 and ITS1-2 (Genbank: KM980195-98) were amplified from the type strains. The ITS1 locus appeared to be very useful for species discrimination since we found a length difference of around 70 nt between the two clades of honey bee trypanosomatids, clearly envisioned by 1.5% agarose gel or capillary electrophoresis (Figure 1). Although the ITS1-2 locus differed by around 200 nucleotides (~930 bp for *C. mellificae* and ~1,140 bp for *L. passim*), it could not be assigned unambiguously using 1.5% agarose gel electrophoresis and this marker was not readily amplified in infected honey bee samples. Also, the ITS1-2 marker did not allow easy differentiation between the *C. bombi* and *C. expoeki* type strains due to the small fragment length polymorphism of 22 nt. The 18S rRNA fragments showed no usable fragment length polymorphisms (Figure A1), but possess several nucleotide differences between the bee trypanosomatids. By mapping published *C. mellificae* sequences from China, Japan, Spain, Switzerland and Turkey (Genbank: AB738082, AB745487, AB745488, GU321196, KF607064, KJ704242-KJ704251) onto the 18S rRNA of the type strains, we could assign them as previously discussed (Cepero et al., 2014; Schwarz et al., 2015).

While the ITS1 locus was easily acquired from the type strains, we could not successfully amplify it from all infected honey bee samples. This was also observed using the cytochrome *b* and gGAPDH markers (Schwarz et al., 2015). Nonetheless, we obtained ITS1 sequences from nine Belgium honey bee samples, seven Japanese samples, three Swiss samples but none of the Spanish and Turkish samples.

To assess the specificity of the observed ITS1 polymorphism, we performed a phylogenetic analysis which included our unique sequences and published sequences from *C. bombi*, *C. expoeki*, *C. mellificae* and *L. passim*. All ITS1 amplicons from Japan (Genbank: KP133004-KP133018), Switzerland (Genbank: KP132992-KP133003) and almost all Belgium honey bee samples (Genbank: KP133019, KP133024-KP133035) could be assigned to the *L. passim* clade (strain BRL/SF) by phylogenetic analysis (Figure 2). This was confirmed for a Swiss sample using the cytochrome *b* marker (Genbank:

KM98018), as already employed for the Belgian and Japanese samples (Morimoto et al., 2013; Schwarz et al., 2015).

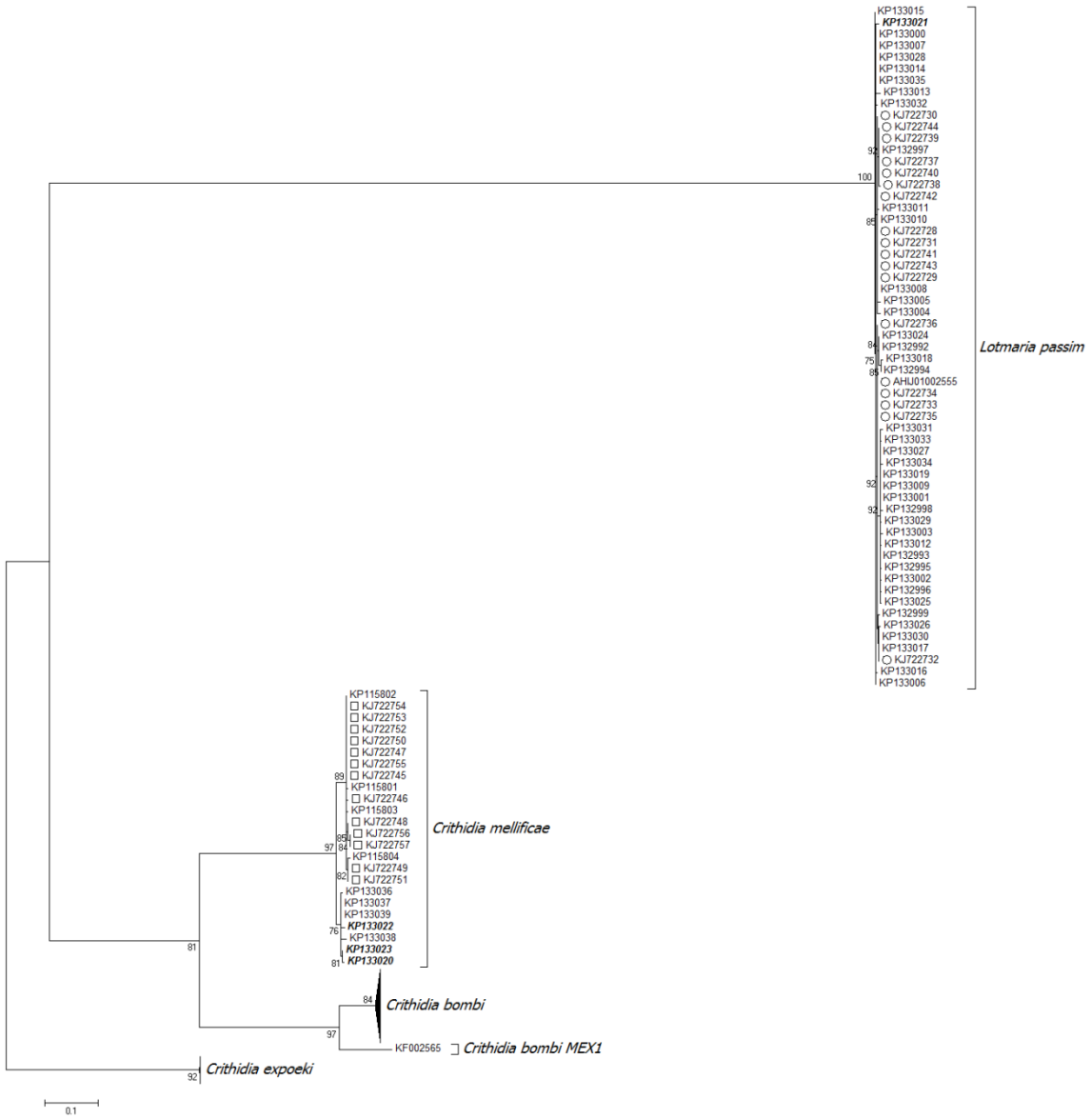


Figure 2*: Phylogenetic analysis of trypanosomatid ITS1 clones. Each isolate is indicated by its accession number, but those of* C. bombi *and* C. expoeki *are compressed. The clades are visualized by named brackets. Branch support is indicated by aLRT statistics, although only values higher than 70% are shown. Accessions of the* C. mellificae *types are indicated by squares and those of the* L. passim *types by circles. The sequences of the co-infected sample from Belgium are shown in bold italic.*

Surprisingly, sequences of *C. mellificae* were detected in two Belgian honey bee samples. One sample was solely infected with this species (Genbank: KP133036-KP133039), but the other sample

(Genbank: KP133020, KP133022, KP133023) was co-infected with *L. passim* (Genbank: KP133021) (Figure 2). Investigation of single honey bee specimens from this sample with mixed infection revealed that *L. passim* was detected in all bee specimens. Only one of five bees was co-infected with *C. mellificae*. In addition to honey bees, we could detect *C. mellificae* in trypanosomatid cultures derived from *O. cornuta* and *O. bicornis* (Genbank: KP115801-KP115804). The 18S rRNA and the ITS1 marker from both infected solitary bees were identical to the type strain *C. mellificae* ATCC-30254.

A Belgian honey bee sample infected with *L. passim* was used to characterize multiple loci, which were compared to *L. passim* strain SF. Sequencing of the 18S rRNA (Genbank: KM980188) and 28S rRNA (Genbank: KM980189-90) amplicons indicated only few nucleotide differences with the *L. passim* strain SF genome (Genbank: AHIJ01002555), resulting in ~99% identity. The complete ITS1-2 (Genbank: KM980191-94) region was also very similar (98% nucleotide identity). The cytochrome *b* amplicon (0.8 kb) of the Belgian isolate (Genbank: KM980180) was even completely identical (Genbank: AHIJ01002387). On the other hand, the virulence factor gp63 (Genbank: KM980179) was largely identical (93.6% nucleotide and 91.1% amino acid similarity) to strain SF (Genbank: AHIJ01002023) but a small variable region of 10 amino acids was present (Figure 3). The predicted gp63 protein of strain SF contains the conserved Peptidase_M8 (leishmanolysin) superfamily domain. Two additional gp63 proteins were retrieved from *Crithidia fasciculata* (Genbank: AAA30319, Q06031) which had around 70% amino acid similarity with those of *L. passim*.

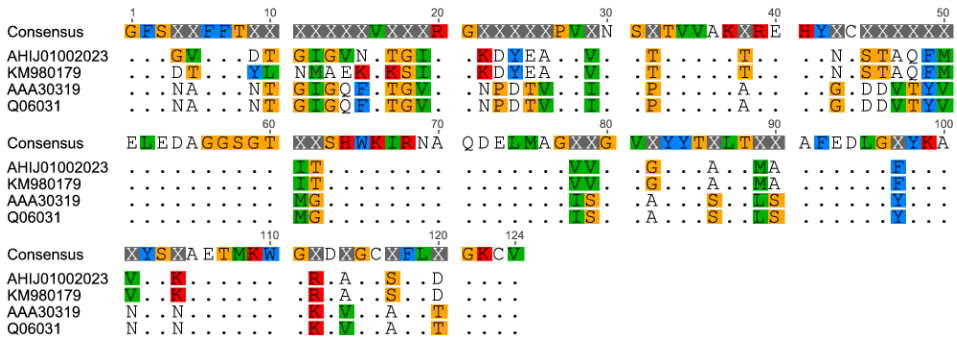


***Figure 3**: Alignment of gp63 amino acid sequences from the trypanosomatids* Crithidia fasciculata *(Genbank: AAA30319, Q06031) and* L. passim *strain SF (Genbank: AHIJ01002023 contig 298) and the obtained sequence from an infected Belgian honey bee sample (Genbank: KM980179).*

6. Discussion

In this study, we wanted to design a convenient method to diagnose infections of the trypanosomatid parasites *C. mellificae* and *L. passim* in honey bee samples, without the need to sequence amplicons. HRM proved to be a simple technique to classify genotypes of *Leishmania donovani* and *Trypanosoma cruzi* (Higuera et al., 2013; Zackay et al., 2013) and was also used to assess the strain diversity of Deformed wing virus (Martin et al., 2012). While HRM analysis of the type strains of *C. mellificae*, *C. bombi*, *C. expoeki* and *L. passim* appeared successful, we were not able to genotype infected honey bee samples. This can be caused by the different extraction methods and elution buffers.

Another method to discriminate between two trypanosomatids is the amplification of a locus with a fragment length polymorphism. We succeeded in amplifying the ITS1 marker in the trypanosomatid type strains, which revealed a fragment length polymorphism between *C. mellificae* and *L. passim*, as already demonstrated for *C. bombi* and *C. expoeki* (Schmid-Hempel et al., 2010). The ITS1-2 marker was also amplified, but this was less usable than the ITS1 marker.

The ITS1 marker could be amplified in a subset of the infected honey bee samples from a widespread origin. We confirmed the omnipresence of *L. passim* (Schwarz et al., 2015) and the rare detection of *C. mellificae*, since the latter is only known from Australia (Langridge et al., 1967), Belgium (Schwarz et al., 2015) and the USA (origin of *C. mellificae* ATCC-30254 and ATCC-30862). Its presence in Australia is not certain since no molecular data are available. Surprisingly, we found a coincidence of both parasites in one Belgian honey bee sample. Further research demonstrated that *C. mellificae* was only very scarcely detected in this colony. The presence of *C. mellificae* in this sample was not demonstrated before based on the 18S rRNA, cytochrome *b* and gGAPDH markers (Schwarz et al., 2015). Furthermore, we provide evidence for *O. cornuta* as another hymenopteran host of *C. mellificae*, in addition to *A. mellifera*, *O. bicornis* and *Vespula squamosa* (Schwarz et al., 2015). Since another Belgian honey bee sample was infected exclusively with *C. mellificae*, we can presume that this trypanosomatid still infects hymenopterans nowadays. It is possible that the

presence of *C. mellificae* is underestimated in honey bees due to its apparent low prevalence. Moreover, some primer sets do not detect both trypanosomatids (Cepero et al., 2014) but broad range primers for both honey bee trypanosomatids are now available for several loci like the 18S rRNA, cytochrome *b*, gGAPDH and ITS1 (this study and (Cepero et al., 2014; Ravoet et al., 2014; Schwarz et al., 2015)). Phylogenetic analysis of the ITS1 sequences validated the fragment length polymorphism. Remarkably, *C. mellificae* isolates from Belgian honey bees formed a separate subclade, while those from the Belgian mason bees clustered with the type strains.

We could corroborated assignment of published 18S rRNA sequences from China, Japan, Switzerland, Spain and Turkey to *L. passim* (Schwarz et al., 2015). This parasite was also detected in all investigated samples. However, amplicons obtained with the primers SE-F and SE-R appeared to be too conserved while sequences amplified with the primer sets 609F-706R or 18SF-18SR contained the most indels and point mutations. This implicates that the latter primer sets can be used for species assignment of *C. mellificae* and *L. passim* in addition to primers targeting the cytochrome *b* and gGAPDH loci (Cepero et al., 2014; Schwarz et al., 2015), although sequencing remains necessary in contrast to the ITS1 marker.

In search of intraspecific variation, we compared several loci of *L. passim* strain SF and a Belgian honey bee sample infected with *L. passim*. The almost complete 18S rRNA, 28S rRNA and ITS1-2 regions were almost identical, but they do not encode for proteins. The gp63 virulence factor on the other hand showed a variable amino acid stretch. This protein belongs to a multi-gene family (Mauricio et al., 2007) and has been widely detected in trypanosomatids (d'Avila-Levy et al., 2014; Etges, 1992). Its role in insect parasites is understudied, but they are probably involved in cell adhesion and nutrition (d'Avila-Levy et al., 2014).

We can conclude that an easy differentiation method between both honey bee trypanosomatids was found using the ITS1 marker. The fragment length polymorphism in this region can also reveal the presence of the rarely detected *C. mellificae*, which can be missed using other

common markers like the 18s rRNA, cytochrome *b* and gGAPDH. Sequence variation was found in gp63 virulence factor, which might be a good indicator of intraspecific variation.

7. Acknowledgements

This study was supported by the Research Foundation Flanders (FWO-Vlaanderen, research grant G.0628.11). We thank Ilse Coene and Dr. Kim de Leeneer (Center for Medical Genetics, Ghent University Hospital) for the help with the HRM analyses.

8. References

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55**,** 539-552.

Brown, M.J.F., Loosli, R., Schmid-Hempel, P., 2000. Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91**,** 421-427.

Brown, M.J.F., Schmid-Hempel, R., Schmid-Hempel, P., 2003. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. J Anim Ecol 72**,** 994-1002.

Cepero, A., Ravoet, J., Gomez-Moracho, T., Bernal, J.L., Del Nozal, M.J. and others, 2014. Holistic screening of collapsing honey bee colonies in Spain: a case study. BMC Res Notes 7**,** 649.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D. and others, 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7**,** e43562.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

d'Avila-Levy, C.M., Altoe, E.C., Uehara, L.A., Branquinha, M.H., Santos, A.L., 2014. Gp63 function in the interaction of trypanosomatids with the invertebrate host: facts and prospects. Subcell Biochem 74**,** 253-270.

Etges, R., 1992. Identification of a surface metalloproteinase on 13 species of Leishmania isolated from humans, Crithidia fasciculata, and Herpetomonas samuelpessoai. Acta Trop 50**,** 205-217.

Gegear, R.J., Otterstatter, M.C., Thomson, J.D., 2005. Does parasitic infection impair the ability of bumblebees to learn flower-handling techniques? Anim Behav 70**,** 209-215.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59**,** 307-321.

Hamilton, P.B., Stevens, J.R., Gaunt, M.W., Gidley, J., Gibson, W.C., 2004. Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int J Parasitol 34**,** 1393-1404.

Higuera, S.L., Guhl, F., Ramirez, J.D., 2013. Identification of Trypanosoma cruzi discrete typing units (DTUs) through the implementation of a high-resolution melting (HRM) genotyping assay. Parasit Vectors 6**,** 112.

Langridge, D.F., Mcghee, R.B., 1967. Crithidia mellificae n. sp.: An acidophilic trypanosomatid of honey bee Apis mellifera. J Protozool 14**,** 485-&.

Lipa, J.J., Smirnoff, W.A., 1971. Crithidia cimbexi sp. n., a new flagellate parasite of Cimbex americana Leach (Hymenoptera: Tenthredinidae). B Acad Pol Sci Biol 19**,** 269-274.

Lipa, J.J., Triggiani, O., 1988. Crithidia bombi sp. n.: A flagellated parasite of a bumblebee Bombus terrestris L. (Hymenoptera, Apidae). Acta Protozool 27**,** 287-290.

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitsaz, F., Derbyshire, M.K. and others, 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res 39**,** D225-D229.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E. and others, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336**,** 1304-1306.

Maser, P., Grether-Buhler, Y., Kaminsky, R., Brun, R., 2002. An anti-contamination cocktail for the in vitro isolation and cultivation of parasitic protozoa. Parasitol Res 88**,** 172-174.

Maslov, D.A., Lukes, J., Jirku, M., Simpson, L., 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol Biochem Parasitol 75**,** 197-205.

Maslov, D.A., Votypka, J., Yurchenko, V., Lukes, J., 2013. Diversity and phylogeny of insect trypanosomatids: all that is hidden shall be revealed. Trends Parasitol 29**,** 43-52.

Maslov, D.A., Westenberger, S.J., Xu, X., Campbell, D.A., Sturm, N.R., 2007. Discovery and barcoding by analysis of spliced leader RNA gene sequences of new isolates of Trypanosomatidae from Heteroptera in Costa Rica and Ecuador. J Eukaryot Microbiol 54**,** 57-65.

Mauricio, I.L., Gaunt, M.W., Stothard, J.R., Miles, M.A., 2007. Glycoprotein 63 (gp63) genes show gene conversion and reveal the evolution of Old World Leishmania. Int J Parasitol 37**,** 565-576.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B. and others, 2013. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. Environ Microbiol Rep 5**,** 74-77.

Popp, M., Lattorff, H.M., 2011. A quantitative in vitro cultivation technique to determine cell number and growth rates in strains of Crithidia bombi (Trypanosomatidae), a parasite of bumblebees. J Eukaryot Microbiol 58**,** 7-10.

Ravoet, J., De, S.L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D.C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J Invertebr Pathol 122**,** 55-58.

Ravoet, J., Maharramov, J., Meeus, I., De, S.L., Wenseleers, T. and others, 2013. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. Plos One 8**,** e72443.

Ruiz-Gonzalez, M.X., Brown, M.J.F., 2006. Honey bee and bumblebee trypanosomatids: specificity and potential for transmission. Ecol Entomol 31**,** 616-622.

Runckel, C., DeRisi, J., Flenniken, M.L., 2014. A draft genome of the honey bee trypanosomatid parasite Crithidia mellificae. Plos One 9**,** e95057.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal Analysis of the Honey Bee Microbiome Reveals Four Novel Viruses and Seasonal Prevalence of Known Viruses, Nosema, and Crithidia. Plos One 6**,** e20656.

Salathe, R., Tognazzo, M., Schmid-Hempel, R., Schmid-Hempel, P., 2012. Probing mixed-genotype infections I: extraction and cloning of infections from hosts of the trypanosomatid Crithidia bombi. Plos One 7**,** e49046.

Schmid-Hempel, P., Reber, F.C., 2004. The distribution of genotypes of the trypanosome parasite, Crithidia bombi, in populations of its host, Bombus terrestris. Parasitology 129**,** 147-158.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. J Eukaryot Microbiol 57**,** 337-345.

Schwarz, R.S., Bauchan, G., Murphy, C., Ravoet, J., de Graaf, D.C., Evans, J.D. Characterization of two species of Trypanosomatidae from the honey bee Apis mellifera: Crithidia mellificae Langridge and McGhee, 1967 and Lotmaria passim n. gen., n. sp. Journal of Eukaryotic Microbiology . 2015.
Ref Type: In Press

Schwarz, R.S., Evans, J.D., 2013. Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. Dev Comp Immunol 40**,** 300-310.

Simpson, A.G., Stevens, J.R., Lukes, J., 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol 22**,** 168-174.

Smirnoff, W.A., Lipa, J.J., 1970. Herpetomonas swainei sp. n., a new flagellate parasite of Neodiprion swainei (Hymenoptera: Tenthredinidae). J Invertebr Pathol 16**,** 187-195.

Tyc, J., Votypka, J., Klepetkova, H., Sulakova, H., Jirku, M., Lukes, J., 2013. Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): frequent cosmopolitism and moderate host specificity. Mol Phylogen Evol 69**,** 255-264.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E. and others, 2009. Colony Collapse Disorder: A Descriptive Study. Plos One 4**,** e6481.

Votypka, J., Sukova, E., Kraeva, N., Ishemgulova, A., Duzi, I. and others, 2013. Diversity of trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing fleas (Insecta: Siphonaptera) and description of a new genus Blechomonas gen. n. Protist 164**,** 763-781.

Wallace, F.G., 1966. The trypanosomatid parasites of insects and arachnids. Exp Parasitol 18**,** 124-193.

Westenberger, S.J., Sturm, N.R., Yanega, D., Podlipaev, S.A., Zeledon, R. and others, 2004. Trypanosomatid biodiversity in Costa Rica: genotyping of parasites from Heteroptera using the spliced leader RNA gene. Parasitology 129**,** 537-547.

Zackay, A., Nasereddin, A., Takele, Y., Tadesse, D., Hailu, W. and others, 2013. Polymorphism in the HASPB repeat region of East African Leishmania donovani strains. PLoS Negl Trop Dis 7**,** e2031.

9. Supplementary information

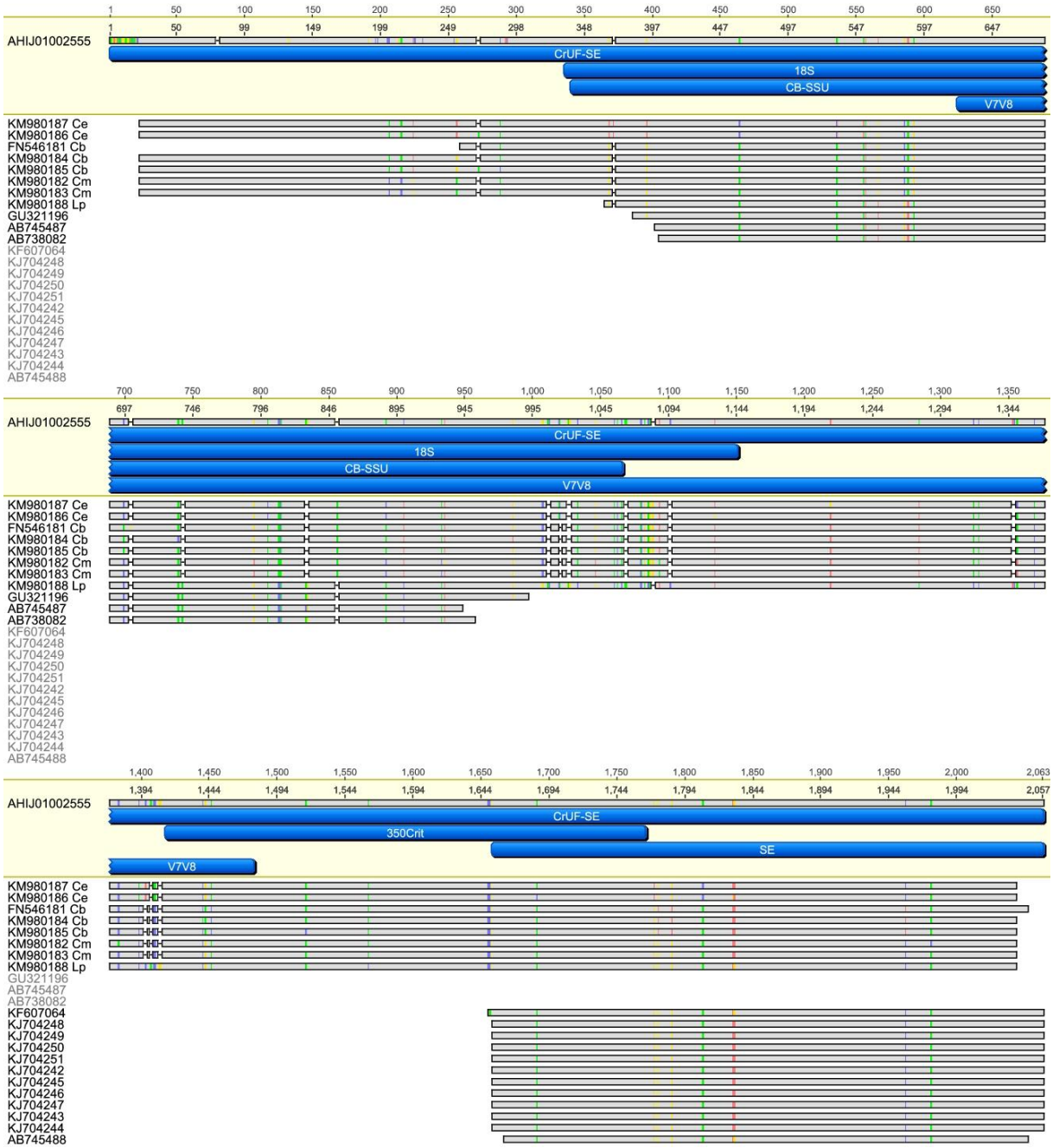


Figure S1*: Mapping of 18S rRNA sequences from* C. bombi*,* C. expoeki*,* C. mellificae *and* L. passim *isolates, indicated by their accession numbers. The genome draft of* L. passim *strain SF was used as reference sequence. The primers used in this study to amplify 18S rRNA fragments are indicated in blue.*

***Table A1**: List of PCR primers used for trypanosomatid fragment detection in this study. Most targets have a small size difference, due to some indels between* C. mellificae *and* L. passim *sequences. The mentioned sizes are referring to those of* L. passim*.*

Target	Primers	Sequence (5'-3')	Size (bp)	Reference
18S rRNA	CB-SSUrRNA-F2	CTTTTGACGAACAACTGCCCTATC	~717	Schmid-Hempel and Tognazzo, 2010
	CB-SSUrRNA-B4	AACCGAACGCACTAAACCCC		
	18S-F*	GGCGTCTTTTGACGAACAAC	~817	Schwarz et al., 2015
	18S-R*	TACGTTCTCCCCCGAACTAC		
	609F	CACCCGCGGTAATTCCAGC	~842	Maia Da Silva et al., 2004
	706R	CTGAGACTGTAACCTCAA		
	350Crith-F	CCCACGGGAATATCCTCAGC	350	This study
	350Crith-R	GGTAAAACCCGCCGATGAGT		
	SE-F	CTTTTGGTCGGTGGAGTGAT	406	Meeus et al., 2010
	SE-R	GGACGTAATCGGCACAGTTT		
	CrU-F1	TTGAGATCTGGTTGATTCTGC	~2040#	Meeus et al., 2010
28S rRNA	S-1842	GGGTCTAGAGTAGGAAGACCGATAGC	~1008	Maslov et al., 1996
	S-1843	GTGGTACCGGTGGATTCGGTTGGTGAG		
ITS1	IR1	GCTGTAGGTGAACCTGCAGCAGCTGGATCATT	variable	Maia Da Silva et al., 2004
	5.8R	GGAAGCCAAGTCATCCATC		
ITS1-2	Cr-ITS-F	CCTGCAGCTGGATCATTTTC	~1100	This study
	Cr-ITS-R	TTTCTTTTCCTCCGCTGAGT		
Cyt b	HRM-cytb-F	GCCATTTAGGTTTTGTAATACGAA	187	This study
	HRM-cytb-R	CGCACATATCCAATAAAGCCT		
	SF-cytb-F	AAAGCGGAGAAAGAAGAAAAG	830	This study
	SF-cytb-R	CAGGCACAGCTTTTAAGAAAC		
	Tryp-cytb-F	TGTGGWGTKTGTTTAGC	490	Ravoet et al., 2014
	Tryp-cytb-R	CRTCWGAACTCATAAAATAATG		
Gp63	Cr-gp63-F	ACGAGATCGCACATTCCCTC	414	This study
	Cr-gp63-R	GGAACTGTGTCACGTTGTCG		

* *These primers were used for screening purposes.*

\# *Size is referring to the primer combination CrU-F1 and SE-R. This was used for the* C. bombi*,* C. expoeki *and* C. mellificae *ATCC-30862 strains*

To span the 18S rRNA amplicons, we used published primer sets and designed another primer pair based on the *Crithidia bombi* partial 18S rRNA gene (Genbank: FN546181). Primers for the ITS 1-2 region (partial 18S, entire ITS 1, 5.8S rRNA, ITS-2, partial 28S rRNA) were designed using published sequences of *C. mellificae* and *L. passim* (Genbank: KJ722728- KJ722744). ITS 1, 28S rRNA and cytochrome b regions were amplified with published primers. An alignment of *L. passim* cytochrome *b* sequences (Genbank: AB744129, AB716358, AB716358) served as a template for the design of

HRM primers. The complete *L. passim* cytochrome *b* gene (Genbank: AHIJ01002387, contig 625) and a partial *Crithidia fasciculata* metalloproteinase gene (Genbank: M94364) served also as template to design primers.

Maia Da Silva, F., Noyes, H., Campaner, M., Junqueira, A.C., Coura, J.R., Anez, N., Shaw, J.J., Stevens, J.R., Teixeira, M.M., 2004. Phylogeny, taxonomy and grouping of Trypanosoma rangeli isolates from man, triatomines and sylvatic mammals from widespread geographical origin based on SSU and ITS ribosomal sequences. Parasitology 129**,** 549-561.

Maslov, D.A., Lukes, J., Jirku, M., Simpson, L., 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol. Biochem. Parasitol. 75**,** 197-205.

Meeus, I., de Graaf, D.C., Jans, K., Smagghe, G., 2010. Multiplex PCR detection of slowly-evolving trypanosomatids and neogregarines in bumblebees using broad-range primers. Journal of applied microbiology 109**,** 107-115.

Ravoet, J., De, S.L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D.C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J Invertebr Pathol 122**,** 55-58.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. J Eukaryot Microbiol 57**,** 337-345.

Schwarz, R.S., Bauchan, G., Murphy, C., Ravoet, J., de Graaf, D.C., Evans, J.D. 2015. Characterization of two species of Trypanosomatidae from the honey bee Apis mellifera: Crithidia mellificae Langridge and McGhee, 1967 and Lotmaria passim n. gen., n. sp. Journal of Eukaryotic Microbiology. In Press.

CHAPTER 9

GENOME SEQUENCE HETEROGENEITY OF LAKE SINAI VIRUS FOUND IN HONEY BEES AND ORF1/RDRP-BASED POLYMORPHISMS IN A SINGLE HOST

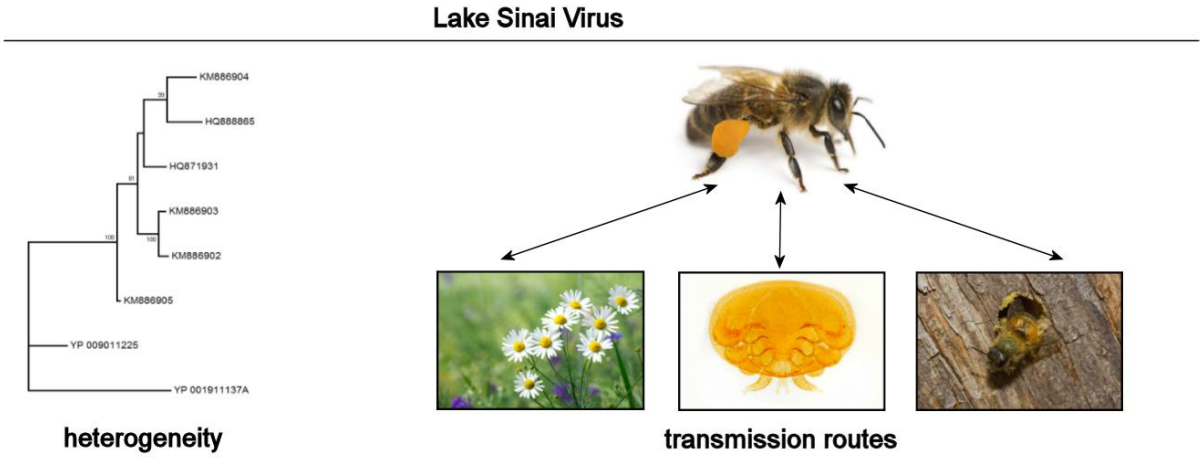
The work presented in Chapter 9 was adapted from the following manuscript:

J. Ravoet, L. De Smet, T. Wenseleers, D.C. de Graaf, Genome sequence heterogeneity of Lake Sinai Virus found in honey bees and Orf1/RdRP-based polymorphisms in a single host. Virus Research, 2015, In Press. doi:10.1016/j.virusres.2015.02.019

Contributions

J. Ravoet designed the study, assisted by D.C. de Graaf, and performed the experimental work. The manuscript was written by D.C. de Graaf and J. Ravoet, assisted by L. De Smet and T. Wenseleers. The final version was accepted by all co-authors.

1. Graphical abstract



2. Abstract

Honey bees (*Apis mellifera*) are susceptible to a wide range of pathogens, including a broad set of viruses. Recently, next-generation sequencing has expanded the list of viruses with, for instance, two strains of Lake Sinai Virus. Soon after its discovery in the USA, LSV was also discovered in other countries and in other hosts. In the present study, we assemble four almost complete LSV genomes, and show that there is remarkable sequence heterogeneity based on the Orf1, RNA-dependent RNA polymerase and capsid protein sequences in comparison to the previously identified LSV 1 and 2 strains. Phylogenetic analyses of LSV sequences obtained from single honey bee specimens further revealed that up to three distinctive clades could be present in a single bee. Such superinfections have not previously been identified for other honey bee viruses. In a search for the putative routes of LSV transmission, we were able to demonstrate the presence of LSV in pollen pellets and in *V. destructor* mites. However, negative-strand analyses demonstrated that the virus only actively replicates in honey bees and mason bees (*Osmia cornuta*) and not in *Varroa* mites.

3. Introduction

Honey bees (*Apis mellifera*) are susceptible to a wide range of pathogens, including viruses (Evans and Schwarz, 2011). The first honey bee viruses were described in 1963 (Bailey et al., 1963), and many others were discovered later. At present, more than 20 bee viruses have been reported, but some of them, such as Arkansas Bee Virus (Bailey and Woods, 1974), have not been characterized in detail at the biochemical or genomic level. Other viruses, such as Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV) have been extensively studied over recent decades, as they have been found to have important effects on honey bee health, including associations with honey bee colony collapses (reviewed by de Miranda et al. (de Miranda et al., 2010; de Miranda and Genersch, 2010)).

Generally, replication has been regarded as a prerequisite of pathogenicity, and an overt DWV infection is correlated with replication in honey bees and mites (Gisder et al., 2009; Yue and Genersch, 2005). Replication of positive single-stranded RNA viruses, which include most of the honey bee viruses, is indicated by the production of a negative-strand intermediate. In the particular case of DWV, the predominant site of replication coincides with the site of deformities, representing the typical clinical sign of the illness, i.e. wing deformities (Boncristiani et al., 2009).

Several metagenomic studies were recently performed to elucidate the cause of declining numbers of honey bee colonies (Cornman et al., 2012; Cox-Foster et al., 2007; Granberg et al., 2013; Runckel et al., 2011). This resulted in the detection of new honey bee viruses, such as Aphid Lethal Paralysis Virus strain Brookings (ALPV), Big Sioux River Virus (BSRV) and two strains of Lake Sinai Virus (LSV 1 and LSV 2) (Runckel et al., 2011). Whereas ALPV and BSRV are members of the common Dicistroviridae family, the LSVs are unclassified, but related to Anopheline-associated C virus (AACV) and Chronic Bee Paralysis Virus (CBPV)

(Cook et al., 2013). They have a different genome organization, leading to the proposition of the genus Sinaivirus for the LSVs and Chroparavirus for AACV and CBPV (Kuchibhatla et al., 2014). The capsid protein from Mosinovirus (MoNV) is also related to the LSVs, although this virus is taxonomically placed within the Nodavirirdae based on its RdRP protein (Schuster et al., 2014).

Interpreting the clinical significance of these newly discovered viruses is far from simple, especially in regard to subtle syndromes or chronic disease, and when morphological deformities, paralysis or even sudden death are absent. In the case of the plant viruses that were recently discovered in honey bees (Cornman et al., 2012; Granberg et al., 2013), an indication of putative clinical relevance was provided by the negative-strand detection of Tobacco Ringspot Virus in honey bees (Li et al., 2014).

Soon after its discovery in the USA, LSV was also found in Belgian (Ravoet et al., 2013) and Spanish apiaries (Granberg et al., 2013). The Belgian bee health study revealed a prevalence of 14.6% (Ravoet et al., 2013). LSV was also found in the solitary bees *A. vaga*, *A. ventralis*, *O. bicornis* and *O. cornuta* (Ravoet et al., 2014). Previous sequence analyses pointed to a remarkable heterogeneity among the identified LSV strains (Cornman et al., 2012; Ravoet et al., 2013; Runckel et al., 2011).

The present study was aimed at further exploring the LSV sequence heterogeneity. Furthermore, we investigated (1) polymorphisms within single host (honey bee) specimens, (2) putative routes of transmission and (3) virus replication in bees. These results should be of great value to elucidate the effects of LSV on honey bee health.

4. Material and methods

4.1 Sampling and RNA isolation

We used honey bee samples, collected in Belgium in July 2011, which were previously screened for LSVs (Ravoet et al., 2013). Ten LSV positive samples were selected for further genetic characterization of the LSV genome. Their viral RNA was isolated using the QiaAmp Viral RNA mini kit (Qiagen), as described previously (De Smet et al., 2012). Briefly, ten bees per sample were homogenized in 5 ml PBS by mechanical agitation in a TissueLyser for 90 sec at 30 Hz. After centrifugation at 13,300 g for 5 minutes, the viral RNA was extracted from 140 µl of the supernatant.

To investigate virus replication and possible routes of transmission, we used additional samples of LSV-positive honey bees, solitary bees (*A. vaga*, *A. ventralis*, *O. bicornis* and *O. cornuta*, collected in 2012 (Ravoet et al., 2014)), pollen pellets from the corbiculae of forager bees (collected in July 2011) and *Varroa destructor* mites (collected in July 2012). All samples were obtained at the apiary of Ghent University (campus Sterre, Ghent, Belgium). In these cases, total RNA was extracted using the RNeasy Lipid Tissue mini kit (Qiagen). Ten mites per sample were first manually ground in 500 µl PBS. For the pollen samples, 100 mg pollen pellets were collected and mixed with 500 µl QIAzol. Total RNA was isolated from the bees using 200 µl of the suspension (of the PBS crushed bees) and 1 ml QIAzol reagent. Single specimens of an infected honey bee sample were directly homogenized in 1 ml QIAzol by mechanical agitation in the presence of glass beads (2 mm). The RNA was extracted according to the manufacturer's instructions.

4.2. Reverse transcriptase-PCR

Using random hexamer primers, 5 µl viral RNA or 1 µg total RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). All PCR reaction

mixtures contained: 2 µM of each primer (Table S1), 1.0 mM $MgSO_4$, 1.25 U Hotstar Taq HiFidelity DNA polymerase (Qiagen) and 1 µl cDNA. The following cycling conditions were used: 95°C for 5 min; 35 cycles of [94°C for 30 s, 56°C for 30 s, 72°C for 45 s or 1 m 40 s (for amplicons > 1 kb)]; final elongation 72°C for 10 min; hold at 4°C. All PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and visualized under UV light.

4.3. Genome sequence

All sequence analyses were performed in Geneious R7. Conserved nucleotide sequences were determined by aligning the genomes of two American LSV strains (LSV 1, Genbank: HQ871931; LSV 2, Genbank: HQ888865) using the MUSCLE plugin. Several degenerate primer pairs were designed to amplify the majority of the Orf1, RdRP and capsid genes and overlapping fragments (Figure 1, Table S1). Amplicons of the expected size were gel-extracted and cloned into a pGEM-T Easy vector (Promega). The purified plasmids were sequenced using M13 primers and internal primers (Table S1). The obtained genome sequences were assembled *de novo* and mapped onto the LSV 1 genome as a reference.

4.4. Genome analysis and phylogenetics

The resulting Orf1, RdRP and capsid genes and proteins from the honey bee samples were aligned with those of LSV strain 1 and 2 using the MUSCLE plugin. The gene alignments served as templates to design primers for negative-strand detection using the Primer3 plugin. The proteins were aligned to assess the amino acid similarity. Furthermore, the whole RdRP proteins of all LSVs were aligned with those of AACV (Genbank: YP_009011225), CBPV (Genbank: YP_001911137A), MoNV (Genbank: AIO11151) and the Nodaviridae types Nodamura virus (Genbank: NP_077730) and Striped Jack nervous necrosis virus (Genbank:

NP_599247). The LSV capsid proteins were aligned with those of AACV (Genbank: AGW51753), CBPV (Genbank: YP_001911140) and MoNV (Genbank: AIO11154).

To investigate LSV polymorphisms, we analysed an Orf1/RdRP fragment (primers LSV1765-F and LSV2368-R) originating from four single bee specimens. Five clones per bee were obtained and aligned with sequences retrieved from the same apiary (Genbank: KF768348- KF768351).

The capsid and RdRP protein alignments (of AACV, CBPV, LSVs, MoNV and Nodaviridae) and the Orf1/RdRP gene alignment were used for phylogenetic analyses. In the capsid and RdRP protein alignments, poorly aligned blocks were first removed with Gblocks (Talavera and Castresana, 2007), which retained 66% (485/733) and 47% (630/1325) of the amino acids, respectively. Selection of the best fitted maximum likelihood models was based on the Bayesian information criterion (BIC), as implemented in MEGA6 (Tamura et al., 2013). The phylogenetic analyses for the capsid, RdRP and the Orf1/RdRP alignments were performed with the Whelan And Goldman model with a discrete gamma distribution (WAG+G), the Le-Gascuel model with a discrete gamma distribution (LG+G) and the Kimura 2-parameter model with invariable sites (K80+I), respectively, using PhyML 3.0 (Guindon et al., 2010). The branch reliability was assessed using approximate likelihood-ratio tests based on a Shimodaira-Hasegawa-like (aLRT SH-like) procedure (Anisimova and Gascuel, 2006).

4.5. Negative-strand detection

LSV replication was investigated in honey bees and solitary bees using strand-specific RT-PCR, following the tagged cDNA procedure described in the COLOSS BEEBOOK (de Miranda et al., 2013). We synthesized cDNA using 1 µg total RNA (from honey bees, *A. vaga*, *A. ventralis*, *O. bicornis* and *O. cornuta*) and 20 pmol of the tagged negative-strand-specific forward primer (TAG-repLSV2158-F). Later on, the cDNA was purified using the GeneJET PCR

Purification Kit (Thermo Scientific) to remove unincorporated primers, which could cause false positive results. PCR reactions were performed using 2 µl of purified cDNA, 2 µM of the tag-specific forward primer TAG-F and 2 µM of the LSV-specific reverse primer repLSV2490-R. The following cycling conditions were used: 95°C for 5 min; 35 cycles of [94°C for 30 s, 58°C for 30 s, 72°C for 45 s]; final elongation 72°C for 10 min; hold at 4°C. To validate this PCR-based negative-strand detection method, the purified cDNA was amplified in a PCR reaction with addition of only the primer repLSV2490-R (and no TAG-F). This ensures the complete removal of unincorporated TAG-repLSV2158-F primers.

5. Results

5.1 Genome analysis

Our genome sequencing strategy covered almost the entire LSV genomes and consisted of the cloning and sequencing of 3 gene-specific amplicons and 3 overlapping fragments that spanned the gaps between these genes (Figure 1). Only the untranslated regions at the termini were incomplete. This approach resulted in the successful assembly of four almost complete LSV genomes from honey bee samples, designated as LSV strains VBP022, VBP166, VBP256 and exp10, which are deposited in Genbank under the accession numbers KM886902-KM886905.

The identified nucleotide sequences of these strains were between 5,187 and 5,192 nt long. They have a variable spacer (19-23 nucleotides) between the RdRP and capsid genes. A similar spacer of 18 nt was found in LSV 2, whereas LSV 1 shows a gene overlap of 125 nt (Runckel et al., 2011). The 6 LSV genomes (2 American and 4 Belgian) have a very similar GC content, varying between 50.7% and 51.7%.

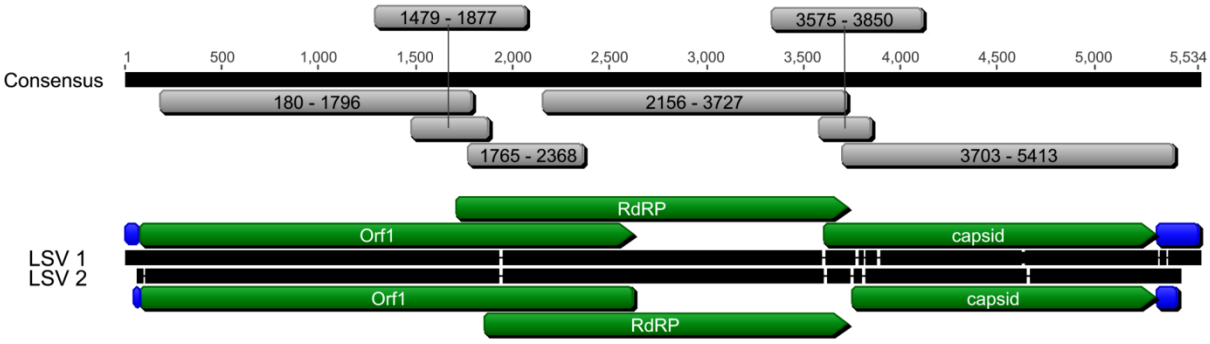


***Figure 1**: Graphical overview of the amplified fragments (grey boxes), based on an alignment of LSV 1 (HQ871931) and 2 (HQ888865). The primers are indicated by numbers, corresponding to the positions in the LSV 1 genome. The LSV proteins are shown in green boxes, and the UTRs are shown in blue.*

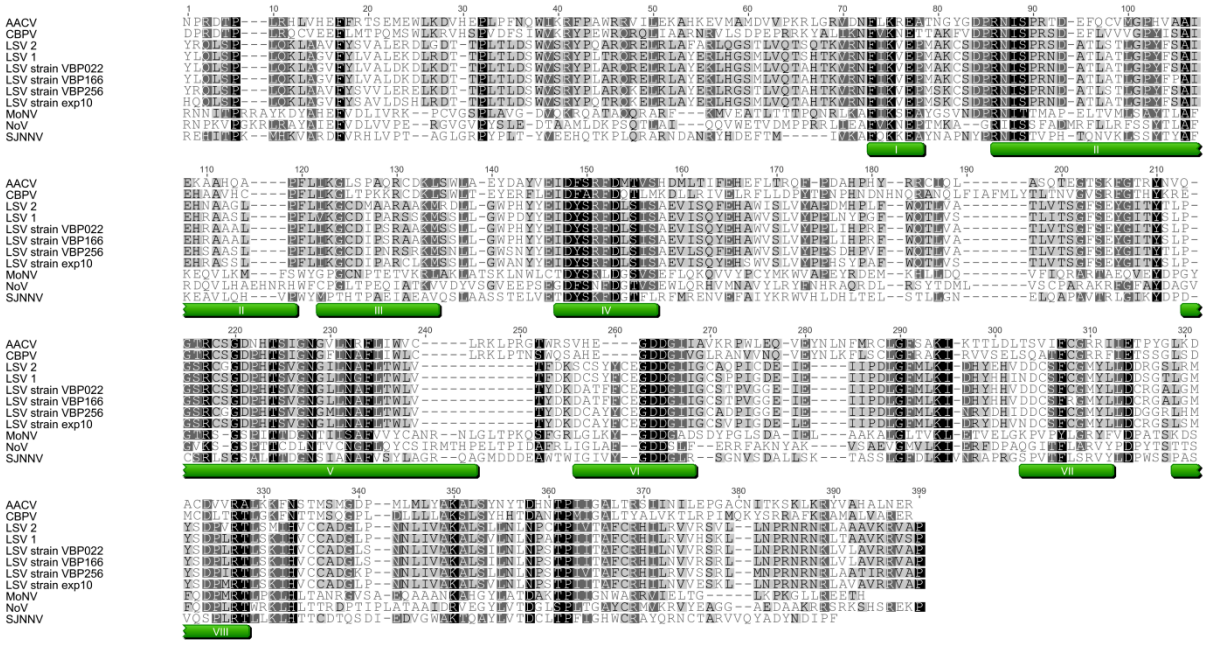


***Figure 2**: Trimmed alignment of the RdRP proteins from AACV (Genbank: YP_009011225), CBPV (Genbank: YP_001911137A), LSV strains from the USA (LSV 1 and 2; Genbank: AEH26187, AEH26192) and Belgium (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905), MoNV (Genbank: AIO11151) and the Nodaviridae types Nodamura virus (Genbank: NP_077730) and Striped Jack nervous necrosis virus (Genbank: NP_599247). The eight conserved viral RdRP domains (Koonin et al., 1993) are shown below the alignment in green boxes.*

***Table 1**: Similarity matrix of the whole Orf1, RdRP and capsid genes and proteins from American (Genbank: HQ871931, HQ888865) and Belgian LSV strains (Genbank: KM886902- KM886905), expressed in percent identity. The nucleotide identify is given first and the amino acid identity after the semicolon.*

Orf1						
	HQ871931	HQ888865	KM886902	KM886903	KM886904	KM886905
HQ871931		73% : 72%	71% : 71%	76% : 75%	72% : 71%	72% : 73%
HQ888865	73% : 72%		79% : 83%	72% : 70%	76% : 78%	78% : 81%
KM886902	71% : 71%	79% : 83%		74% : 71%	74% : 75%	92% : 87%
KM886903	76% : 75%	72% : 70%	74% : 71%		70% : 69%	73% : 71%
KM886904	72% : 71%	76% : 78%	74% : 75%	70% : 69%		79% : 84%
KM886905	72% : 73%	78% : 81%	92% : 87%	73% : 71%	79% : 84%	
RdRP						
	HQ871931	HQ888865	KM886902	KM886903	KM886904	KM886905
HQ871931		76% : 80%	74% : 82%	76% : 83%	77% : 80%	75% : 84%
HQ888865	76% : 80%		93% : 80%	76% : 77%	74% : 82%	77% : 78%
KM886902	74% : 82%	93% : 80%		76% : 91%	74% : 78%	78% : 83%
KM886903	76% : 83%	76% : 77%	76% : 91%		74% : 80%	78% : 85%
KM886904	77% : 80%	74% : 82%	74% : 78%	74% : 80%		80% : 85%
KM886905	75% : 84%	77% : 78%	78% : 83%	78% : 85%	80% : 85%	
Capsid						
	HQ871931	HQ888865	KM886902	KM886903	KM886904	KM886905
HQ871931		69% : 71%	74% : 71%	65% : 87%	66% : 69%	66% : 91%
HQ888865	69% : 71%		70% : 94%	60% : 70%	60% : 83%	62% : 72%
KM886902	74% : 71%	70% : 94%		65% : 69%	67% : 82%	67% : 86%
KM886903	65% : 87%	60% : 70%	65% : 69%		72% : 68%	71% : 86%
KM886904	66% : 69%	60% : 83%	67% : 82%	72% : 68%		79% : 69%
KM886905	66% : 91%	62% : 72%	67% : 86%	71% : 86%	79% : 69%	

The genome sequence heterogeneity is reflected in the variance of the sequence identity for the different genes: 70.1-92.1% for Orf1, between 74.0-92.6% for RdRP and between 60.1-78.7% for capsid (Table 1) was confirmed. At the protein level, the most extreme values of amino acid sequence identity were found in the capsid, which varied between 67.9% and 94.4% (Table 1). Nevertheless, many conserved regions were observed in the three viral proteins (Figures S1-S3). All RdRP genes encode the DxSRFD and SG amino acid motifs, a conserved domain in the NTP binding pocket of some viral families (Runckel et al., 2011). The RdRP proteins even share conserved regions with those of the related viruses AACV,

CBPV, MoNV and Nodaviridae (Figure 2). Moreover, all eight conserved viral RdRP domains (Koonin and Dolja, 1993) were found. Based on this RdRP alignment, a phylogenetic tree was constructed (Figure S4). This was also performed on an alignment of the capsid proteins of AACV, CBPV, LSVs and MoNV (Figure S5).

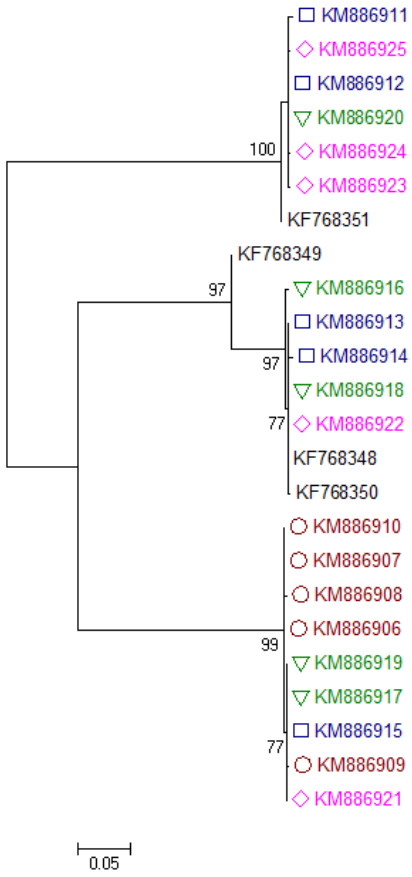


***Figure 3**: Phylogenetic analysis of LSV clones from single bee specimens. LSV strains from the same apiary, shown in black, were included in the analysis. Each isolate is indicated by its accession number. LSV clones isolated from individual bees (1-4) are designated by colour and shape (red circle, blue square, green triangle and fuchsia rhombus). Branch support for each node is designated by aLRT (approximate Likelihood-Ratio Test) values (>70%).*

4.2 Orf1/RdRP-based polymorphism in a single bee

During our genome assembly, it became evident that multiple LSV strains occur within the pooled honey bee samples. We evaluated the virus heterogeneity in single honey bee specimens, and phylogeny of the identified strains revealed that up to three distinct clades

were present in a single bee (Figure 3). The obtained Orf1/RdRP sequences were deposited in Genbank under the accession numbers KM886906-KM886925.

4.3 Transmission routes and viral replication

In our search for the putative routes of LSV transmission, we were able to demonstrate the presence of LSV in pollen pellets and in *V. destructor* mites (Figure 4). Moreover, we were able to detect the negative-strand intermediate in honey bees and in *O. cornuta* (Figure 4). Nevertheless, we did not detect viral replication in the other solitary bees (*A. vaga*, *A. ventralis* and *O. bicornis*) or in *V. destructor* mites.

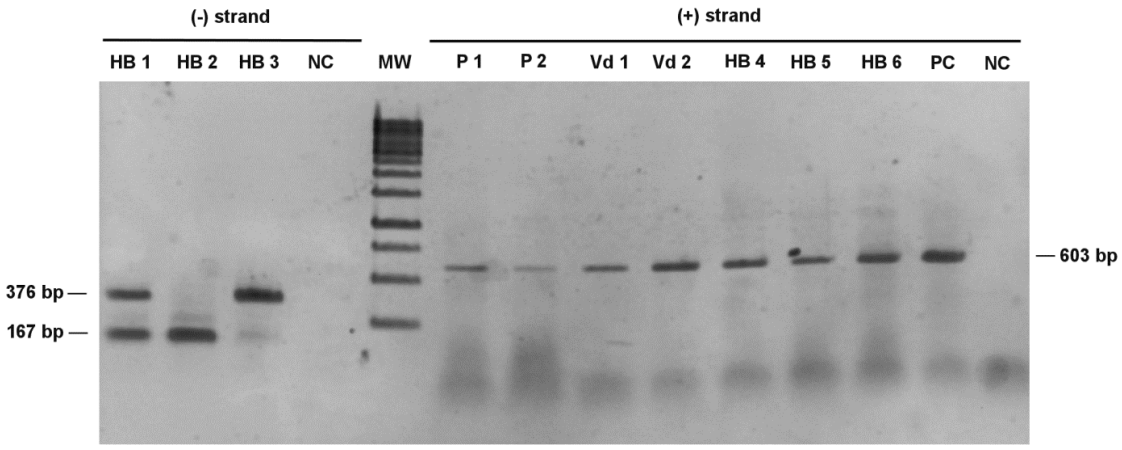


Figure 4*: Molecular detection of the positive- and negative-strands of LSV in different hosts. LSV sequences were amplified from pooled honey bee, pollen and V. destructor mite samples using strand-specific RT-PCR, and visualized by gel-electrophoresis. These amplicons are verified by cloning and sequencing; LSV negative-strand-specific PCR product = 376 bp, LSV positive-strand-specific PCR product size = 603 bp. No templates were added in both negative controls. A diluted plasmid was used as a positive control. The pooled honey bee samples used for negative-strand detection were positive for LSV using positive-strand PCR.*

HB: honey bee samples, MW: molecular weight marker (Generuler 1 kb DNA ladder, Thermo Scientific), N: negative control, P: pollen samples, PC: positive control, Vd: Varroa destructor *mite samples. The numbers indicate different samples. The amplicon size of the positive and negative-strand PCR reactions, respectively 603 bp and 376 bp, are shown on both sides of the gel. The non-specific product of 167 bp in honey bee samples 1-3 corresponded to the* Apis mellifera *retinoid – and fatty acid-binding glycoprotein (Genbank: XM_006561492).*

5. Discussion

Recently, several insect viruses that are related to the Nodaviridae family received considerable attention. Although the four studied LSV genomes show a similar organization as LSV 2, with variable spacers between the RdRP and capsid genes, the sequence identity of the capsid gene could be as low as 60.1% with LSV 2. This confirms the genome sequence heterogeneity of numerous LSV strains (Cornman et al., 2012). Remarkably, this sequence heterogeneity seems to have no geographic link, as the phylogenetic tree of the RdRP protein from the American and Belgian LSV strains showed no clustering by country (Figure S4). In contrast, several geographic lineages were identified in the bee virus Israeli Acute Paralysis Virus (IAPV) (Chen et al., 2014; Palacios et al., 2008). The phylogenetic tree of the capsid protein also demonstrated no geographic clustering, but confirmed the close relationship of the MoNV capsid to the LSVs (Figure S5).

The LSV genomes encode three genes: Orf1, RdRP and capsid. The RdRP gene encodes a RNA-dependent RNA polymerase that is strongly conserved in the different LSV strains. A Tetravirus-like capsid protein is predicted to be encoded by the capsid gene (Runckel et al., 2011). Although the function of Orf1 remains unclear, it contains a domain homologous to the Alphavirus methyltransferase-guanyltransferase, a putative membrane protein. This is also present in the ORF1 of CBPV RNA 1 (Kuchibhatla et al., 2014).

In addition to the sequence heterogeneity between samples, we also observed an Orf1/RdRp-based polymorphism in a single bee. Sequence analysis and subsequent phylogeny of numerous clones from individual bees revealed the presence of multiple LSV strains in the same specimen. This high level of intra-individual variation has not yet been revealed for a honey bee virus, although some DWV sequence polymorphisms were already reported in the variable leader protein (Lp) gene of pupae infested with the mite

Tropilaelaps mercedesae (Forsgren et al., 2009). DWV is part of a complex, together with the related viruses Kakugo Virus and *Varroa destructor* Virus-1. The nucleotide differences are concentrated in the 5' UTR and the Lp (de Miranda et al., 2010; Lanzi et al., 2006). Honey bees can be infected by another viral complex, formed by the related viruses APBV, IAPV and Kashmir Bee Virus (de Miranda et al., 2010). Their nucleotide differences are situated in the 5' UTR. Although a low genetic variability was assumed for these members, a high frequency of nucleotide polymorphisms was observed at the population level for DWV and IAPV (Chen et al., 2014; Cornman et al., 2013).

Honey bee viruses can be transmitted by several routes (Chen et al., 2006), and the most basic mode is probably oral uptake, for instance by contaminated pollen (Singh et al., 2010). Nevertheless, the hematophagous mite *V. destructor* represents by far the most important vector of honey bee viruses, as it delivers the virus directly into the hemocoel by puncturing the integument during nourishment (Rosenkranz et al., 2010). It has been demonstrated that some honey bee viruses, for instance DWV and IAPV, even replicate within this mite (Di Prisco et al., 2011; Ongus et al., 2004). We found no evidence that this is also the case for LSV. Nevertheless, even horizontal transmission alone could have important consequences for the population dynamics and epidemiology of honey bee viruses. In fact, in the Hawaiian islands, the introduction of *Varroa* has been shown to have led to the establishment of just one single, virulent strain of DWV (Martin et al., 2012).

Our discovery that LSV can be found in pollen and *Varroa* mites is an important step to fully elucidate the transmission routes of this new honey bee virus. It implies that horizontal transmission of LSV can occur via infected bees, via the vectoring mite or via contaminated pollen. Although we do not provide causal evidence of transmission, cross-

species transmission of honey bee viruses and subsequent infection has previously been experimentally demonstrated (Furst et al., 2014; Mazzei et al., 2014; Singh et al., 2010).

Detection of viral replication was based-negative strand analyses, which might bear some pitfalls. Nevertheless, we included several controls to avoid them, e.g. purification of the tagged cDNA. The ability of LSV replication in honey bees (Runckel et al., 2011) was confirmed, suggesting that LSV infections are not entirely harmless. Moreover, the demonstration of virus replication in the solitary bee *O. cornuta* suggests that LSV is a multi-host virus, akin to other honey bee viruses such as DWV and IAPV. This solitary bee is also susceptible to DWV infection (Mazzei et al., 2014), but replication of these viruses is demonstrated in several pollinators (Levitt et al., 2013; Li et al., 2011; Zhang et al., 2012). Even clinical symptoms of an overt DWV infection, such as crippled wings, have been observed in bumble bees (Genersch et al., 2006). Our results indicate that LSV is a common honey bee virus, which might represent an infection risk for other pollinators as well.

6. Acknowledgements

This study was supported by the Research Foundation Flanders (FWO-Vlaanderen, research grant G.0628.11).

7. References

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Syst Biol 55**,** 539-552.

Bailey, L., Gibbs, A.J., Woods, R.D., 1963. Two viruses from adult honey bees (Apis mellifera Linnaeus). Virology 21**,** 390-395.

Bailey, L., Woods, R.D., 1974. Three previously undescribed viruses from the honey bee. J Gen Virol 25**,** 175-186.

Boncristiani, H.F., Di Prisco G., Pettis, J.S., Hamilton, M., Chen, Y.P., 2009. Molecular approaches to the analysis of deformed wing virus replication and pathogenesis in the honey bee, Apis mellifera. Virol J 6**,** 221.

Chen, Y.P., Evans, J.D., Feldlaufer, M., 2006. Horizontal and vertical transmission of viruses in the honeybee, Apis mellifera. J Invertebr Pathol 92**,** 152-159.

Chen, Y.P., Pettis, J.S., Corona, M., Chen, W.P., Li, C.J. and others, 2014. Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health. PLoS Pathog 10**,** e1004261.

Cook, S., Chung, B.Y., Bass, D., Moureau, G., Tang, S. and others, 2013. Novel virus discovery and genome reconstruction from field RNA samples reveals highly divergent viruses in dipteran hosts. PLoS One 8**,** e80720.

Cornman, R.S., Boncristiani, H.F., Dainat, B., Chen, Y.P., Vanengelsdorp, D. and others, 2013. Population-genomic variation within RNA viruses of the Western honey bee, Apis mellifera, inferred from deep sequencing. BMC Genomics 14.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D. and others, 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7**,** e43562.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

de Miranda, J.R., Bailey, L., Ball, B.V., Blanchard, P., Budge, G.E. and others, 2013. Standard methods for virus research in Apis mellifera. J Apicult Res 52.

de Miranda, J.R., Cordoni, G., Budge, G., 2010. The Acute Bee Paralysis Virus-Kashmir Bee Virus-Israeli Acute Paralysis Virus complex. J Invertebr Pathol 103**,** S30-S47.

de Miranda, J.R., Genersch, E., 2010. Deformed Wing Virus. J Invertebr Pathol 103 Suppl 1**,** S48-S61.

De Smet, L., Ravoet, J., de Miranda, J.R., Wenseleers, T., Mueller, M.Y. and others, 2012. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One 7**,** e47953.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H.F., Evans, J.D., Chen, Y., 2011. Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. J Gen Virol 92**,** 151-155.

Evans, J.D., Schwarz, R.S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol 19**,** 614-620.

Forsgren, E., de Miranda, J.R., Isaksson, M., Wei, S., Fries, I., 2009. Deformed wing virus associated with Tropilaelaps mercedesae infesting European honey bees (Apis mellifera). Exp Appl Acarol 47**,** 87-97.

Furst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506**,** 364-366.

Genersch, E., Yue, C., Fries, I., de Miranda, J.R., 2006. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. J Invertebr Pathol 91**,** 61-63.

Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites (Varroa destructor). J Gen Virol 90**,** 463-467.

Granberg, F., Vicente-Rubiano, M., Rubio-Guerri, C., Karlsson, O.E., Kukielka, D. and others, 2013. Metagenomic detection of viral pathogens in spanish honeybees: co-infection by aphid lethal paralysis, Israel acute paralysis and lake sinai viruses. PLoS One 8**,** e57459.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59**,** 307-321.

Koonin, E.V., Dolja, V.V., 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit Rev Biochem Mol 28**,** 375-430.

Kuchibhatla, D.B., Sherman, W.A., Chung, B.Y., Cook, S., Schneider, G. and others, 2014. Powerful sequence similarity search methods and in-depth manual analyses can identify remote homologs in many apparently "orphan" viral proteins. J Virol 88**,** 10-20.

Lanzi, G., de Miranda, J.R., Boniotti, M.B., Cameron, C.E., Lavazza, A. and others, 2006. Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.). J Virol 80**,** 4998-5009.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K. and others, 2013. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 176**,** 232-240.

Li, J., Peng, W., Wu, J., Strange, J.P., Boncristiani, H., Chen, Y., 2011. Cross-species infection of Deformed Wing Virus poses a new threat to pollinator conservation. J Econ Entomol 104**,** 732-739.

Li, J.L., Cornman, R.S., Evans, J.D., Pettis, J.S., Zhao, Y. and others, 2014. Systemic spread and propagation of a plant-pathogenic virus in European honeybees, Apis mellifera. MBio 5**,** e00898-13.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E. and others, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336**,** 1304-1306.

Mazzei, M., Carrozza, M.L., Luisi, E., Forzan, M., Giusti, M. and others, 2014. Infectivity of DWV associated to flower pollen: experimental evidence of a horizontal transmission route. PLoS One 9**,** e113448.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. J Gen Virol 85**,** 3747-3755.

Palacios, G., Hui, J., Quan, P.L., Kalkstein, A., Honkavuori, K.S. and others, 2008. Genetic analysis of Israel acute paralysis virus: distinct clusters are circulating in the United States. J Virol 82**,** 6209-6217.

Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D.C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J Invertebr Pathol 122**,** 55-58.

Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T. and others, 2013. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS One 8**,** e72443.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. J Invertebr Pathol 103 Suppl 1**,** S96-119.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Schuster, S., Zirkel, F., Kurth, A., van Cleef, K.W., Drosten, C. and others, 2014. A unique nodavirus with novel features: mosinovirus expresses two subgenomic RNAs, a capsid gene of unknown origin, and a suppressor of the antiviral RNA interference pathway. J Virol 88**,** 13447-13459.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA viruses in hymenopteran pollinators: Evidence of inter-Taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5**,** e14357.

Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 56**,** 564-577.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30**,** 2725-2729.

Yue, C., Genersch, E., 2005. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). J Gen Virol 86**,** 3419-3424.

Zhang, X., He, S.Y., Evans, J.D., Pettis, J.S., Yin, G.F., Chen, Y.P., 2012. New evidence that Deformed Wing Virus and Black Queen Cell Virus are multi-host pathogens. J Invertebr Pathol 109**,** 156-159.

8. Supplementary information

***Table S1**: List of PCR primers used for LSV fragments in this study. The position of the primers and the ampicon size are referring to LSV 1 (Genbank: HQ871931). The sequence of the tag used in the negative strand detection is indicated in bold. [#]: primers used for screening of the positive strand, [¥]: primers used for screening of the negative strand*

Target gene	Primers	Sequence (5'-3')	Size (bp)
Orf1	LSV0180-F [1]	ACGATGTGCAGYYATGAGTA	1616
Orf1	LSV1796-R [1]	CGWGGCCTCAGCACGA	
Orf1	LSV0931-F [1]	CGCCTGAYCAGTTGGCC	N.A.
Orf1	LSV0950-R [1]	GAGGCCAACTGRTCAGG	
Orf1 – RdRP	LSV1765-F [#]	TCAAYCTKGAGCGATTTCGTGCTG	603
Orf1 – RdRP	LSV2368-R [2, #]	GAGGTGGCGGCGCSAGATAAAGT	
Orf1 – RdRP	LSV1479-F	CTSGACTTCATYATCCATCTGTG	398
Orf1 – RdRP	LSV1877-R	GTCACMARRCTTGATATCATGTT	
RdRP	LSV2156-F	GTGCGGACCTCATTTCTTCATG	1571
RdRP	LSV3727-R	TTYACGCGTAAAGAACAGACCT	
RdRP	LSV2481-F	AGATTTARGGGATACGACACCTTTGA	860
RdRP	LSV3341-R	ACRATCAAATTGTTGGGWAGACCAT	
RdRP – Capsid	LSV3575-F	GCATATCTCCTGCGTTGCA	275
RdRP – Capsid	LSV3850-R	GTTACGGCGCCTACGATT	
Capsid	LSV3703-F	TTYAGGTCTGTTCTTTACGCGT	1710
Capsid	LSV5413-R	CGACTGATTACCAGTAACCACAC	
Capsid	LSV4028-F	ATGGKTCTGCTGTYACCACATG	1210
Capsid	LSV5238-R	ACCRAGCCAGTTCCACGC	
Capsid	LSV4480-F	CTTGARACTCAGGGATTYGTCACMGC	751
Capsid	LSV5231-R	CAGTTCCACGCMGGCTTGATGAG	
RdRP	TAG-repLSV2158-F	**GGCCGTCATGGTGGCGAATAA**GCGGACCTCATTTCTTCATG	
	TAG-F [3,¥]	**GGCCGTCATGGTGGCGAATAA**	376
RdRP	repLSV2490-R [¥]	CCAAGGTCAAAGGTGTCGTATCC	

[1]: Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., de Graaf, D. C., 2014. Widespread occurrence of honey bee pathogens in solitary bees. J Invertebr.Pathol. 122, 55-58.

[2]: Ravoet, J., Maharramov, J., Meeus, I., De Smet, L., Wenseleers, T., Smagghe, G., de Graaf, D. C., 2013. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS ONE 8, e72443.

[3]: Plaskon, N.E., Adelman, Z.N., Myles, K.M., 2009 Accurate Strand-Specific Quantification of Viral RNA. PLoS ONE 4: e7468.

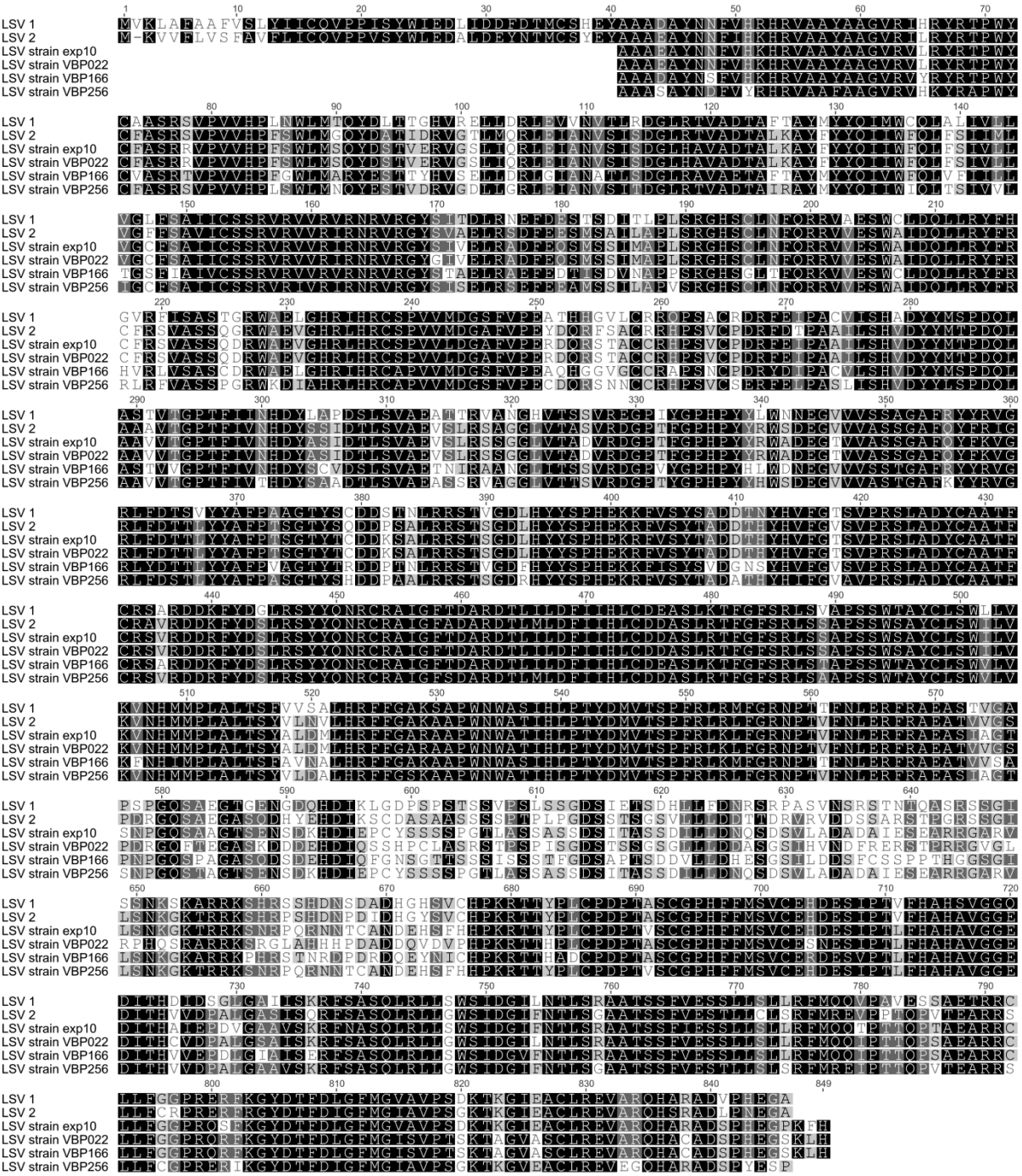


***Figure S1**: Alignment of the complete Orf1 proteins from the USA (LSV 1 and 2; Genbank: AEH26187, AEH26192) and Belgium (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905).*

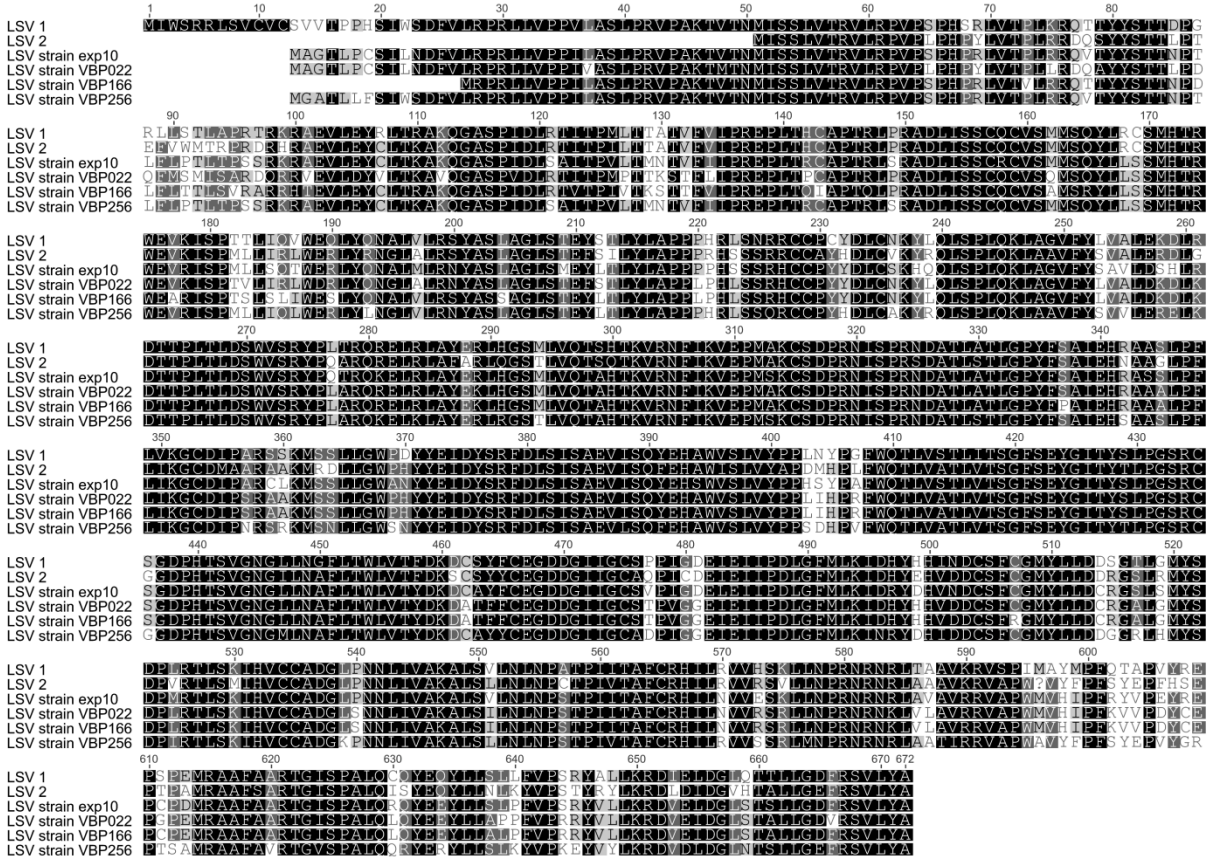


***Figure S2**: Alignment of the complete RdRP proteins from the USA (LSV 1 and 2; Genbank: AEH26187, AEH26192) and Belgium (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905).*

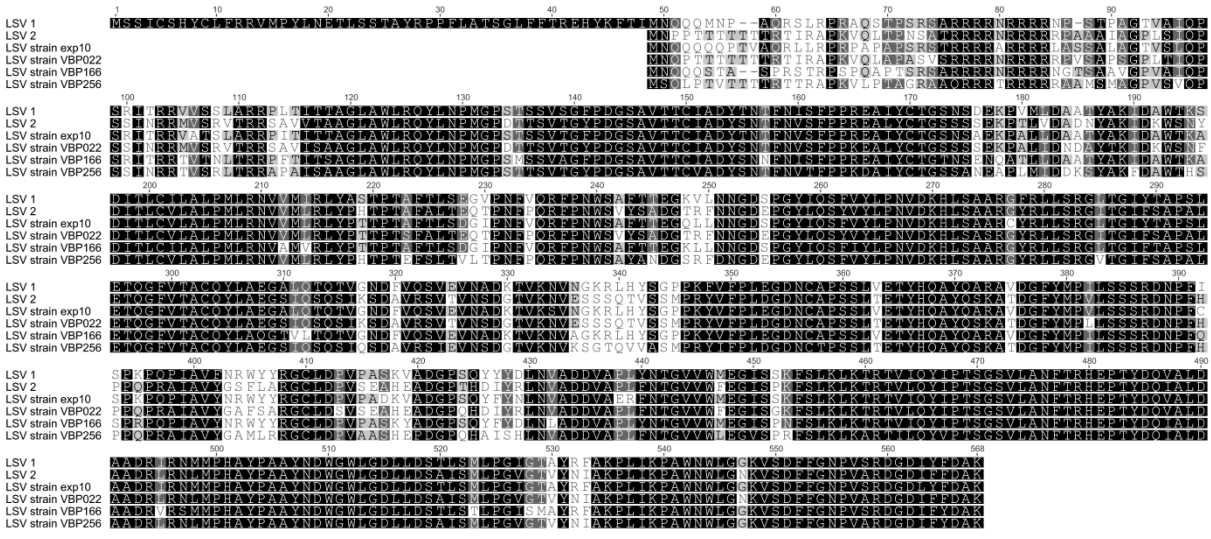


***Figure S3**: Alignment of the complete capsid proteins from the USA (LSV 1 and 2; Genbank: AEH26194, AEH26188) and Belgium (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905).*

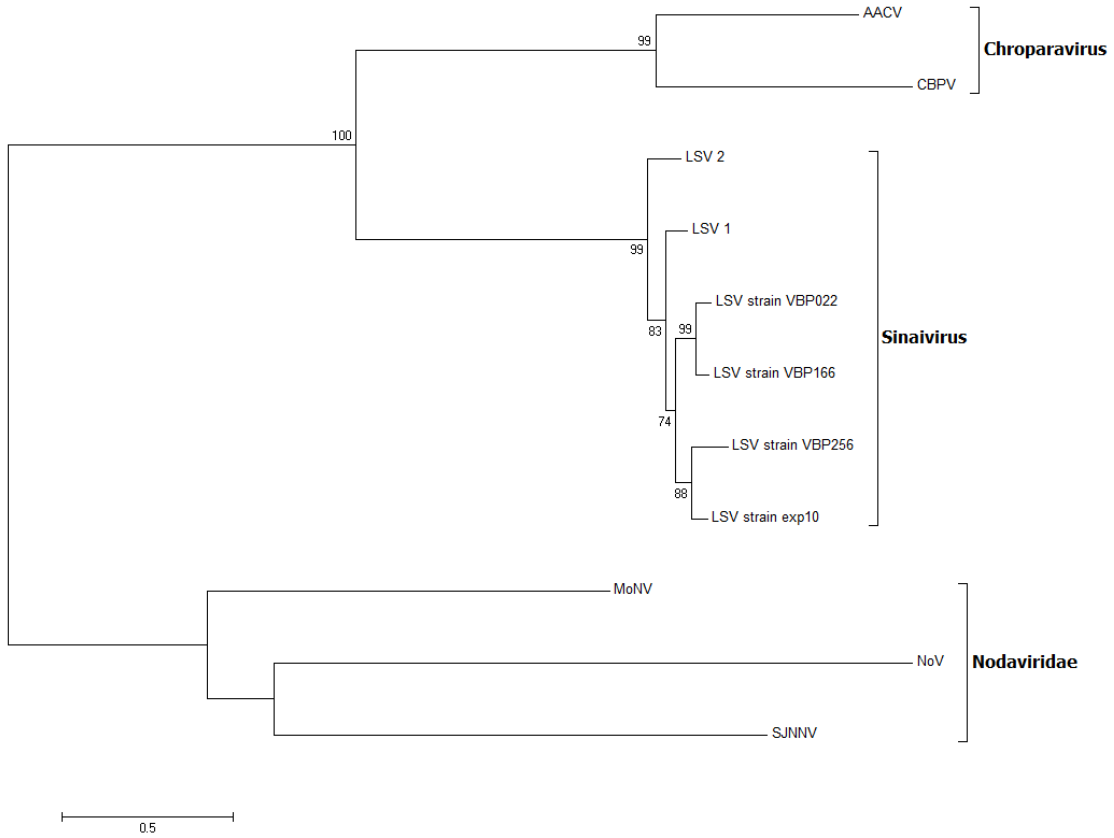


***Figure S4**: Phylogeny of the RdRP proteins from American (LSV 1 and 2; Genbank: AEH26187, AEH26192) and Belgian (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905) LSVs, AACV (Genbank: YP_009011225), CBPV (Genbank: YP_001911137A), MoNV (Genbank: AIO11151) and the Nodaviridae types Nodamura virus (Genbank: NP_077730) and Striped Jack nervous necrosis virus (Genbank: NP_599247). Each isolate is indicated by its accession number and the clades are designated by genus or family names. Branch support for each node is designated by aLRT (approximate Likelihood-Ratio Test) values (>70%).*

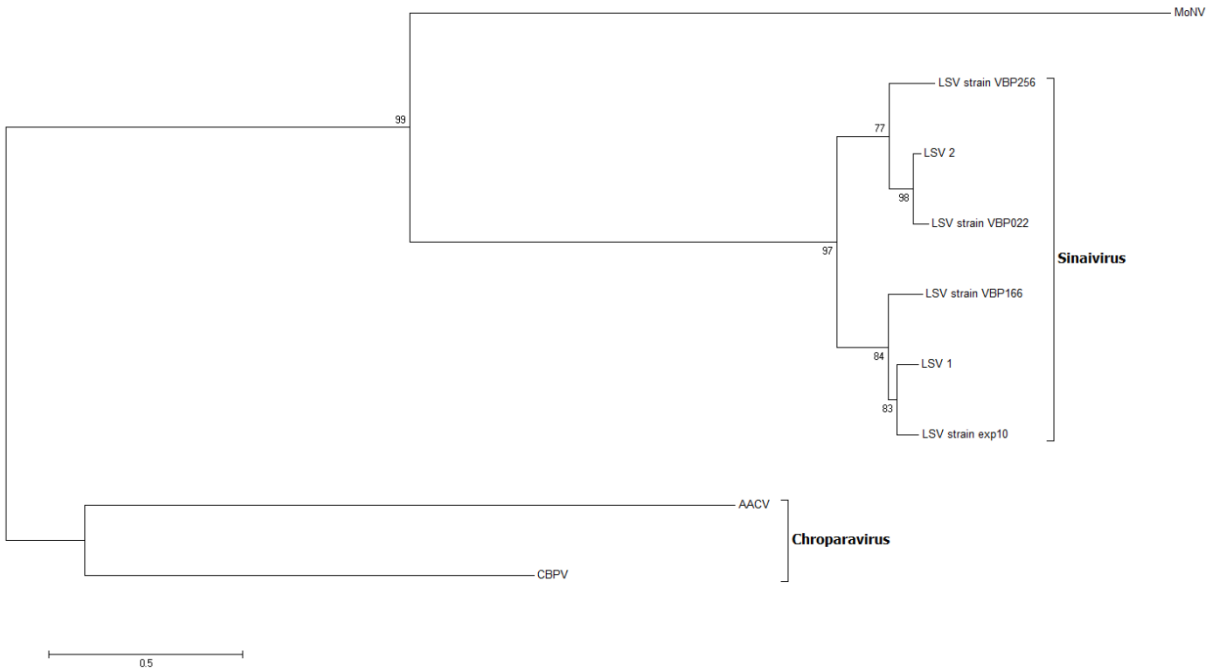


***Figure S5**: Phylogeny of the capsid proteins from American (LSV 1 and 2; Genbank: AEH26194, AEH26188) and Belgian (LSV strains VBP022, VBP166, VBP256, exp10; Genbank: KM886902-KM886905) LSV strains and MoNV (Genbank: AIO11154). The capsids of AACV (YP_009011225) and CBPV (YP_001911137A) were used as outgroups. Each isolate is indicated by its accession number and the clades are designated by genus names. Branch support for each node is designated by aLRT (approximate Likelihood-Ratio Test) values (>70%).*

PART IV

DISCUSSION

CHAPTER 10

GENERAL DISCUSSION

1. The relation between parasites and colony losses

Unusually high colony collapses were reported almost a decade ago. Several pathogens like DWV, *N. ceranae* and *V. destructor* have been suggested as main culprits (Higes et al., 2008; Highfield et al., 2009; Rosenkranz et al., 2010). Nowadays, an emerging hypothesis is that synergistical interactions among various parasites can lead to these colony declines (Figure 1) (Cornman et al., 2012; Cox-Foster et al., 2007).

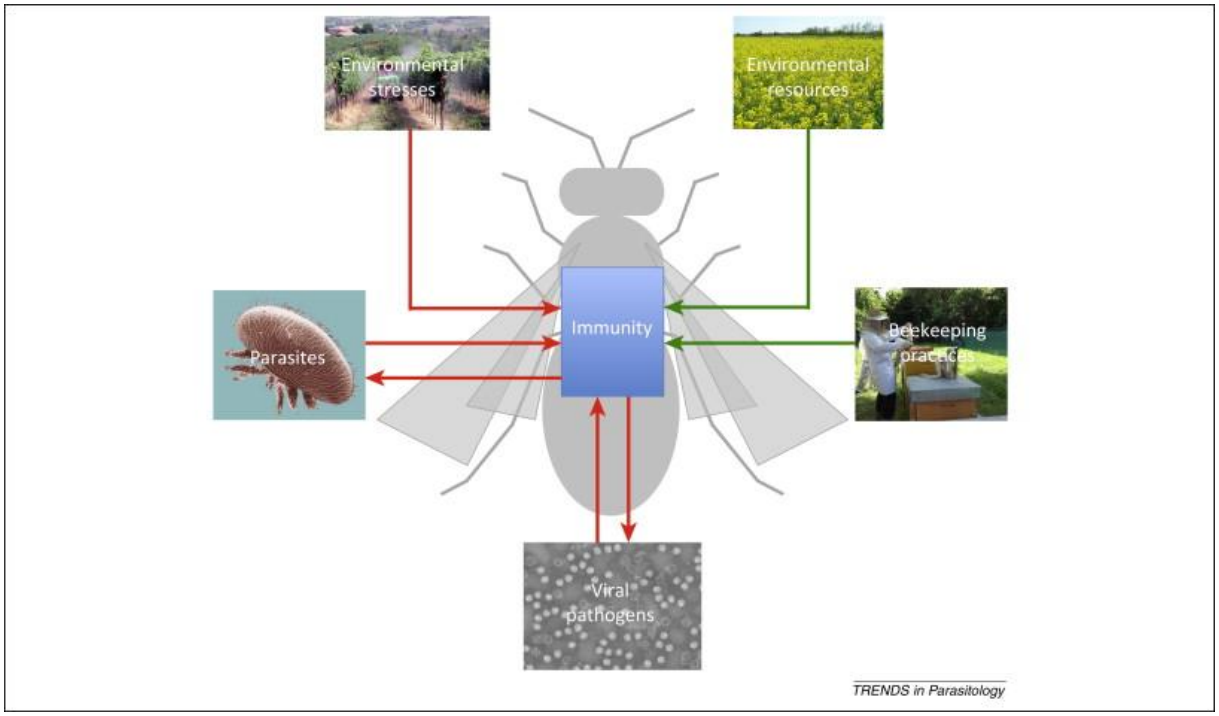


***Figure 1**: Multiple interactions between honeybees and environmental factors. Green and red arrows denote positive and negative interactions, respectively (Nazzi and Pennacchio, 2014).*

An extensive model is proposed by Francesco Nazzi and colleagues (Nazzi et al., 2012; Nazzi et al., 2014). This model proposes that *V. destructor* infestation causes an immune suppression in the honey bee, leading to very high DWV titers. Eventually this virus cannot be controlled by the bee,

therefore contributing to the bee mortality and colony collapse. An additional issue is the viral replication within the bee, but also within the mite (Ongus et al., 2004; Yue and Genersch, 2005). Moreover, other factors such as pesticides (Di Prisco et al., 2013) can also deplete the immune system of the honey bee.

2. The honey bee pathosphere

The first honey bee pathogens were published more than a century ago, e.g. *V. jacobsoni* in 1904 and *N. apis* in 1909. Smaller parasites like viruses were described several decades later. Although the first evidence for the viral nature of a paralysis causing agent was reported in 1945 (Burnside, 1945), conclusive evidence was only twenty years later published (Bailey et al., 1963). Later on, numerous honey bee viruses were characterised in a relative small time period. This was caused by the rise of techniques like electron microscopy and immune-diffusion tests. Today we see the same thing happening. Sequencing of PCR amplicons revealed the existence of *N. ceranae* (Fries et al., 1996) and cryptic species in the mite genera *Varroa* and *Tropilaelaps* (Anderson and Morgan, 2007; Anderson and Trueman, 2000). Next-generation sequencing, combined with RACE (Rapid amplification of cDNA ends) and qPCR, has further boosted our knowledge of honey bee pathology. Also several new honey bee viruses were discovered since the start of this PhD thesis (Runckel et al., 2011). As a result the honey bee patosphere, the total assembly of parasites, has been expanded and comprises more than 30 taxa (Figure 2).

Most of the viruses described in the 20th century were discovered due to the clinical symptoms that they cause in honey bees. One of the most famous examples is DWV, which cause shrivelled wings (see Part I, Chapter 1). Nevertheless, the clinical differences between several viruses can be subtle such as between ABPV and CBPV. The methodology of virus preparation from sick bees and subsequent propagation can cause contamination, but can also lead to a serendipitous discovery of novel pathogens e.g. TRSV (Li et al., 2014).

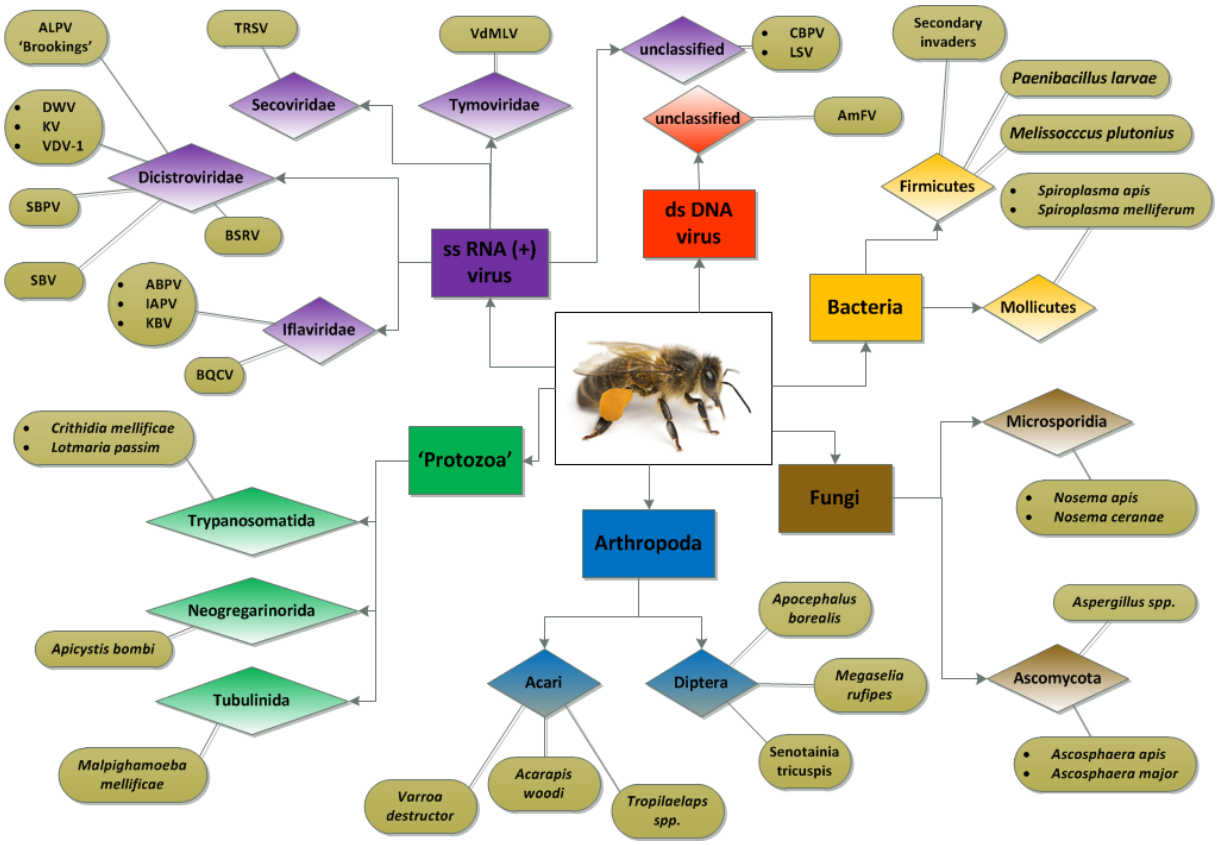


***Figure 1**: Overview of all known* Apis mellifera *pathogens, with their taxonomic ranking. The bee lice (*Braula coeca*), the small hive beetle (*Aethina tumida*) and wax moths (*Achroia grisella *and* Galleria mellonella*) are not included since they are no true endo- or ectoparasitoids. Some obscure viruses like Arkansas Bee Virus and Berkeley Bee Picorna-like Virus are not included since nucleotide information is lacking.*

Previous screenings of honey bee parasites were based on single PCR assays and included only small numbers of samples because of the cost price and labour intensity. This implicated that each pathogen was separately surveyed (Tentcheva et al., 2004), but multiplex PCR assays were later on developed (e.g. (Martin-Hernandez et al., 2007; Meeus et al., 2012; Sguazza et al., 2013)). Also microarray based detection methods were lately published (Glover et al., 2011; Runckel et al., 2011). However, the MLPA technology allows the screening of numerous viruses in one PCR reaction, without the need for a fluorescent microarray scanner or the possibility of non-specific PCR products from a multiplex PCR reaction. This technology was already used for the detection of respiratory viruses (Berning et al., 2014; Reijans et al., 2008) and sexually transmitted pathogens (Muvunyi et al.,

2011). We could adapt this technology for the detection of 10 bee viruses, which resulted into the development of the BeeDoctor tool (Part II, Chapter 3). The BeeDoctor enables to survey numerous honey bee pathogens in one sample and thus a fast and comprehensive screening without high expenses.

The pathosphere of Belgian honey bees was poorly studied during the last decade. Only few studies reported the common viruses ABPV, BQCV, CBPV, DWV and SBV (de Graaf et al., 2008; Nguyen et al., 2011), but they took only a limited number of colonies into account. Several other pathogens were known to be present, but their prevalence remained uncertain, with exception of *P. larvae* (de Graaf et al., 2001).

To clarify this situation, a survey of a large number of pathogens in more than 300 honey bee samples was conducted (Figure 3; Part II, Chapters 3 en 4). Quite a few of them were reported for the first time from Belgium: *A. bombi*, *A. borealis*, *C. mellificae*, LSV and VdMLV. Some of these neglected pathogens appeared to have an abundant prevalence but were previously only occasionally detected in other countries.

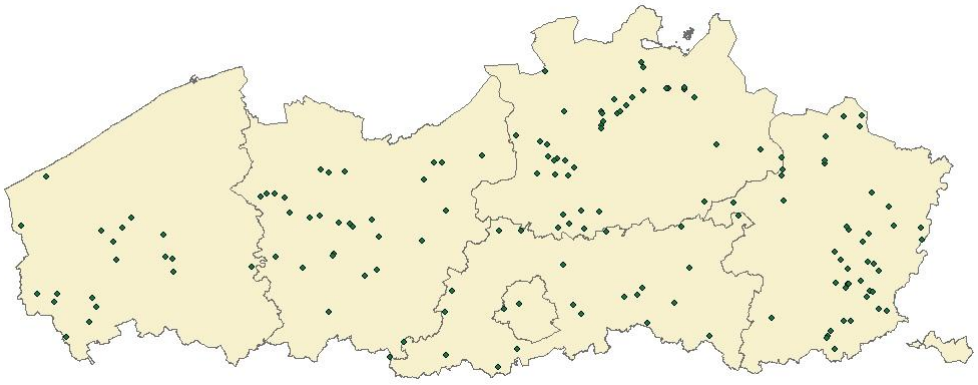


Figure 3*: Overview of the screened apiaries in the Flemish part of Belgium*

While this study comprised one of the early detections of the viruses LSV and VdMLV. The former was first reported from the USA (Runckel et al., 2011). Although two strains were originally described, later on it became clear that other strains were present in the USA (Cornman et al., 2012) and Spain (Granberg et al., 2013). In this PhD thesis we were able to design a PCR assay that amplifies

all known LSV variants, based on their sequence data. Subsequent to the analysis of the Belgian LSVs, one strain was detected in different honey bee samples which we designated LSV strain 4 (Genbank: JX878492). Other sequence variants were detected as well (Part II, Chapter 4), which drew our attention to these puzzling complex of viral genotypes. The almost complete genomes of four strains were obtained by combining amplification of large fragments and subsequent primer walking (Part III, Chapter 9). Next-generation sequencing can possibly reveal other complete LSV genomes, although this is not always successful (Granberg et al., 2013). The LSV virus was also detected in *V. destructor* mites.

Honey bee pathogens can be transmitted via several routes (Figure 4), which are rather well known (Chen et al., 2006). This includes both horizontal transmission and vertical transmission. The former concerns all pathogens, but the latter is only applicable to viruses (and possibly honey bee spiroplasmas).

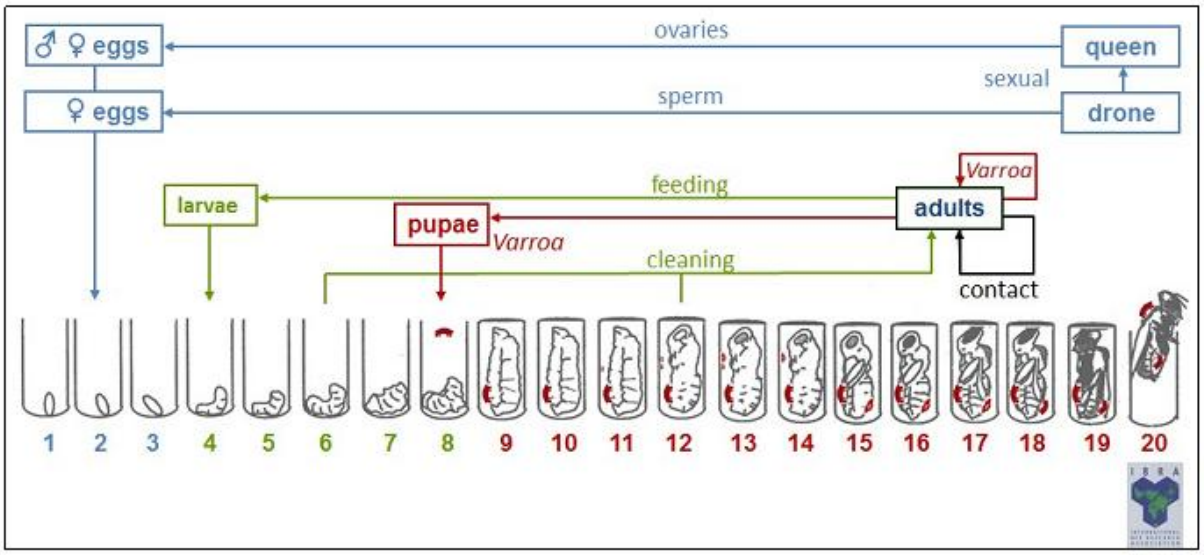


***Figure 4**: Illustration of the different transmission routes for honey bee pathogens (de Miranda et al., 2013).*

Many honey bee viruses were already detected in eggs, semen, spermatheca and/or ovaries. Nevertheless, possible vertical transmission was not yet investigated for the recently described viruses. In this PhD work, we proved that ALPV strain Brookings, LSV and VdMLV can infect eggs (Part

II, Chapter 6) and thus are also vertically transmitted. Moreover, we detected the negative-strand of SBV within the eggs. Since they were not surface-sterilised, it is also possible that this originates from transovum transmission, such as surface contamination with sperm containing negative-strand RNAs. It can be expected that a replicating virus in honey bee eggs can have consequences for the development into a queen, resulting in a clinical relevance for queen breeding. A broad sanitary screening of the breeder queens seems advisory. The colonies of these queens are screened for a limited number of pathogens, like American foulbrood, but only in case of import or export of these colonies. Horizontal transmission is mainly mite-vectored or between honey bees mutually via feeding, cleaning etc. The vectoring via mites is demonstrated for several viruses (Bowen-Walker et al., 1999; Celle et al., 2008; Di Prisco et al., 2011; Ongus et al., 2004) and we could extend this with LSV (Part III, Chapter 9). The impact of viruses can be seriously affected by a shift in transmission. Before the introduction of *V. destructor* in European honey bee colonies was DWV a rather harmless honey bee virus. The ability to replicate within the mite (Ongus et al., 2004) causes high viral concentrations which can be lethal for honey bees. Moreover, this led to a tremendous decrease in the DWV strain diversity and increase in DWV prevalence (Martin et al., 2012), and ultimately a virulent strain has prevailed (Ryabov et al., 2014).

Pollen pollets can contain multiple viruses (Singh et al., 2010) which are infectious towards honey bees and other pollinators. Therefore we wondered if other pollinators are also involved in virus transmission, a very dynamic process. Few other studies demonstrated the presence of honey bee pathogens in other insects (Celle et al., 2008; Evison et al., 2012; Furst et al., 2014; Singh et al., 2010). Even replication (Levitt et al., 2013) and clinical symptoms (Genersch et al., 2006) were already demonstrated in other bees. Although commercial bumble bee colonies seem to be an important sink of pathogen spillover towards wild bees (Graystock et al., 2013; Murray et al., 2013; Otterstatter and Thomson, 2008), honey bees are also considered as a likely source (Furst et al., 2014). However, only a limited number of parasites have been detected in wild bees. In a case study we sampled five solitary bee species around an apiary and screened them for many parasites (Part II,

Chapter 5). As a result, we could detect numerous pathogens including AmFV, LSV and VdMLV which were previously restricted to honey bees. Equally surprising was the finding of the protist *C. bombi*, thought to be restricted to bumble bees.

This PhD study (Part II, Chapter 5) and some recent studies (Furst et al., 2014; Levitt et al., 2013; McMahon et al., 2015) revealed that many honey bee parasites are not restricted to honey bees, but infect also other pollinators. This generalism can have important effects on the virulence and pathogenicity in the different hosts (Woolhouse et al., 2001). Transmission of bee parasites can occur via mites or beetles (Eyer et al., 2009; Forsgren et al., 2009; Ongus et al., 2004), but also via flowers (Durrer and Schmidhempel, 1994) as demonstrated for a bumble bee protist.

Honey bees can be infected by only a few protists such as amoebas, neogregarines and trypanosomatids. Some of them were lately detected in several countries (Morimoto et al., 2013; Runckel et al., 2011; Yang et al., 2013). Contrarily to neogregarines (*A. bombi)*, trypanosomatids became a popular subject. Molecular detection methods were reported, which soon led to sequence data of multiple loci like the 18S rRNA, cytochrome *b* and gGAPDH (Runckel et al., 2011; Schmid-Hempel and Tognazzo, 2010). The correlation of *C. mellificae* with winter losses and the synergistic effect of *N. ceranae* in Belgium (Part II, Chapter 4) boosted further research. When new cell cultures were established, it became evident that a second trypanosomatid species, namely *L. passim*, can infect honey bees (Part III, Chapter 7). Moreover we could develop an easy detection method between these two trypanosomatid species, based on a fragment length polymorphism of the ITS 1 locus (Part III, Chapter 8). This is more reliable than sequencing of other loci, especially in case of co-infection.

3. Critical note on the apicultural industry

The high pathogen load found in honey bees (Part II) forces us to reflect the current apicultural industry. Several parasites have been correlated with the recent colony collapses (Higes et al., 2008;

Highfield et al., 2009) and even the number of parasites can be correlated (Part II, Chapter 4). This should urge the apicultural industry to alter some beekeeping practices, like breeding and mobility.

Currently, honey bee breeding is focussed on characteristics like honey yield, low aggression and low swarming activity. Moreover, the kept honey bee races in Belgium (and several other countries) are no endemic subspecies but were imported. The mobility of honey bee colonies is also reflected in the transportation of hives for pollination services. These extensive trades in bees poses the main risk for disease dissemination around the world (Gordon et al., 2014; Mutinelli, 2011). There are plenty of examples that demonstrate this, such as the global spread of *Varroa destructor* (reviewed by Rosenkranz and colleagues (Rosenkranz et al., 2010)) and the recent introduction of the small hive beetle (*Aethina tumida*) (Palmeri et al., 2014) in Italy. Once established the further spread of a disease can occur extremely fast: for instance, the spread of the *Varroa*-mite reached a maximum progression 12 km/year in New Zealand (Stevenson et al., 2005) and 40 km/year in Madagascar (Rasolofoarivao et al., 2013). A similar pattern of spread was seen when fluvalinate-resistant mites were spread throughout Europe (Martin, 2004). One can assume that new pathogens, brought to us by international trade of bees, can become omnipresent in no time.

The restrictions on the import of honey bee colonies from outside the European Union is regulated by the European Directive 92/65/EEC and amended by the Commission Decision 2010/270/EU. Nevertheless, the obliges health certificate for intra-Union trade concerns only American foulbrood and two exotic parasites (*Aethina tumida* and *Tropilaelaps* spp.), which is far too less than needed. This certificate should be extended with qualitative analyses of ABPV, DWV and *N. ceranae*, which can cause colony collapses when high titers are present (Berthoud et al., 2010; Dainat et al., 2012; Higes et al., 2008; Nguyen et al., 2011).

The apicultural industry should also shift its focus more on the honey bee itself. The breeding of honey bees is nowadays focused on the manageability and the production. This is part of the problem concerning the intolerance towards *Varroa* infestation and parasite

infections in general. The tolerance of *Apis cerana* towards *Varroa* mites consists of many aspects, such as hygienic behaviour, grooming and disturbance of the mite population dynamics (Rosenkranz et al., 2010). Natural selection of *Apis mellifera* has been reported from few isolated locations in Europe (Fries et al., 2006; Le Conte et al., 2007). Besides, selective cross-breeding of different subspecies resulted in *Varroa* tolerant honey bee strains like Russian (Primorsky) honey bees. Also the aggressive Africanized honey bees, a cross between African and European *A. mellifera* subspecies, appears to be less infested by mites and viruses (Hamiduzzaman et al., 2014; Medina-Flores et al., 2014; Yanez et al., 2014). Recently, the molecular mechanisms of this honey bee defence behaviour were partly elucidated (Behrens et al., 2011; Haddad et al., 2015; Spotter et al., 2012). This can result in the selective breeding of honey bee subspecies that are tolerant to viruses and mites, without focussing on the manageability and the production.

The individual beekeeper can also improve some of its beekeeping management by limiting the stress on the honey bee colonies. A stressed colony is more susceptible to diseases, resulting in a higher chance to collapse. This stress can result from pesticides (both from agricultural use and beekeeping practices), food deficiency and beekeeping practices like queen supersedure, colony dividing and honey harvesting.

4. Future perspectives

Although some parasites were neglected for a long time, the spotlight has fallen on a few of them. Since the start of this PhD work, our knowledge about honey bee trypanosomatids and Lake Sinai Virus has been greatly expanded. Nevertheless, other pathogens remain obscure. For instance, we know little about the gregarines and amoebas that infect honey bees. Despite numerous next-generation sequencing projects of honey bees there is still no molecular data available for the amoeba *Malpighamoeba mellificae*. Recent studies proposed that more than one neogregarine and

tracheal mite can infect honey bees (Cepero et al., 2014a; Cepero et al., 2014b). In addition, the number of honey bee viruses had been expanded since the start of this PhD work. This includes 'true' bee viruses like ALPV, but also plant-pathogenic viruses such as TRSV. Some other plant viruses (e.g. Turnip Ringspot Virus) were also discovered in honey bees (Cornman et al., 2012; Granberg et al., 2013), but their pathogenicity towards honey bees has not been demonstrated. Nevertheless, the pathogenicity of TRSV was also not expected. Concluding, several new honey bee pathogens are described lately with advanced molecular techniques so others will probably follow in the next few years.

5. References

Anderson, D.L., Morgan, M.J., 2007. Genetic and morphological variation of bee-parasitic Tropilaelaps mites (Acari : Laelapidae): new and re-defined species. Exp Appl Acarol 43**,** 1-24.

Anderson, D.L., Trueman, J.W.H., 2000. Varroa jacobsoni (Acari: Varroidae) is more than one species. Exp Appl Acarol 24**,** 165-189.

Bailey, L., Woods, R.D., Gibbs, A.J., 1963. Two Viruses from adult honey bees (Apis mellifera Linnaeus). Virology 21**,** 390-&.

Behrens, D., Huang, Q., Gessner, C., Rosenkranz, P., Frey, E. and others, 2011. Three QTL in the honey bee Apis mellifera L. suppress reproduction of the parasitic mite Varroa destructor. Ecol Evol 1**,** 451-458.

Berning, L., Aberle, S.W., Simon, B., Luger, C., Apfalter, P. and others, 2014. Evaluation of a multiplex ligation-dependent probe amplification assay for the detection of respiratory pathogens in oncological patients. J Clin Virol 60**,** 141-146.

Berthoud, H., Imdorf, A., Haueter, M., Radloff, S., Neumann, P., 2010. Virus infections and winter losses of honey bee colonies (Apis mellifera). J Apicult Res 49**,** 60-65.

Bowen-Walker, P.L., Martin, S.J., Gunn, A., 1999. The transmission of deformed wing virus between honeybees (Apis mellifera L.) by the ectoparasitic mite Varroa jacobsoni Oud. J Invertebr Pathol 73**,** 101-106.

Burnside, C.E., 1945. The cause of paralysis of honeybees. Am Bee J 354-355.

Celle, O., Blanchard, P., Olivier, V., Schurr, F., Cougoule, N. and others, 2008. Detection of Chronic bee paralysis virus (CBPV) genome and its replicative RNA form in various hosts and possible ways of spread. Virus Res 133**,** 280-284.

Cepero, A., Martin-Hernandez, R., Prieto, L., Gomez-Moracho, T., Martinez-Salvador, A. and others, 2014a. Is Acarapis woodi a single species? A new PCR protocol to evaluate its prevalence. Parasitol Res.

Cepero, A., Ravoet, J., Gomez-Moracho, T., Bernal, J.L., del Nozal, M.J. and others, 2014b. Holistic screening of collapsing honey bee colonies in Spain: a case study. BMC Research Notes 7**,** 649.

Chen, Y.P., Evans, J.D., Feldlaufer, M., 2006. Horizontal and vertical transmission of viruses in the honeybee, Apis mellifera. J Invertebr Pathol 92**,** 152-159.

Cornman, R.S., Tarpy, D.R., Chen, Y., Jeffreys, L., Lopez, D. and others, 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7**,** e43562.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D. and others, 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318**,** 283-287.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012. Predictive Markers of Honey Bee Colony Collapse. PLoS One 7**,** e32151.

de Graaf, D.C., Brunain, M., Imberechts, H., Jacobs, F., 2008. First molecular confimation of deformed wing virus infections of honeybees from a Belgian apiary reveals the presence of black queen cell virus and Varroa destructor virus 1. Vlaams Diergen Tijds 78**,** 101-105.

de Graaf, D.C., Vandekerchove, D., Dobbelaere, W., Peeters, J.E., Jacobs, F.J., 2001. Influence of the proximity of American foulbrood cases and apicultural management on the prevalence of Paenibacillus larvae spores in Belgian honey. Apidologie 32**,** 587-599.

de Miranda, J.R., Bailey, L., Ball, B.V., Blanchard, P., Budge, G.E. and others, 2013. Standard methods for virus research in Apis mellifera. J Apicult Res 52.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E. and others, 2013. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. Proc Natl Acad Sci USA 110**,** 18466-18471.

Di Prisco, G., Pennacchio, F., Caprio, E., Boncristiani, H.F., Evans, J.D., Chen, Y.P., 2011. Varroa destructor is an effective vector of Israeli acute paralysis virus in the honeybee, Apis mellifera. J Gen Virol 92**,** 151-155.

Durrer, S., Schmidhempel, P., 1994. Shared use of flowers leads to horizontal pathogen transmission. P Roy Soc B-Biol Sci 258**,** 299-302.

Evison, S.E., Roberts, K.E., Laurenson, L., Pietravalle, S., Hui, J. and others, 2012. Pervasiveness of parasites in pollinators. PLoS One 7**,** e30641.

Eyer, M., Chen, Y.P., Schafer, M.O., Pettis, J., Neumann, P., 2009. Small hive beetle, Aethina tumida, as a potential biological vector of honeybee viruses. Apidologie 40**,** 419-428.

Forsgren, E., de Miranda, J.R., Isaksson, M., Wei, S., Fries, I., 2009. Deformed wing virus associated with Tropilaelaps mercedesae infesting European honey bees (Apis mellifera). Exp Appl Acarol 47**,** 87-97.

Fries, I., Feng, F., daSilva, A., Slemenda, S.B., Pieniazek, N.J., 1996. Nosema ceranae n sp (Microspora, Nosematidae), morphological and molecular characterization of a microsporidian parasite of the Asian honey bee Apis cerana (Hymenoptera, Apidae). Eur J Protistol 32**,** 356-365.

Fries, I., Imdorf, A., Rosenkranz, P., 2006. Survival of mite infested (Varroa destructor) honey bee (Apis mellifera) colonies in a Nordic climate. Apidologie 37**,** 564-570.

Furst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J., Brown, M.J.F., 2014. Disease associations between honeybees and bumblebees as a threat to wild pollinators. Nature 506**,** 364-366.

Genersch, E., Yue, C., Fries, I., de Miranda, J.R., 2006. Detection of Deformed wing virus, a honey bee viral pathogen, in bumble bees (Bombus terrestris and Bombus pascuorum) with wing deformities. J Invertebr Pathol 91**,** 61-63.

Glover, R.H., Adams, I.P., Budge, G., Wilkins, S., Boonham, N., 2011. Detection of honey bee (Apis mellifera) viruses with an oligonucleotide microarray. J Invertebr Pathol 107**,** 216-219.

Gordon, R., Bresolin-Schott, N., East, I.J., 2014. Nomadic beekeeper movements create the potential for widespread disease in the honeybee industry. Aust Vet J 92**,** 283-290.

Granberg, F., Vicente-Rubiano, M., Rubio-Guerri, C., Karlsson, O.E., Kukielka, D. and others, 2013. Metagenomic detection of viral pathogens in spanish honeybees: co-infection by aphid lethal paralysis, Israel acute paralysis and lake sinai viruses. PLoS One 8**,** e57459.

Graystock, P., Yates, K., Evison, S.E., Darvill, B., Goulson, D., Hughes, W.O.H., 2013. The Trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. J Appl Ecol 50**,** 1207-1215.

Haddad, N.J., Batainh, A.M., Migdadi, O.S., Saini, D., Krishnamurthy, V. and others, 2015. Next generation sequencing of Apis mellifera syriaca identifies genes for Varroa resistance and beneficial bee keeping traits. Insect Sci.

Hamiduzzaman, M.M., Guzman-Novoa, E., Goodwin, P.H., Reyes-Quintana, M., Koleoglu, G. and others, 2014. Differential responses of Africanized and European honey bees (Apis mellifera) to viral replication following mechanical transmission or Varroa destructor parasitism. J Invertebr Pathol.

Higes, M., Martin-Hernandez, R., Botias, C., Bailon, E.G., Gonzalez-Porto, A.V. and others, 2008. How natural infection by Nosema ceranae causes honeybee colony collapse. Environ Microbiol 10**,** 2659-2669.

Highfield, A.C., El, N.A., Mackinder, L.C., Noel, L.M., Hall, M.J. and others, 2009. Deformed wing virus implicated in overwintering honeybee colony losses. Appl Environ Microb 75**,** 7212-7220.

Le Conte, Y., de Vaublanc, G., Crauser, D., Jeanne, F., Rousselle, J.C., Becard, J.M., 2007. Honey bee colonies that have survived Varroa destructor. Apidologie 38**,** 566-572.

Levitt, A.L., Singh, R., Cox-Foster, D.L., Rajotte, E., Hoover, K. and others, 2013. Cross-species transmission of honey bee viruses in associated arthropods. Virus Res 176**,** 232-240.

Li, J.L., Cornman, R.S., Evans, J.D., Pettis, J.S., Zhao, Y. and others, 2014. Systemic spread and propagation of a plant-pathogenic virus in European honeybees, Apis mellifera. MBio 5**,** e00898-13.

Martin, S.J., 2004. Acarcide (pyrethroid) resistance in Varroa destructor. Bee World 85**,** 67-69.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E. and others, 2012. Global honey bee viral landscape altered by a parasitic mite. Science 336**,** 1304-1306.

Martin-Hernandez, R., Meana, A., Prieto, L., Salvador, A.M., Garrido-Bailon, E., Higes, M., 2007. Outcome of colonization of Apis mellifera by Nosema ceranae. Appl Environ Microb 73**,** 6331-6338.

McMahon, D.P., Furst, M.A., Caspar, J., Theodorou, P., Brown, M.J., Paxton, R.J., 2015. A sting in the spit: widespread cross-infection of multiple RNA viruses across wild and managed bees. J Anim Ecol.

Medina-Flores, C.A., Guzman-Novoa, E., Hamiduzzaman, M.M., Arechiga-Flores, C.F., Lopez-Carlos, M.A., 2014. Africanized honey bees (Apis mellifera) have low infestation levels of the mite Varroa destructor in different ecological regions in Mexico. Genet Mol Res 13**,** 7282-7293.

Meeus, I., Vercruysse, V., Smagghe, G., 2012. Molecular detection of Spiroplasma apis and Spiroplasma melliferum in bees. J Invertebr Pathol 109**,** 172-174.

Morimoto, T., Kojima, Y., Yoshiyama, M., Kimura, K., Yang, B. and others, 2013. Molecular detection of protozoan parasites infecting Apis mellifera colonies in Japan. Environ Microbiol Rep 5**,** 74-77.

Murray, T.E., Coffey, M.F., Kehoe, E., Horgan, F.G., 2013. Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. Biol Conserv 159**,** 269-276.

Mutinelli, F., 2011. The spread of pathogens through trade in honey bees and their products (including queen bees and semen): overview and recent developments. Revue Scientifique et Technique-Office International des Epizooties 30**,** 257-271.

Muvunyi, C.M., Dhont, N., Verhelst, R., Crucitti, T., Reijans, M. and others, 2011. Evaluation of a new multiplex polymerase chain reaction assay STDFinder for the simultaneous detection of 7 sexually transmitted disease pathogens. Diagn Microbiol Infect Dis 71**,** 29-37.

Nazzi, F., Brown, S.P., Annoscia, D., Del Piccolo, F., Di Prisco, G. and others, 2012. Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. PLoS Pathog 8**,** e1002735.

Nazzi, F., Pennacchio, F., 2014. Disentangling multiple interactions in the hive ecosystem. Trends in Parasitology 30**,** 556-561.

Nguyen, B.K., Ribiere, M., Vanengelsdorp, D., Snoeck, C., Saegerman, C. and others, 2011. Effects of honey bee virus prevalence, Varroa destructor load and queen condition on honey bee colony survival over the winter in Belgium. J Apicult Res 50**,** 195-202.

Ongus, J.R., Peters, D., Bonmatin, J.M., Bengsch, E., Vlak, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. J Gen Virol 85**,** 3747-3755.

Otterstatter, M.C., Thomson, J.D., 2008. Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? PLoS One 3**,** e2771.

Palmeri, V., Scirto, G., Malacrino, A., Laudani, F., Campolo, O., 2014. A scientific note on a new pest for European honeybees: first report of small hive beetle Aethina tumida, (Coleoptera: Nitidulidae) in Italy. Apidologie 1-3.

Rasolofoarivao, H., Clemencet, J., Ravaomanarivo, L.H., Razafindrazaka, D., Reynaud, B., Delatte, H., 2013. Spread and strain determination of Varroa destructor (Acari: Varroidae) in Madagascar since its first report in 2010. Exp Appl Acarol 60**,** 521-530.

Reijans, M., Dingemans, G., Klaassen, C.H., Meis, J.F., Keijdener, J. and others, 2008. RespiFinder: a new multiparameter test to differentially identify fifteen respiratory viruses. J Clin Microbiol 46**,** 1232-1240.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. J Invertebr Pathol 103 Suppl 1**,** S96-119.

Runckel, C., Flenniken, M.L., Engel, J.C., Ruby, J.G., Ganem, D. and others, 2011. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, Nosema, and Crithidia. PLoS One 6**,** e20656.

Ryabov, E.V., Wood, G.R., Fannon, J.M., Moore, J.D., Bull, J.C. and others, 2014. A virulent strain of deformed wing virus (DWV) of honeybees (Apis mellifera) prevails after Varroa destructor-mediated, or in vitro, transmission. PLoS Pathog 10**,** e1004230.

Schmid-Hempel, R., Tognazzo, M., 2010. Molecular divergence defines two distinct lineages of Crithidia bombi (Trypanosomatidae), parasites of bumblebees. J Eukaryot Microbiol 57**,** 337-345.

Sguazza, G.H., Reynaldi, F.J., Galosi, C.M., Pecoraro, M.R., 2013. Simultaneous detection of bee viruses by multiplex PCR. J Virol Methods 194**,** 102-106.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N. and others, 2010. RNA Viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-Apis hymenopteran species. PLoS One 5.

Spotter, A., Gupta, P., Nurnberg, G., Reinsch, N., Bienefeld, K., 2012. Development of a 44K SNP assay focussing on the analysis of a varroa-specific defence behaviour in honey bees (Apis mellifera carnica). Mol Ecol Resour 12**,** 323-332.

Stevenson, M.A., Benard, H., Bolger, P., Morris, R.S., 2005. Spatial epidemiology of the Asian honey bee mite (Varroa destructor) in the North Island of New Zealand. Preventive Veterinary Medicine 71**,** 241-252.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F. and others, 2004. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl Environ Microb 70**,** 7185-7191.

Woolhouse, M.E., Taylor, L.H., Haydon, D.T., 2001. Population biology of multihost pathogens. Science 292**,** 1109-1112.

Yanez, O., Tejada, G., Neumann, P., 2014. First detection of viruses in africanized honey bees from Peru. Virol Sin 29**,** 321-323.

Yang, B., Peng, G.D., Li, T.B., Kadowaki, T., 2013. Molecular and phylogenetic characterization of honey bee viruses, Nosema microsporidia, protozoan parasites, and parasitic mites in China. Ecol Evol 3**,** 298-311.

Yue, C., Genersch, E., 2005. RT-PCR analysis of Deformed wing virus in honeybees (Apis mellifera) and mites (Varroa destructor). J Gen Virol 86**,** 3419-3424.

CURRICULUM VITAE

PERSONAL DETAILS

Name:	Ravoet Jorgen
Date of birth:	23 October 1985
Nationality:	Belgian
E-mail:	jorgen.ravoet@gmail.com

EDUCATION

PhD candidate, Biochemistry and Biotechnology, Ghent University (Dehousse grant)

- Dissertation topic: Exploring the involvement of honey bee pathogens in colony collapses from epidemiological data, pathogen genotyping and host immune responses.
- Advisor: Prof. Dirk C. de Graaf

Master of Science in Biology, Vrije Universiteit Brussel, cum laude (2010)

- Dissertation topic: Geoptimaliseerde aanmaak van nanobody-conjugaten door inbrengen van een enkele cysteïne aan het oppervlak in nanobodies, Laboratory of Cellular and Molecular Immunology.
- Advisor: Prof. Serge Muyldermans

Preparatory Programme Master of Science in Biology, Vrije Universiteit Brussel (2008)

- Dissertation topic: Effect van *Bacillus thuringiensis* op *Bombus terrestris*. Laboratory of Cellular Genetics
- Advisor: Prof. Guy Smagghe

Bachelor in Biomedical Laboratory Technology, option in Pharmaceutical and Biological Laboratory Techniques, Katholieke Hogeschool Leuven (2007)

- Onderzoek naar leucinerijke G-proteïne gekoppelde receptoren bij Drosophila melanogaster, Molecular Developmental Physiology and Signal Transduction.

SCIENTIFIC OUTPUT

A1 peer reviewed publications

1: Ravoet J, De Smet L, Wenseleers T, de Graaf DC. Vertical transmission of honey bee viruses in a Belgian queen breeding program. BMC Veterinary Research, 2015, In Press.

2: Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, de Graaf DC, Evans JD. Characterization of Two Species of Trypanosomatidae from the Honey Bee Apis mellifera: Crithidia mellificae Langridge and McGhee, 1967 and Lotmaria passim n. gen., n. sp. J Eukaryot Microbiol. 2015, In Press. doi: 10.1111/jeu.12209.

3: Ravoet J, De Smet L, Wenseleers T, de Graaf DC. Genome sequence heterogeneity of Lake Sinai Virus found in honey bees and Orf1/RdRP-based polymorphisms in a single host. Virus Res. 2015, In Press. doi: 10.1016/j.virusres.2015.02.019.

4: Ravoet J, De Smet L, Meeus I, Smagghe G, Wenseleers T, de Graaf DC. Widespread

occurrence of honey bee pathogens in solitary bees. J Invertebr Pathol. 2014;122:55-8. doi: 10.1016/j.jip.2014.08.007.

5: Cepero A, Ravoet J, Gómez-Moracho T, Bernal JL, Del Nozal MJ, Bartolomé C, Maside X, Meana A, González-Porto AV, de Graaf DC, Martín-Hernández R, Higes M. Holistic screening of collapsing honey bee colonies in Spain: a case study. BMC Res Notes. 2014;7:649. doi: 10.1186/1756-0500-7-649.

6: Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC. Comprehensive bee pathogen screening in Belgium reveals Crithidia mellificae as a new contributory factor to winter mortality. PLoS One. 2013 ;8(8):e72443. doi: 10.1371/journal.pone.0072443.

7: De Smet L, Ravoet J, de Miranda JR, Wenseleers T, Mueller MY, Moritz RF, de Graaf DC. BeeDoctor, a versatile MLPA-based diagnostic tool for screening bee viruses. PLoS One. 2012;7(10):e47953. doi: 10.1371/journal.pone.0047953.

Participation at international conferences

Ravoet J., De Smet L., Verleyen P., Wenseleers T.H., de Graaf D.C., The prevalence of honeybee pathogens in the Flemish part of Belgium screened using a MLPA method. Eurbee 5 2012, Halle an der Saale, Germany (poster).

Ravoet J., Reybroeck W., Wenseleers T.H., de Graaf D.C., Beeswax residue analysis points to a repugnant historical contamination: a Flemish case study. 60. AG-Tagung der Institute für Bienenforschung e.V. 2013, Würzburg, Germany (poster).

Ravoet J., Maharramov J., Meeus I., De Smet L., Wenseleers T., Smagghe G., de Graaf D.C., Honey bee health screening reveals a broad set of pathogens, from which *Crithidia mellificae* correlated with winter losses. Eurbee 6 2014, Murcia, Spain (oral presentation).

GUIDANCE OF STUDENTS

Practical courses

- Integrated practical exercises 1st Bachelor in Medicine and Dentistry: Aspects of peripheral blood research, Prof. Dr. Frans Jacobs
- Practical exercises Biomedical physiology (2nd Bachelor in Biochemistry and Biotechnology), Prof. Dr. Dirk de Graaf

Bachelor projects

- Dario Priem, Dorien De Vlieger, Jasmien Vercruysse (2011-2012) Detectie van virussen en bacteriën in de honingbij en Varroa mijt, 3de bachelor in Biochemistry and Biotechnology, Ghent University.
- Dieter Hofman, Sam Debrouwer, Linde Van Landuyt (2012-2013) Temporal Distribution of Bee Pathogens in Honey Bee Colonies, 3de bachelor in Biochemistry and Biotechnology, Ghent University.
- Sara Phlypo, Eline Soetens , Sander Stevens (2013-2014) Validatie van lateral flow device in de diagnose van virusziekten bij de honingbij, 3de bachelor in Biochemistry and Biotechnology, Ghent University.

Master projects

- Emmelien Vancaester (2013-2014) Moleculair onderzoek rond trypanosomatidae in de honingbij, 1ste Master in Biochemistry and Biotechnology, Ghent University.

Master thesis

- Tine Descamp (2012-2013) Ontluikende bijenpathogenen: verspreiding en invloed op het immuunantwoord van de honingbij, 2nd master in Biochemistry and Biotechnology, Ghent University.