Comprehensive Bee Pathogen Screening in Belgium Reveals *Crithidia mellificae* as a New Contributory Factor to Winter Mortality

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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 occurrence 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.


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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 [1].

However, over the last decade unusually high honey bee colony losses have been reported, mainly in North-America [2] and Europe [3]. 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 [2,4,5].

The ectoparasitic mite *Varroa destructor* is almost certainly a key player in causing the observed elevated colony losses [6–11]. 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 [12]. The mite weakens the bees by sucking hemolymph from both adult bees and pupae [13]. In addition, they can transmit many of the known honey bee viruses [14–18] and cause a reactivation of covert virus infections due to host immune suppression [19]. The mite destabilizes the within-host dynamics of viruses due to this immune suppression, which can then reach lethal levels [20]. Further, *V. destructor* and Deformed Wing Virus (DWV) will reduce the life span of winter bees, which can cause a colony collapse [21].

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 [22]. IAPV was initially identified as a predictive marker for colony losses in the USA [23]. An expanded study could not confirm this result [5]. Moreover, a retrospective study revealed that this virus was already present before the first colony collapse disorders were ever reported [24]. In Europe, ABPV was linked with colony losses in Belgium [25], Germany [10] and Switzerland [26]. Furthermore, DWV has been linked to winter mortality in both Switzerland [26] and Germany [10].

The role of the Microsporidian fungus *Nosema ceranae*, another parasite that originates from the Asian honey bee [27], in causing colony collapse is still controversial. Sudden colony collapses in Spain were attributed to *N. ceranae* infection [28,29], but these
observations could not be confirmed by later independent studies in and outside Spain [2,23,30–32]. Recently, a prospective study revealed the presence of the little reported pathogens *Crithidia melliteae*, *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 Virus (ALPV) strain Brookings, Big Sioux River Virus (BSRV), the USA. Furthermore, the novel viruses (Aphid Lethal Paralysis *C. mellificae* eighties [34,35]. Also descriptions of spiroplasmas in honey bees go back to the early [33]. Earlier reported pathogens *Crithidia melliteae* has been little studied since its first description in 1967 [36], even though the related *Crithidia bombi* is known to have serious effects on bumble bees, particularly under starvation conditions [37,38]. 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 [39]. As shortly afterwards several neglected and new honey bee pathogens were described in the USA [33], we decided to re-examine these samples in order to type them for several of these other known honeybee pathogens. Based on this, we here provide the first molecular evidence for the presence of parasitic phorid flies in honey bee samples in the Palaearctic region, and demonstrate the presence of two *Spiroplasma* spp., *Apicystis bombi*, ALPV strain Brookings, *C. melliteae*, different LSV strains and *Varroa destructor* Macula-like Virus (VdMLV) in Belgian honey bees. 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.

### Materials and Methods

**Honey Bee Sampling and Preparation**

For detailed description of the worker bee sampling procedure we refer to our previous paper [39]. 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 [40].

**MLPA Analysis**

BeeDoctor [39], 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 [33], 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 [39], starting from 1 µl RNA. All

### Table 1. Honey bee pathogen incidences.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Type</th>
<th>Prevalences</th>
<th>Associations</th>
<th>With winter losses*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Overall</td>
<td>Surviving colonies*</td>
<td>Collapsed colonies*</td>
</tr>
<tr>
<td>ALPV</td>
<td>Dictistroviridae</td>
<td>3.3% (12/363)</td>
<td>3.3% (4/122)</td>
<td>3.7% (4/107)</td>
</tr>
<tr>
<td>ABPV</td>
<td>Dictistroviridae</td>
<td>56.2% (204/363)</td>
<td>59.0% (72/122)</td>
<td>54.2% (58/107)</td>
</tr>
<tr>
<td><em>Apicystis bombi</em></td>
<td>Ophryocystidae</td>
<td>40.8% (148/363)</td>
<td>41.8% (51/122)</td>
<td>41.1% (44/107)</td>
</tr>
<tr>
<td><em>Apocephalus borealis</em></td>
<td>Phoridae</td>
<td>31.1% (118/363)</td>
<td>32.8% (40/122)</td>
<td>33.6% (36/107)</td>
</tr>
<tr>
<td>BQCV</td>
<td>Dictistroviridae</td>
<td>13.5% (49/363)</td>
<td>10.7% (13/122)</td>
<td>14.0% (15/107)</td>
</tr>
<tr>
<td>CBPV</td>
<td>Unclassified RNA virus</td>
<td>1.7% (6/363)</td>
<td>0.0% (0/122)</td>
<td>1.9% (2/107)</td>
</tr>
<tr>
<td><em>Crithidia melliteae</em></td>
<td>Trypanosomatidae</td>
<td>70.5% (256/363)</td>
<td>71.3% (87/122)</td>
<td>81.3% (87/107)</td>
</tr>
<tr>
<td>DWV</td>
<td>Ifflaviridae</td>
<td>69.4% (252/363)</td>
<td>61.5% (75/122)</td>
<td>67.3% (72/107)</td>
</tr>
<tr>
<td>LSV complex</td>
<td>Unclassified RNA virus</td>
<td>14.6% (43/363)</td>
<td>17.2% (21/122)</td>
<td>15.0% (16/107)</td>
</tr>
<tr>
<td><em>Nosema apis</em></td>
<td>Nosematidae</td>
<td>10.2% (37/363)</td>
<td>13.1% (16/122)</td>
<td>10.3% (11/107)</td>
</tr>
<tr>
<td><em>Nosema ceranae</em></td>
<td>Nosematidae</td>
<td>92.6% (336/363)</td>
<td>89.3% (109/122)</td>
<td>94.4% (101/107)</td>
</tr>
<tr>
<td><em>Nosema</em> spores</td>
<td>Nosematidae</td>
<td>75.2% (273/363)</td>
<td>71.3% (87/122)</td>
<td>72.9% (78/107)</td>
</tr>
<tr>
<td>SBV</td>
<td>Ifflaviridae</td>
<td>19.0% (69/363)</td>
<td>17.2% (21/122)</td>
<td>21.5% (23/107)</td>
</tr>
<tr>
<td><em>Spiroplasma apis</em></td>
<td>Spiroplasmataceae</td>
<td>0.3% (1/363)</td>
<td>0.0% (0/122)</td>
<td>0.0% (0/107)</td>
</tr>
<tr>
<td><em>Spiroplasma melliferum</em></td>
<td>Spiroplasmataceae</td>
<td>4.4% (16/363)</td>
<td>3.3% (4/122)</td>
<td>6.5% (7/107)</td>
</tr>
<tr>
<td><em>Varroa destructor</em></td>
<td>Varroidae</td>
<td>93.7% (313/334)</td>
<td>91.0% (111/122)</td>
<td>95.3% (102/107)</td>
</tr>
<tr>
<td>VdMLV</td>
<td>Tymoviridae</td>
<td>84.3% (306/363)</td>
<td>79.5% (97/122)</td>
<td>84.1% (90/107)</td>
</tr>
</tbody>
</table>

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.

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the MLPA reagents were obtained from MRC-Holland. The amplified MLPA products were analysed using 4% high resolution agarose gel electrophoresis.

**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 MgCl₂; 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 [39], 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 [41], based on the 16S

![](https://example.com/image.png)

**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.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Probit coefficients</th>
<th>Marginal odds ratios</th>
<th>LR $^2$</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Best model based on main effects only$^a$ (AIC 317.21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>−0.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Crithidia mellificae</em></td>
<td>0.37</td>
<td>1.3</td>
<td>3.57</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Varroa destructor</em></td>
<td>0.49</td>
<td>1.4</td>
<td>2.11</td>
<td>0.07</td>
</tr>
<tr>
<td>(b) Best model based on most important main effects and their 1st order interactions$^b$ (AIC 316.11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>−5.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Crithidia mellificae</em></td>
<td>5.09</td>
<td>1.3</td>
<td>6.16</td>
<td>0.01</td>
</tr>
<tr>
<td><em>Nosema ceranae</em></td>
<td>4.97</td>
<td>1.4</td>
<td>4.88</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Varroa destructor</em></td>
<td>0.49</td>
<td>1.4</td>
<td>2.10</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Crithidia mellificae</em> x <em>Nosema ceranae</em></td>
<td>−4.80</td>
<td>0.8</td>
<td>3.37</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$^a$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 [10,26], as well as their first order interaction effects.

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*.

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**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*.

doi:10.1371/journal.pone.0072443.g001
and Gel Extraction Kit (Thermo Scientific) and sequenced for use. Amplicons around 1 kb were extracted using the GeneJET Cloning and Sequencing 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: JQ440620) RNA polymerases and Orf1 genes were generated with Geneious 5.6.4.

Nosema Spore Counting
We determined the Nosema spore levels in the extracts from 10 bees using light microscopy and a haemocytometer according to Cantwell [44]. This extract was diluted when necessary.

Statistics
The multiple-kind lottery model of [45] 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 [46], 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 supersede. 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 [10,26]. 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-values were used, since pathogens a priori are expected to increase colony mortality. The predicitive power of our resulting models was assessed using function CVbinary in R package DAAG.

Results
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
of our samples. The 15 sequenced amplicons showed 100% identity and 6 sequences with only a single nucleotide substitution).

The little reported trypanosomatid *A. borealis* was found in 75.2% (273/363) of the samples, and ranged from 105 to 183 spores (mean = 148 ± 363) 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.

Table 2 and Figure 1). The significant interaction effect was due a markedly but stabilizes around 50% at higher numbers of 3 to 6 (from 5.9% to 52%), the predicted winter mortality goes up markedly but stabilizes around 50% at higher numbers of detected 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 = 316.12), and resulted in a significantly positive first order effect (p = 0.02) and a significantly negative second order effect (AIC = 316.93, p = 0.03). In addition, the use of a third order probit model further increased the accuracy of the fit to the data (AIC = 316.1, p = 0.001). These data had 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 = 316.12), 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.

The spiroplasmas *S. apis* and *S. melliferum* were found 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 [47], was present in 40.8% (148/363) of the samples. The 15 sequenced amplicons fully matched a partial 18S ribosomal RNA sequence (Genbank: JF808447).

### 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 [33]. 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 [48], 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: JX578492). 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.

### 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.
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 [7] (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 [36], China [49], France [50], Japan [51], Switzerland [52] and USA [33]. Besides, the related *C. bombi*, also reported from Asian honey bees [53], has serious effects on the survival of bumble bees under stress conditions [37]. 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 [54]. In addition, an association between both pathogens was reported in the USA [33]. Possibly, the controversial role of *N. ceranae* [23,29,55] 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 [5]. Collapsing colonies, induced by e.g. *V. destructor* and *C. mellificae*, are probably more vulnerable to a diverse set of parasites [48], which elucidate this correlation. Moreover, it appeared that several pathogens can act synergistically and eventually cause a collapse of the honey bee colony [48]. The outcome of these pathogen interactions can vary between regions [48], 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 [33,48] and Spain [56]. 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 [33] and Spanish honey bees [56]. Remarkable was its rather high incidence in the present study [56.2%; 204/363], akin to similar observations in different regions in the USA [33]. 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 [57]. 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 [34,35], including Asian honey bees [58]. They seem to be uncommon in honey bees, with a sudden incidence in the summer [33] 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 Palaeartic region. This phorid fly was recently described as a new honey bee pathogen which alters the host behaviour by hive abandonment, eventually causing death [59].

Besides viruses, bacteria and fungi, honey bees can also be parasitized by *Nosema*. Our study revealed a high prevalence of *Apis cerana* 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 [46]. However, real empirical data is missing to describe the pathology of *A. bombi*. After its detection in honey bees in Finland [47], *A. bombi* was also reported in honey bees in Japan [51] and Argentina [60].

The presence in Argentina is probably induced by spillover from invasive *Bombus terrestris* [61], an introduced pollinator outside the West Palaeartic area [62]. 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 [23,48].

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 trypanosomid 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. From our data it seems advisable to look at a broader range of pathogens in nationwide monitoring programs.

Supporting 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. (TIF)

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' phosphorlated (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.

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**References**


**Author Contributions**

Conceived and designed the experiments: JR LD TG DS CDG. Performed the experiments: JR JM IM. Analyzed the data: JR IM TW. Wrote the paper: JR IM LD TG WS DGCD.
50. Dainat B, Evans JD, Gauthier L, Neumann P (2012) Crithidia mellifcae is widespread in Europe and can be used as a predictive marker of honeybee colony losses. 5th European Conference of Apidology.


