Red River Apiarists' Association

The Bee Cause

Volume 10, Issue 8 November 2013

- Next general meeting is 7:30 Tuesday, November 12**th** at the **River Heights Community Centre, 1370 Grosvenor Ave., Winnipeg.**
- **(in room right off maindoor)**

Speaker: Gadget Night, and alternative ways to improve your beekeeping and use materials and things you might have and not think would help in your beekeeping practice.

Inside this issue:

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

August 26, 2013

The interesting insight in this article is the wider scope and sensitivity of recognition analysis to the biological impacts on bees over and above those of chemicals.

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.

Citation: Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, et al. (2013) Comprehensive Bee Pathogen Screening in Belgium Reveals *Crithidia mellificae* as a New Contributory Factor to Winter Mortality. PLoS ONE 8(8): e72443. doi:10.1371/journal.pone.0072443

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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.

However, over the last decade unusually high honey bee colony losses have been reported, mainly in North-America and Europe. 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.

The ectoparasitic mite *Varroa destructor* is almost certainly a key player in causing the observed elevated colony losses. This mite jumped from the Asian honey bee (*Apis cerana*) to the European honey bee (*Apis mel-*

2013 Executive

President: Charles Polcyn
Ph 204-284-7064
231 Buxton Road
Winnipeg,MB R3T 0H4
Email: charles_polcyn@ymail.com.

1st Vice President: John Badiuk
Ph .204-943-0166
128 Victoria Ave W
Winnipeg, MB R2C 1S5
Email:honeyb@mymts.net

2nd Vice President: Armand St Hilaire
Ph 204-427-2757
P0 Box 93
Roseau River, MB. R0A 1P0
Email: asthil@mymts.net

Secretary: Art Quanbury
Ph 204-489-6994
35 Cordova St.
Winnipeg, MB R3N 0Z9
Email: quanbury@shaw.ca

Treasurer: John Speer
Ph 204-222-3007
Box 16, Group 555, RR 5
Winnipeg, MB R2C 2Z2
Email: jurnss@mymts.net

MBA Delegate: Jim Campbell
Ph 204-467-5246
Box 234
Stonewall, MB R0C 2Z0
Email: jaycam@mts.net

RRAA web site administrator: Jim Campbell
Ph 204-467-5246
Box 234
Stonewall, MB R0C 2Z0
Email: jaycam@mts.net

Newsletter Editor: Ken Rowes
Ph 204-755-3427
Cloverleaf Box 758
RR1 Anola, MB R0E 0A0
Email: Roweskd@mymts.net

Presidents Comments -- November 2013

It has been a strange fall with several overly warm days followed by very cool days for a short time. The bees are still flying on a regular basis, but are not finding very much due to the cool weather.
One wonders if it is time to put the bees away for the year.

It was a great contrast at our October meeting in "Outside Wintering" bee styles. One of the Provincial Apiarists at our RRAA meeting went through the details of Outdoor Wintering using insulated plastic blankets either in two packs or four. One of our members described his method of using a Styrofoam box inserted over a pair of hives. Both methods mentioned the importance of air movement at the base of the hive as well as a Vent Hole near the top of the hive to evacuate the interior warmed up moist air. What was common to both of them was location of the hives over the winter that provided wind and weather protection.

There is a request to CFIA from a group of MBA beekeepers to permit the import of package bees in the spring from the USA to replace their increasing winter losses. There has been no official decision yet, but it would be applicable to all the provinces across Canada. One wonders if this might lead to the movement of summer colonies from the USA across the Canadian border in all provinces. That could cause a territorial problem in the prairie provinces as is there enough yard locations and space. for everyone.

The mortality rate of all the pollinators in Canada seems to be increasing...Many question the role a variety of the pesticides that are being used by farmers especially those that have a " Neonic " basis. An email sent to one of our members referred to a " Neonic Type of Pesticide " that is being utilized on Winter Wheat seed without any notice to the growers of the Wheat. Their questions on this were answered by the seed seller that all seeds sold have this type of " Neonic " treatment in one form or another.
This is another example of a large corporation determining the quality of the food produced. There are possible side effects that may affect the pollinators and indirectly ourselves. Many countries in Europe have banned a variety of the ":Neonic " pesticides for several years and the honeybee populations seemed to have improved. Is there a relationship or just an example of how things can change over time.

Our final meeting of the year is on November 12th, which has its main feature " Gadget Night " where members bring there own creations for display and discussion.

There is also a need to form an Executive Search Committee for next years Executive.. Fortunately all Exec. Members have agreed to serve for another year. thus a Search Committee is not needed at this time.. However there can be nominations for any position from the floor at the January RRAA meeting. All that is required is that you are a dues paying RRAA member for at least a year, have a supporting seconder for your nomination and that you have more floor votes than the Executive Nominee.

There will be an opportunity for any member to share BEE stories of 2013. and finally there will be a variety of light snacks catered by our first Vice- President, who is a trained chef.

I will not be at the November RRAA meeting as there has been a request to CESO from 2 Universities in the Philippines to do a feasibility study for future

Apiculture programs. I will leave at the end of October and return to Manitoba in late November.
I am wishing that all of you and your families have a good holiday season and that Christmas arrives with its traditional snowfall.

Yours in Beekeeping-
Charles Polcyn RRAA 2013 President
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Minutes of the RRAA General Meeting
River Heights Community Club October 8, 2013

Chair: Charles Polcyn
Recording Secretary: Art Quanbury

Approval of Minutes of September 10, 2013
Moved: Chris Argiriou
Seconded: Ken Rowes
Carried

Other Business/Members comments
Charles commented that the Honey show was well attended and given good coverage in a couple of newspapers. One of the main questions from the public to the volunteers had to do with the health of the honey bee and what were the reasons for its decline.
Charles thanked Ken Rowes for a very informative article in the Newsletter and the comment was made that the RRAA Newsletter was better than the MBA Newsletter.
Ron Rudiak mentioned that a special program on Lyme disease would be presented on the David Suzuki program on Thursday.

MBA Report
Jim Campbell reported that there was still no word from the Dept. of Agriculture on whether bees would be imported from the USA.

David Ostermann – wintering colonies
David gave a presentation on wintering colonies that included many interesting facts, observations and suggestions. Some of the highlights included:
Outdoor vs. Indoor wintering
In 2008-09 44.5% of bees in Manitoba were wintered outdoors (14647 colonies) vs. 55.5% indoors (18255 colonies). Some colonies are shipped to BC for wintering. It was felt that indoor wintered colonies were not as strong as ones wintered outdoors.
Good Internet site on wintering: www.capabees.com
Winter losses: 16% in 2011/12 and 46% in 2012/13. The long winter could account for some of the high losses lat year. It is an indication that beekeepers cannot account for all the factors affecting bee health.

Hive Wraps: A 4 colony wrap seems to be most effective as the grouping helps keep the bees warm. A top opening of at least 1 inch is important for ventilation and to let moisture escape. The bottom entrance should be reduced. It is time to insulate when daytime temperatures are consistently below -5 degrees C. Pink insulation covered with tar paper or black plastic is effective. Four pack wraps will cost about $100.00 when using pink fiberglass insulation. Bees will also live under snow cover up to a depth of 6 feet.
Site Selection: Protection from the North is vital. Some East-West ventilation is important. Other factors to consider are: wind protection, open to sun, layout, protection from pests such as skunks and mice (put mouse poison under the top wrap), stocking rate, water supply.

Indoor wintering: Move colonies indoors the end of October to 10th of November. Indoor temperature should be 5 to 10 degrees C.

Freeing up frames: to recover frames immerse in hot water. Plastic foundation will not warp at temperatures up to 80 degrees C. Beeswax melts at 62-64 degrees C. David found that warping occurred at temperature of 90 degrees after 14-17 minutes.

Waldemar Damert: Outdoor wintering system
Waldemar brought his insulation system made from rigid 2 inch extruded polystyrene insulation. The size covers two single hives. The sides are wrapped with black pallet wrap and the top is taped with building wrap tape (Tyvek tape). There is a layer of vapour barrier on the top. The hives also have a 2 inch base of the same insulation that is always under them and the cover fits down over this bottom base. There is a top and bottom opening and the top opening is ¼ inch larger than the bottom entrance. He closes the bottom entrance in the spring and reopens it in mid-May. The cover is slightly higher than the hive so there is some headroom above the hive top.

Door prizes:
Chris Argiriou: floral honey guide
Doug Buckingham: pumpkin
Alex Remkes: Time magazine article
Tim Kennedy: package of GORP
Keith Bamford: soft squeezy bee
Doug Buckingham: pumpkin
Duane Versluis: honey
Nelson Szwaluk: artificial honey
Keith Bamford: borage honey
John Speer: pumpkin

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MBA Report Nov 2013

Jim Campbell, MBA Representative

Manitoba Beekeepers' Association (MBA) has just received feedback from the Federal Government on their request to permit importation of package bees from continental USA. As one can imagine, this position is not fully adopted in some other provinces. Canadian Food Inspection Agency (CFIA) completed the Risk Assessment process, releasing the report at end October. Directors of Canadian Honey Council met with several groups in Ottawa the week of Oct 28-30 to discuss various issues affecting the industry. Among the face-to-face meetings was with Dr. Connie Razman (CFIA Bee Stock Imports/Exports), Debbie Fishbien (CFIA Honey) and Matt McBain (Policy advisor to Min Ritz).

In the 2013 report, two areas seem to be consistent with earlier Risk Assessments. Africanized honey bees, antibiotic-resistant American foulbrood, small hive beetle and amitraz-resistant varroa mite are identified as hazards associated with the importation of honey bees from the U.S. Key risk factors considered in the assessment are the distribution and prevalence of honey bee diseases in the USA, the extensive migratory beekeeping industry, the overwintering of colonies in the southern part of USA, the lack of interstate movement controls, and an absence of a national honey bee management program. MBA and others have until November 25, 2013 to comment on the report

MBA directors reviewed a beekeepers' proposal to establish a Honey Bee Diagnostics Lab in Winnipeg. This would help, as currently there isn't a provincial budget for this work any more. Directors plan to present the idea for consideration at their 12 November Annual Meeting, and determine members interest in proceeding. Things like costs and liability insurance issues need further review.

MBA has partnered with Alberta Commission to perform a disease profile study of bees across Canada. This project will take considerable funding, and we hope to have other provinces come on board. In the meantime, MBA awaits grant-funding approval from Ag Canada before any work can be done.

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Bad Beehaviour

The Entomoloist April 2013

The strange case of the bandit bumblebees

To most people, bumblebees are charming slightly absurd creatures that blunder through garden and meadow with neither the steely determination of the honeybee nor malevolent intention of the wasp. If you are a plant, though things look rather different-for from the point of view of some flowering plants many bumblebees are nothing more than thieve. They rob then of their nectar and give nothing in return.

Nectar robbery, in which a bumblebee carves a hole in the side of a flower as a bank robber might cut his way into a vault, was discovered by Charles Darwin. This technique lets bees get at the nectar of flowers whose shapes have evolved to encourage their pollination by insects with long tongues, which can reach down narrow tubes.

Some bumblebees do have such tongues. But some do not. Short – tongued bees are, however, unwilling to deny themselves the bounty of nectar inside these flowers. Hence the hole-cutting. By breaking in in this way, though, a bumblebee nullifies the 100m-year-old pact between flowering plants and insects that the plant feeds the insect in exchange for the insect pollinating the plant.

The question about nectar robbery that has intrigued biologists from Darwin onwards is whether the behaviour is innate or learnt. Darwin, though he originated the idea that many behaviour patterns are products of evolution by natural selection, suspected that it is learnt. Insects, in other words, can copy what other insects get up to. Only now, though, has somebody proved that this is true.

The observations were made by David Goulson (then at the University of Stirling now at the University of Sussex), and his colleagues. To test his ideas he had to go to Switzerland for only there could he find a flower of the shape to conduct the study.

His crucial observation was that when the flowers of an alpine plant called the yellow rattle are robbed, the entry holes—because of the structure of the flower tend to be unambiguously on either the right-hand side or the left-hand side. Moreover, preliminary observation suggested that the holes in flowers in a single meadow are often all made on the same side. This led him to speculate that bumblebees in a particular area do indeed learn the art of nectar robbery from one another. And then copy the technique with such fidelity that they always attack a flower from the same side.

Crime and nourishment

His team monitored 13 alpine meadows during the summers of 2009 and 2011. They painstakingly recorded the sites of robbery holes in yellow-rattle flowers, and studied the behaviour of 168 bumblebees. They tried to follow each bee until it had visited 20 flow, though they lost sight of some insects before they reached this score. If they could, they then captured the insect so as not to follow it again on another occasion.

Dr Goulson found, as he reports in Behaviour Ecology and Scoiobiology, that two short-tongued bumbles species which live in the area, ***Bombus lucoru*** and ***Bombus wurflenii***, demonstrated handedness when they robbed flowers. Moreover, if one species was behaving in (say) a left-handed manner in a particular meadow, the other species was likely to do the same. This suggests that one species can learn form another—a trick previously thought to be confined to vertebrates.

(continued on Pg 10)

lifera) more than fifty years ago and has since become an almost cosmopolitan pest. The mite weakens the bees by sucking hemolymph from both adult bees and pupae. In addition, they can transmit many of the known honey bee viruses and cause a reactivation of covert virus infections due to host immune suppression. The mite destabilizes the within-host dynamics of viruses due to this immune suppression, which can then reach lethal levels . Further, *V. destructor* and Deformed Wing Virus (DWV) will reduce the life span of winter bees, which can cause a colony collapse.

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

The role of the Microsporidian fungus *Nosema ceranae*, another parasite that originates from the Asian honey bee, in causing colony collapse is still controversial. Sudden colony collapses in Spain were attributed to *N. ceranae* infection, but these observations could not be confirmed by later independent studies in and outside Spain.

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. Earlier descriptions of spiroplasmas in honey bees go back to the early eighties. Also *C. mellificae* has been little studied since its first description in 1967, even though the related *Crithidia bombi* is known to have serious effects on bumble bees, particularly under starvation conditions. 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. As shortly afterwards several neglected and new honey bee pathogens were described in the USA, 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. mellificae*, 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. 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.

MLPA Analysis

BeeDoctor, 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 . Because of the high similarities of the two described LSV strains, 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, 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.

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.

Red River Apiarists' Association

Editor's Note by Ken Rowes

My apologies for such a scientific intense headliner. It has been a area of fisheries diagnostic work I was involved in. It shows the depth and sensitivity we have come to in answering factors contributing to bee death.

The latest CFIA (Canadian Food Inspection Agency) report for US border opening to bees is that their risk assessment affirms the present restrictions except opening it to queens that can be individually inspected for signs of disease before import into Canada.

Co-operator this summer reported Health Canada saying it is not delaying be action on neonectoid chemicals Alex Binkley). It reports success in drilling seeds directly into the soil. My understanding is that process uses air and it would send the lubricant dust into the air creating another problem.

Well here we are into November and no snow but the ponds are frozen over. Temperatures still a little high so as not to wrap outdoor colonies, at least here in the south. Although some have taken bees indoors.

Since our last meeting I have like a few beekeepers have made outdoor wintering boxes out of Styrofoam similar to Waldermar in his latest seminar. Outdoor wintering allows for upgrades in the honey house and wintering room. An opportunity to pull out equipment for check-over and repairs.

It also allows for making the 2% new frames for next years replacement, for me that's about 50 frames. And its time to take the old material to the McGregor wax works.

I have been told that they are not into the candle grade wax making but are still accepting wax.

Blessing wishing the fall & Christmas season bee you well.

The Bee Cause is the official publication of the Red River Apiarists' Association for distribution to its members and their colleagues in the beekeeping industry. It is published eight times a year on a monthly basis except December and the summer months of June, July, and August when membership meetings do not occur.

Articles can be best submitted in word documents as email attachments. Though they may be edited for spelling and basic grammar, no changes will be made to their contents, message and opinions. They are those of their originator and not of the Red River Apiarist Association.

Deadline for any submission to this newsletter is the second Saturday preceding the membership meeting to allow for publishing and mailing delays. Regular membership meetings are normally scheduled 7:30 PM on the second Tuesday of every month at the River Heights Community Centre located at 1370 Grosvenor Avenue in Winnipeg except the months as noted above.

The Red River Apiarists' Association, formed in 1963, represents the beekeepers of the Red River Valley and environs in southern Manitoba. The association provides a forum for the promotion of sound beekeeping practices through education, networking opportunities, meetings, field days, workshops, presentations by local apicultural experts, as well as the dissemination of this monthly newsletter.

We are on the web!
www.beekeepingmanitoba.com

CLASSIFIEDS

1 For Sale: Complete honey extracting line 48 Frame extractor, uncapping table, sump, tank, pump, pipes. **Contact Lance at 204-712-6783, lancewld@gmail.com**

3 For Sale: For sale : heavy frames of pollen - $60 per super of ten frames, 15 supers of plastic frames - $34 ea. Wrecking 2005 F-350 4x4 – asking $4,000 OBO Booking spring colonies – minimum 4 frames of brood – mid May - $250 30 honey supers with plastic comb - $32 each Winter wraps made to your specifications - $45 to $65 each Interlake Honey Producers Ltd. Interlake Honey Producers, Fisher Branch, MB 204-372-6920 . Can deliver to Winnipeg. Supers are in good to average shape and all the frames are fully drawn out plastic frames. We have no AFB history. **Paul Gregory paul@interlakeforageseeds.com**

5 Wanted: Looking for good used Cowen type horizontal 28 to 60 frame extractor, plus sump and pump. Call **Don Friesen, Rosenfeld, at 204-746-8863 or e-mail stonefield71@hotmail.com**

6 For Sale: 12 hive top feeders, 19 queen excluders, 4 super extractor. **Contact Doug at Tel 757-4694 or doug.henry1@gmail.com**

7. For Sale: custom made Bee-gloves $17. **Contact ken Fehler 204-667-9013**

8 For Sale: Man Lake SS Extractor 9/18 frame. Asking $1300, used twice**. Contact Janice at 204-895-9667.**

9 For Sale: Bee Equipment, Nucs, Plastic Feeder Frames, Box & Frame Parts. Contact **Charles Polcyn at (204) 284-7064 or by Email-charles_polcyn@ymail.com**

10 For Sale: 6 hive top feeders, 20 frames with foundation call 204-612-2754 Doug Beck or e-mail doug-janetb@hotmail.com

11 For Sale: 2 frame manual extractor, uncapping knife, bee suit, smoker bellows, hive cover (metal), 5 supers (assembled), 50 frames (plastic & wire), 2 hive bottoms, hive scrapers, and much more for $ 450.00 Please call Adrian at 204-338-7172

Temperature cycles for slowly-evolving trypanosomatids and neogregarines were as described, 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, based on the 16S ribosomal RNA sequence. Due to unspecific bands from *N. ceranae* rRNA around 700 bp, only universal *Spiroplasma* primers were used. Amplicons around 1 kb 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 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. 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.

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.

Nosema Spore Counting

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

Statistics

The multiple-kind lottery model 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, 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. 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.

RESULTS

Survey of Pathogens

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, 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).

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. 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, 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. 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.

Effect of Pathogens on Colony Winter loses

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, we decided to test whether these winter losses could be predicted on the bases of the presence or absence of these pathogens .

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). 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* × *N. ceranae*, significantly contributed to explaining winter mortality in this model ($p = 0.01$, 0.02, 0.07 and 0.03, respectively. The significant interaction effect was due a negative synergy between *C. mellificae* and *N. ceranae* on winter mortality . 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). 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.

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, $p<0.001$).

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. 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. 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, China, France, Japan , Switzerland and USA. Besides, the related *C. bombi*, also reported from Asian honey bees, has serious effects on the survival of bumble bees under stress conditions. 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. In addition, an association between both pathogens was reported in the USA . Possibly, the controversial role of *N. ceranae* 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. Collapsing colonies, induced by e.g. *V. destructor* and *C. mellificae*, are probably more vulnerable to a diverse set of parasites, which elucidate this correlation. Moreover, it appeared that several pathogens can act synergistically and eventually cause a collapse of the honey bee colony. The outcome of these pathogen interactions can vary between regions, 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 . Diverse viral sequences are reported in the USA and Spain. 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 and Spanish honey bees. Remarkable was its rather high incidence in the present study (56.2%; 204/363), akin to similar observations in different regions in the USA. 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. 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, including Asian honey bees . They seem to be uncommon in honey bees, with a sudden incidence in the summer 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.

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. However, real empirical data is missing to describe the pathology of *A. bombi*. After its detection in honey bees in Finland, *A. bombi* was also reported in honey bees in Japan and Argentina.

The presence in Argentina is probably induced by spillover

from invasive *Bombus terrestris*, an introduced pollinator outside the West Palaearctic area . 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.

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 advisory to look at a broader range of pathogens in nationwide monitoring programs.

For Complete article and references see: http://www.plosone.org/article/info:doi/10.1371/journal.pone.0077512#abstract0

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(from Pg 4 Bad Behaviour)

Handedness in any given meadow, Dr, Goulson found, increased as the season progressed. But each summer appeared to sart as a blank slate. The handedness that developed in a meadow in 2009 did not predict its handedness in 2011.

The most reasonable explanation, Dr, Goulson argues, is that each year a few bumblebees which have learnt the trick of nectar robbery in the previous season come out of hibernation and start robbing flowers again. By chance, they make more holes on one side of the flowers than the other, and as the habit is picked up by other, newly hatched bees, a preference for left or right spreads by a process of positive feedback. The bees have, in other words, created a simple culture. It is a criminal culture, admittedly. But no one ever said that nature was pretty.

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