Screening Swedish bees for pathogens shines new light on the parasite *Gregarine*

Nellie Svedin
Abstract

Bees are under pressure from habitat loss, environmental stress and pathogens including viruses. Research have shown viral infections to be one of the major causes of colony losses. The purpose of this study was to screen three viruses; Deformed wing virus, Slow bee paralysis virus and Acute bee paralysis virus but also three parasites; Nosema spp., Crithidia spp., and Gregarine spp. due to their mortality and the lack of knowledge regarding some of the pathogens’ habitat and hosts. During the years of 2015 and 2016 three bee species were collected in a number of 156 samples including honeybees, bumblebees and solitary bees equally divided. Extraction of RNA and DNA was done using only the abdomen which was homogenised by the use of MixerMill and later extracted on a QIAcube. Real-time qPCR was used in this study as a qualitative screening method. DWV was detected primarily in honeybees, infecting as much as 70 %. The parasite known to have bumblebees as its host, namely Crithidia spp. was detected in 23 % of the honeybees collected, 15 % of the bumblebees and 2 % of the solitary bees. According to the results; Crithidia spp. is no longer a specific parasite found in bumblebees but have broaden its host spectrum to both honeybees and solitary bees. Furthermore, the parasite detected in most samples was Gregarine spp. and was detected in 50 % out of the bees collected suggesting that this parasite is common in Swedish bees.

Keywords: SBPV, DWV, Nosema spp., Crithidia spp., Gregarine spp.
Introduction

Bees are found in more than 20000 species all over the world, but there are only 9 species of honeybees. These do not only play the important roll providing humans with nutrition such as honey, but are also important when it comes to assisting bumblebees with the pollination of a variety of food crops. Pollination by bees is needed to sustain biodiversity of wild flora. Honeybees (*Apis mellifera*) produces beeswax, pollen and other hive products. However, to ensure this, a healthy population of bees are essential. Bee colonies consist of one reproducing queen, many thousands of worker bees and a few hundred drones. The worker bees are sterile females and are responsible for all the work in the hive whereas the drones’ only purpose is to mate with and fertilize the virgin queens (McMenamin *et al.*, 2015).

Large-scale losses of managed honeybees have increased during recent years (McMenamin *et al.*, 2015). Bees are under pressure from habitat loss, environmental stress and pathogens including viruses that causes lethal epidemics. Research have shown viral infections to be one of the major causes of colony losses (McMenamin *et al.*, 2015).

The extent of bee viruses have since the 1950s been increasing in Europe but also in North America according to findings of higher virus titres but also new viruses introduced in countries where they have never been found before. One of the major causes of this are because of infections from viruses in the *Iflaviridae* and *Distroviridae* family (McMenamin *et al.*, 2015).

Until the 1980s, viral infections where considered harmless since they did not at that time lead to the collapse of colonies due to low virus titres in the infected bees. From being without concern, there was a worldwide introduction of the ectoparasitic mite *Varroa destructor* which resulted in beekeepers encountering difficulties maintaining their colonies. Many viral
infections were considered harmless until this point but studies showed that *V. destructor* act as a biological vector of these viruses, increasing the virus titre before transferring the virus to the bees (Genersch et al., 2010). *V. destructor* is crab-shaped and the females are reddish brown most common to find while the male is greenish white and not likely to find outside the brood cell. The mite attaches to the body of the bee and feeds on the haemolymph. During this process, viruses spread to the bees. The reproducing female or “mother-mite” enters a brood cell where she first lays an egg that develops into a male. After that she lays between 1-3 eggs that develops into female mites who later mates with their brother (Rosenkrantz et al., 2010). The male serves it purpose and later dies or gets eaten. This is why the females are most common to find (Giuseppina et al., 2015)

Studies show viruses most lethal to bees since the introduction of *V. destructor* belongs to the family *Dicistroviridae* and *Iflaviridae*. Belonging to *Dicistroviridae* is the Acute bee paralysis virus- Kashmir bee virus- Israeli acute paralysis virus complex (ABPV-KBV-IAPV complex). This complex consists of three closely related viruses. It has been discussed wheatears they are three different viruses or in fact only one virus. Furthermore, the family *Iflaviridae* consists of two virus species namely *Deformed wing virus* (DWV), which is given the name due to its symptoms and *Slow bee paralysis virus* (SBPV) who induces paralysis (Genersch et al., 2010; Kalynych et al., 2016)

Pathogens such as mites, fungi, bacteria and viruses can be found in infected or deceased bees. One of the parasitic fungi having negative effect on colony fitness and development of bees belong to *Microsporidia, Nosema* spp which can be found in both honeybees and different species of bumblebees (Vavilova et al., 2015). Moreover, a parasite commonly infecting bumblebees belong to the *Trypanosomoe, Crithidia* spp. (Schlüns et al., 2010). The third parasite belongs to *Gregarines, Gregarine* spp. and is a recently discussed parasite since it has been more common to find in colonies than before. This parasite does however no harm to the gut but they cause the males to lose weight (Bailey and Ball, 1991).
Studies when injecting lethal doses of ABPV, IAPV, and KBV in honeybees showed to causes rapidly progressing paralysis, including symptoms of trembling, shivering wings, darkening, loss of hair from the body and finally developing paralysis leading to their death. The symptoms was not as rapidly progressing for IAPV as for ABPV and KBV. Considering the high virulence of these viruses when injected, it is not surprising that it started to cause problems as an emerging viral disease with the introduction of the mite V. destructor which transfers the viruses when feeding on pupae and adult bees (Hou et al., 2014).

IAPV was originally identified as a bee-specific virus but has today a much broader host spectrum including wild and social solitary bees as well as social wasps (Gisder et al., 2015). ABPV are widely spread in honeybee colonies and is involved in honey bee colony losses, especially if the colonies are highly infested with the V. destructor. The original host of the ABPV-complex is most likely A.mellifera, however it has also been detected in bumblebee species. ABPV causes no obvious symptoms but is extremely virulent when injected into pupae or adult and death will occur within a few days (Bailey, 1965; Furgala et al., 1966; Bailey et al., 1974).

ABPV accumulates in the brain and hypopharyngeal glands of the adult bee and is most likely to be detected in the brood. However, diseased brood is normally rapidly removed or cannibalised by the adult population escaping detection (de Miranda et al., 2010; Reynaldi et al., 2010; Azzami et al., 2012).

Outbreaks of DWV belonging to the family Iflaviridae have been shown to be linked to the ability of V. destructor to act not only as a mechanical vector of but also as a biological vector (Ball et al., 1988; Genersch et al., 2010). DWV replicates in mites prior to its vectoring into pupae which seems to be necessary for the introduction of an overt infection in pupae. The infected pupae later developing into non-viable bees with deformed wings. DWV in a V. destructor infested colony is considered as one of the key players of colony collapses worldwide (Ball et al., 1988).
The main host of DWV is the European honeybee, and has increased since the distribution of *V. destructor*. DWV have also been reported in bumblebees such as *B. terrestris* and *B. pascuorum* (Genersch *et al.*, 2006).

In the absence of *V. destructor*, DWV is a rather benign virus not showing any visible symptoms. With the establishment of *V. destructor* in the European honeybee, DWV infections became more prevalent and signs of symptoms such as deformed wings, shortened abdomens and discoloration started to show. These bees die within 67 hours after infection (Yang *et al.*, 2007).

SBPV belongs to the *Iflaviridae* family together with DWV and was discovered in England in 1974. An injection with SBPV into the abdomen of adult bees induces paralysis of the anterior two pairs of legs in about 10 days (Kalynych *et al.*, 2016).

This virus is extremely rare, having been identified positively only in Britain, Fiji and Western Samoa. Only in Britain has it so far ever been associated with colony mortality (de Miranda *et al.*, 2010).

The parasitic fungi *Microsporidia, Nosema* spp seems to play a role in bee decline in the U.S and Europe. Honeybees can be infected by two different species of *Nosema*: *N. apis* (Bailey, 1955) and *N. ceranae* (Fries, 1993), with *N. apis* being referred to “as-”"the silent killer” due to its lack of overt symptoms (Botías *et al.*, 2012). *N. ceranae* is a comparatively recent parasite of the European honeybee mostly found in the southern parts of Europe (Tantillo *et al.*, 2015), but has also been found in the north including the south of Sweden. Bumblebees are more commonly infected with *N. bombi* (Fantham *et al.*, 1914; McIvor *et al.*, 1995; Gawlson, 2003).

One of the differences between *N. apis* and *N. ceranae* is how quickly *N. ceranae* can cause a colony to die by being aggressive and surviving even the toughest climate (Forsgren *et al.*, 2013).
In addition to *Nosema* spp. there is also *Trypanosome, Crithidia* spp. having the bumblebee as a host. *C. bombi* is perhaps the most prevalent parasite of bumblebees (MacFarlane et al., 1995; Gaulson, 2003). The parasites mentioned above interacts with the intestinal epithelium and are most likely partially responsible for the declining bee populations by causing an intestinal infection, also called microsporidiosis (Botías et al., 2013). This causes the bees to lose their ability to distinguish between flowers that contain nectar and those that do not which causes the bees to starve to death. Furthermore, concerning *Crithidia* spp. it is more common for commercially bred bees to get infected with the parasite in comparison with wild bees, there is no documentation on why that is (Schlüns et al., 2010).

In this study, 156 bees were collected in Uppsala, Sweden, as a part of a European project including countries as Norway, France, Turkey, Germany, Great Britain, Spain, Switzerland, Croatia and Belgium. The main goal with this project was to get a perspective in the distribution of mentioned pathogens in Sweden and the other countries involved by using real-time qPCR. By mapping out where they are located there can be a comparison to earlier studies that may show an increase or a decrease of these pathogens. Furthermore, the study was also about finding out if the viruses and parasites where specific to a certain host or not and also which species are located in the different hosts.

**Method and material**

**Specimen collection**

156 specimens were collected at the Ecology department (EK), Microbiology department (MB) and from the landscaped teaching garden (TG), Uppsala, Sweden in the years of 2015 and 2016. The specimen included bumblebees listed as following: *B. lapidarius, B. terrestis, B. hortorum, B. sylvarum, B. locorum, B. veteranus, B. jonellus, B. ruderarius, B. soroeenis*
and *B. bohemicus* but also honey bees (*A. mellifera*) and solitary bees as: *Melitta*, *Andrena* and *Anthophora*. The number of samples collected were equally divided between honeybees, bumblebees and solitary bees.

An ethical review were not needed for this analysis considering the fact that honey bees and bumblebees are not connected to any individual and are counted not as animals but insects. Because of this, the animal ethics committee was also not involved.

**Preparation of DNA and RNA**

Honeybees, bumblebees and solitary bees were dissected and head, thorax, wings/legs were separated using a single-use scalpel/razor blade. Each sample were stored at -20°C. In this study, the abdomens were used.

TBS buffer (50mM Tris.CL/150mM NaCl (pH 7.5)) were added to each sample in a volume of 100 µL while working on a cooling block. The TBS buffer contained Ambion RNA250 (Thermo Fisher Scientific AM7155) with a concentration of 10 ng/µL which was added to the TBS buffer prior to use. Glass beads where added to each sample with a diameter of 3 mm in a number of 3-5 beads per sample. Following this step, the abdomens were homogenized by using a Mixer Mill (Mixer Mill 400, Retsch) at 30Hz/s for 90 seconds. However, for larger bumblebees this step was done twice. Furthermore, a greater volume of TBS-RNA250 were later added to each sample after homogenization by MixerMill, and the added volume depended on the size of the sample; 700 µL TBS-RNA250 buffer to samples containing bumblebees, 400 µL TBS-RNA250 buffer for honeybees and 200 µL for sweat bees which are more petite than honeybees.

Later on, the homogenized samples, only the liquids were transferred to a QIA-shredder column (included in the Plant RNA extraction kit, QIAGEN) and was centrifuged at 5200 x g for 2 minutes.
From each primary bee abdomen homogenate, 100 µL were transferred from the QIA-shredder to a separate container for the extraction of RNA and 100 µL to a container for the extraction of DNA.

To purify the RNA: 350 µL RLT buffer (Plant RNA purification kit, QIAGEN) where added in each container. The RTL buffer contained 1 % β-mercaptoethanol, which was added separately on the day of use. The samples for RNA purification were mixed for a few seconds on vortex and then placed in the QIAcube for purification following the protocol and reagents of RNeasy® Mini Kit provided by QIAGEN. Samples were diluted in a volume of 50 µL extracted RNA.

For the DNA purification: 180 µL Enzymatic Lysis buffer (20 mM Tris.CL/2mM EDTA/1.2 % Triton x100/ 20mg/mL Lysozyme (pH 8.0)) were added to each container which contained what would become extracted DNA. Each sample were mixed on vortex for a few seconds and then placed in the QIAcube for purification following the protocol and reagents of DNeasy® Blood & Tissue Mini Kit provided by QIAGEN. Samples were eluted in a volume of 200 µL extracted DNA.

In order to determine the concentration of both the RNA and DNA obtained from the QIAcube, NanoDrop was used which uses the spectrophotometer system (ND-1000, Thermo Scientific).

In some samples the concentration of RNA exceeded 100 ng/µL and was adjusted by adding sterile water. This also occurred with some of the DNA samples; in this case the concentration exceeded 20 ng/µL and were adjusted by adding AE buffer (QIAGEN).

In the next stage 10 µL were transferred from each RNA sample to µStrips (Multiply® µStripPro, Sarstedt). The same thing was done with the DNA samples transferring 40 µL instead. The µStrips were stored at -20ºC and the containers with the remaining nucleic acid were stored at -80ºC.
cDNA synthesis
The kit for making cDNA from RNA was purchased from Thermo Scientific (First Strand cDNA Synthesis Kit #K1612). The RT master mix (per 20 µL reaction) was prepared by adding 1 µL Random hexamer primer, 4 µL 5X Reaction Buffer, 2 µL RiboLock Rnase Inhibitor (20U/ µL), 2 µL 10 mM dNTP Mix followed by 2 µL M-MulV Reverse Transcriptase (20U/ µL ). To the prepared mastermix, 10µL of RNA sample where added. For the random hexamer primer synthesis, the samples where incubated using CFXconnect® (BIO-RAD) real-time PCR machine with the following program: 5 minutes at 25ºC followed by 60 minutes at 37ºC. The reaction was terminated by heating at 70ºC for 5 minutes. Moreover, the cDNA (20 µL) was diluted with 180 µL MilliQ water and stored at -70ºC.

Real-Time qPCR
Primers were purchased from Tag Copenhagen A/S and dissolved in MilliQ water according to manufacturer’s protocol to establish a stock solution of 100 µM. Moreover, for this analyse the stock solution was later diluted to 1:10. Target ABPV complex with forward primer KIABPV-F6648 5’-CCT TTC ATG ATG TGG AAA-’3 (Tm 46,8ºC) and reverse primer KIABPV-B6707 5´-CTG AAT AAT ACT GTG GGT ATC-´3 (Tm 48,5ºC). DWV complex forward primer DWV-F8688 5´-GGT AAG GGA TGG TTG TTT G-´3 (Tm 48,9ºC) and reverse primer DWV-B8794 5´-CCG TGA ATA TAG TGT GAG G-´3 (Tm 48,9ºC). SBPV complex forward primer SBPV-F3177 5´-GYG CTT TAG TTC AAT TRC C-´3 (Tm 46,8ºC) and reverse primer SBPV-B3363 5´-ATT ATR GGA CGT GAR AAT ATA C-´3 (Tm 47,4ºC) where the R in the sequences encodes for both the A and G nucleotides. Microsporidia *Nosema* spp. forward primer 5´-TAT GCC GAC GAT GTG ATA TG-´3 (Tm 49,7ºC) and reverse primer 5´-CAC AGC ATC CAT TGA AAA CG-´3 (Tm 49,7ºC). Trypanosome *Crithidia* spp. forward primer 5´-CTT TTG GTC GGT GGA GTG AT-´3 (Tm
51.8°C) and reverse primer 5´-GGA CGT AAT CGG CAC AGT TT-3´ (Tm 51.8°C).

Gregarines *Gregarine* spp. forward primer 5´-CCA GCA TGG AAT AAC ATG TAA GG-3´ (Tm 53.5°C) and reverse primer 5´-GAC AGC TTC CAA TCT CTA GTC G-3´ (Tm 58.4°C).

To establish the master mix for the Real-Time qPCR detecting viruses, SsoFast™ EvaGreen® Supermix (BIO-RAD) was used and mixed according to manufacturer’s protocol (per 10 µL reaction) by adding 2.4 µL MilliQ-H2O, 5 µL SsoFast™ EvaGreen® Supermix final concentration 1 x, 0.3 µL Forward primer (10 µM), final concentration 0.3 µM, Reverse primer (10 µM) final concentration 0.3 µM, and finally adding 2 µL of cDNA template.

The reaction was done using the PCR-protocol shown in table 3 and 4 on CFXconnect® real-time PCR machine with 40 cycles. The data from the reaction was processed using Bio-Rad CFX Manager 3.1. Positive results were not only seen on a melt curve, but also on 1.5 % agarose gel to determine the right size (bp) has been given by the product.

**Results**

Specimens were collected in the years of 2015 and 2016 in a number of 156 samples including honeybees, bumblebees and solitary bees equally divided. Six pathogens were screened in this study divided into three viruses and three parasites which were chosen because of their mortality and some of them since there is a lack in the knowledge regarding their habitat and hosts. One more reason for this study was if the pathogens have broaden their host spectrum and is now today found in more bee species than before.

The method used in this study was real-time qPCR, which is a quantitative method but is in this study used as a qualitative method and provides amplification and detection by the use of fluorescence that gives both a melt curve and a Cq-mean upon detection (figure 1A). One of the samples positive for DWV shown in the figure provided a Cq-mean of 14 cycles whereas
one of the positive samples for SBPV had a Cq-mean of 22 and ABPV a Cq-mean of 32. The melt curve on the right showed one amplicon given for all the samples. Further analysis of the PCR products were done using an 1,5 % agarose gel to further establish the size of the products detected (figure 1B). The PCR products of DWV are the size of 143 bp and is on the gel given a product of 100-200 bp. SBPV-products are 226 bp and given a size of 200-300 bp whereas ABPV-products are 98 bp and is displaying a size of 100-200 bp on the gel.

**Figure 1.** In this study qPCR was used as a qualitative method providing amplification and detection. Three viruses were screened namely DWV, SBPV and ABPV. In this figure; A contains pictures of number of cycles measured upon detection (Cq-mean) and melt curves which provides the number of amplicons given. To further establish the size of the PCR-product; a 1,5% agarose gel was made in B showing the PCR-product of the samples positive for the viruses shown in A.
The viruses screened in this study were DWV, SBPV and ABPV providing negative results in 48 % of the samples collected (figure 2). Out of the honeybees collected (n=57), 30 % did not generate a PCR-product for any of the viruses. The bumblebees (n=52) screened negative in 61 % and solitary bees (n=47) 54 %.

Figure 2. Of the 156 samples collected, not all samples generated a PCR-product which resulted in not all samples being positive for the three viruses screened, namely DWV, SBPV and ABPV. The percentage non-infected (n=76) is representing the samples negative for all the three viruses screened for where n (honeybees) = 17, n (bumblebees) = 32 and n (solitary bees) = 27.

The highly infectious virus DWV have in this study been found in all of the three species collected (figure 3A) and was detected in 70 % of the honeybees, 34 % in bumblebees and 34 % in solitary bees. The second virus, SBPV is a very uncommon virus only found once in Sweden, and is in this study found twice (figure 3B). This time it was detected in 2 % out of the bumblebees collected but was however not found in honeybees or solitary bees. The third virus screened were ABPV and was primarily detected in solitary bees infecting 13 % (figure 3C). The honeybees infected by ABPV resulted in 2 % and the bumblebees 1,7 %.
Three viruses were screened in this study, namely DWV, SBPV and ABPV using real-time qPCR. This figure shows the distribution of bees being infected by each of these viruses in percentage were 100% for DWV, SBPV and ABPV is representing the number of each species of bee collected and n is representing the number of bees infected for every bee species. Where n (DWV) = 73 bees, n (SBPV) = 2 and n (ABPV) = 8.

Real-time qPCR was the method used for screening parasites in the bees collected and provides both a melt curve and a Cq-mean upon detection (figure 4A). One of the samples positive for *Nosema* spp. provided a Cq-mean of 23 whereas one positive sample for *Crithidia* spp. showed a Cq- mean of 25, and *Gregarine* spp. a Cq-mean of 32. The melt curve on the right shows one number of amplicons given for all of the samples. The PCR-products of *Nosema* spp. are the size of 250 bp and is represented on a 1,5 % agarose gel giving a product of 200-300 bp (figure 4B). The size of the PCR-product for *Crithidia* spp. was 417 bp and is
given a size of 300-400 bp and the product of *Gregarine* spp was 260 bp and detected a size of 200-300 bp.

**Figure 4.** Three different parasites were screened in this study namely *Nosema* spp, *Crithidia* spp and *Gregarine* spp. In this figure, A contains pictures of number of cycles upon detection (Cq-mean) and melt curves providing the number of amplicons given. To further establish the size of the PCR-products given; a 1.5% agarose gel was made in B showing the PCR-product of the samples positive for the parasites shown in A.

Parasites can be found in the intestines of the bee and the ones screened for in this study were *Nosema* spp. *Crithidia* spp. and *Gregarine* spp. Out of the bees collected, 41 % did not generate a PCR-product for any of the parasites (figure 5). Honeybees collected (n=57) resulted in 21 % negative for parasites, bumblebees (n=52) 38.5 % and solitary bees (n=47) 66.5 %.
Figure 5. Parasites screened using qPCR were *Nosema* spp, *Crithidia* spp, and *Gregarine* spp. This figure is representing the total amount of bees found negative for any of these parasites n=64. Three bee species were screened; honeybees, bumblebees and solitary bees, and those who were found negative for parasites in each species are shown on the diagram in percentage where n (honeybees) = 12, n (bumblebees) = 20 and n (solitary bees) = 30.

The parasite *Nosema* spp. screened positive for 5% of the honeybees collected (figure 6A), 11.5% in bumblebees and 2% in solitary bees. The second parasite *Crithidia* spp. have been known to have a specific host, namely bumblebees. In this study it was however detected in 23% of the honeybees, 15% in bumblebees and 2% in solitary bees (figure 6B). The earlier uncommon parasite *Gregarine* spp. was detected in 70% of the honeybees collected, 48% in bumblebees and 29% in solitary bees (figure 6C).
Figure 6. The parasites screened using qPCR were *Nosema* spp., *Crithidia* spp. and *Gregarine* spp. with these diagrams showing the percentage of bees infected with each of these parasites. Total percentage for *Nosema* spp., *Crithidia* spp. and *Gregarine* spp. is representing the number of each species of bee collected and n is representing number of bees infected in each bee species. Where n (*Nosema* spp.) = 10 bees, n (*Crithidia* spp.) = 22 and n (*Gregarine* spp.) = 79.

The virus, DWV and the parasite *Gregarine* spp. have in this study both given high numbers of infected bees, and are in most cases found together (figure 7). Total number of bees infected by DWV and *Gregarine* spp. are 152, where this number is representing some bees twice. Out of the total number of samples infected, DWV stands alone on 21 % and *Gregarine* spp. in 25 % giving a percentage of 54 % where they are detected together.
Figure 7. The diagram is representing a connection between DWV and Gregarine spp. where 100% = 152 bees infected by both of these pathogens where \( n \) (DWV) = 32, \( n \) (Gregarine spp.) = 38 and \( n \) (DWV + Gregarine spp) = 82. Screening pathogens was done by using real-time qPCR as a qualitative method.

Discussion

The purpose of the study was to screen three viruses as well as three parasites in the number of bees collected. To extract the RNA and DNA needed, the bees’ abdomens were used for the reason that it contains the highest concentration of pathogens which provides a little loss of detection sensitivity. By homogenising the samples using a MixerMill, exoskeleton are more easily destroyed and more internal fluids can be processed. At first, a manual grinding using a dispensable tip was used instead of the MixerMill. This method was not as simple and MixerMill ended up being the better decision for homogenisation of the samples, although by mixing the samples at 30 Hz/s the samples may have been heated up and therefore caused some loss of RNA or DNA. However, homogenisation with another machine that can provide cooling of the samples during the process would be much more sensitive.

When using the QIAcube for extraction of RNA and DNA, the reagents used are mostly washing buffers and ethanol not interfering with the results. However, when the samples are
in the machine they could end up getting warm, which could lead to loss of RNA and DNA. This machine is invented for extraction of DNA and RNA and one might say the manufacturer could have built in a cooling source in the machine to keep the samples cold when extracting to prevent as much loss as possible.

The concentrations of the samples were measured on a Nanodrop and some of the samples measured low concentrations and that could perhaps be depending on the particular bee considering the sampling where done in 2015 and 2016 where 2016 had overall higher concentration than the samples collected in 2015. However, samples measuring high concentration where later positive for viruses and/or parasites, but this was also true for the samples with low concentrations.

Real-time qPCR was used as a method to screen bees for pathogens providing Cq-means and melt curves showing the number of amplicons given in each sample (figure 1A & 4A). The melt curves suggest that only one amplicon has been amplified in each specific reaction and the Cq-means differed between low and high numbers of cycles upon detection. To further establish the sizes of the PCR-products given, a 1,5 % agarose gel was made (figure 1B & 4B) which could determine the results even further considering the products on the gel being the same size as the expected PCR-product.

Both positive and negative results were obtained from screening viruses and the negative results given (figure 2) suggests that honeybees seem to be more exposed to viral infections, most likely since they are commercial and live in big colonies where infections spread more easily. Moreover, solitary bees were more infected by viruses than bumblebees indicating that there could be other ways for viruses to spread than among the hives. This conclusion is one of the main reason to include solitary bees in to this study.
Negative results concerning parasites (figure 5) were found in 41% out of the bees collected. The species least infected by parasites were solitary bees and the one most infected were the honeybees.

The virus detected in most samples was DWV (figure 3A) having infected as much as 47% out of the bees collected, which is not surprising considering its strong hold in Sweden and is because of this the pathogen most likely to find. The species most infected by DWV were the honeybees with a percentage of 70% infected, strengthening the theory that commercial bees are being more threatened.

SBPV is considered a highly uncommon virus in Sweden having been found once in south of Sweden. In this study it was found in 2 samples, both bumblebees which strengthens the theory about SBPV being specific to bumblebees (Manley et al., 2017). However, to further establish this theory, a much broader study should be made in a country where SBPV is more commonly found.

The third parasite; ABPV was not as commonly found as DWV, most likely since it is considered to be an uncommon virus in Sweden (figure 3C). Having been infecting 8 samples making it still uncommon in Sweden. It was however primarily detected in solitary bees creating a theory regarding if this virus more easily spread outside the hives.

Furthermore, the parasite found positive in most samples were the *Gregarine* spp. (figure 6C) and it was detected in 50% out of the bees collected. This parasite was included in the study because of the small amount of information regarding its habitat and hosts. It was once considered to be an uncommon parasite, and when it was found it had not caused any negative effects on the bees infected. With the information provided in the results, one can surely state that *Gregarine* spp. is not uncommon and has also no specific host. However, the species most infected by the parasite was not surprisingly the honeybees considering the fact that they seem to be more threatened to pathogens than the other species collected.
*Crithidia* spp. have been known to infect a specific host namely bumblebees, however, due to the results given in this study (figure 6B), but also other studies where honeybees were also found infected in high titre makes this statement no longer true (Schwarz, *et al.*, 1967). Regarding this parasite which is supposedly more common to bumblebees it can be shown in this study to infect more than one species giving the theory of an extended host spectrum.

The third parasite *Nosema* spp. are known to infect both honeybees and bumblebees but only in different species of *Nosema*; *N. bombi* has been known to infect bumblebees whereas *N. apis* and *N. ceranae* are most commonly found in honeybees (Forsgren *et al.*, 2010; Fries, 2010; Higes *et al.*, 2006). Infection of these two parasites are common to find together as a mixed infection (Paxton *et al.*, 2007), most likely because competitive advantage has not been found between them (Forsgren *et al.*, 2010). In this study, *Nosema* spp. screened positive in 10 bees (figure 6A) where the bumblebees showed to be the most infected, followed by honeybees and least the solitary bees.

Since there were two pathogens infecting the bees in high numbers they could also be found together in most of the infected samples (figure 7). They were found together in more than 50% of the total percentage of bees infected by both of these pathogens. Because of this, one might build a theory regarding if they are working together and if bees infected with DWV are perhaps more susceptible to other infections such as *Gregarine*. The question one might ask is weather *Gregarine* is getting stronger when infecting a bee with DWV and is no longer as harmless as it was 30 years ago (Bailey *et al.*, 1991).

In summary, this study have helped map out where the most common viruses and parasites are found and in which hosts. This may as well end up rewriting the facts about these pathogens, but also contribute to an interest in more studies being made on solitary bees as well as studies about how and why the host spectrum has broaden.
References


I Fries. Nosema apis - a parasite in the honey bee colony, Bee World. 74 (1993), p. 5–19

I Fries. Nosema ceranae in European honey bees (Apis mellifera), J. Invertebr. Pathol. 103 (2010), p. 73-79


S Gisder and E Genersch. Special Issue: Honey Bee Viruses, Viruses. 7 (2015), p. 5603-5608


