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Nosema Ceranae Interactions with *Nosema apis* and Black Queen Cell Virus

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Abstract: *Nosema ceranae* is a relatively new pathogen of the honeybee (*Apis mellifera*) and the course of type C nosemosis (the disease that it causes) is not entirely known. In order to better understand the course and the consequences of this disease, laboratory experiments were performed. They aimed to compare the course of *N. ceranae* infection with the course of *Nosema apis* infection, taking its influence on the black queen cell virus (BQCV) into account. Determination of the quantity of *N. ceranae* and BQCV genetic material in laboratory tests was performed using real-time PCR. In mixed *Nosema* infections, *N. ceranae* "wins" the competition and manages to outnumber *N. apis* significantly. BQCV exacerbates the course of both A and C nosemoses, but the data shows that in the case of nosemosis C and this viral infection, the mortality rate was the highest from all examined groups. Obtained results show that *N. ceranae* is more pathogenic for *A. mellifera* than *N. apis*, and the course of type C nosemosis is much heavier, which results in the shortened life spans of bees, and in connection with BQCV it becomes even more dangerous to bees.

Keywords: *Nosema ceranae*; *Nosema apis*; black queen cell virus; host-parasite interactions; pathogen-pathogen interactions; *Apis mellifera*

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1. Introduction

Nosema ceranae, originating from the Asian honeybee, *Apis cerana* [1], and quite recently in European honeybees [2], is an emergent pathogen of the honeybee *Apis mellifera*, found almost everywhere in the world [3]. In Poland, its presence was confirmed in 2007 [4].

It is classified as *Microsporidium*, an intracellular parasitic fungus that multiplies in epithelial cells and regeneration crypts in the midgut of the honeybee, and (in consequence) damages them [5]. This parasite is known to cause extensive cell degeneration and lysis (as a consequence of an oxidative stress produced by the honeybees' immune system) [6]. The bee thus suffers from an energetic stress, which alters its basic physiological functions [7].

As a highly virulent pathogen, *N. ceranae* seems to be ousting the long present *N. apis* from colonies in some European countries [8,9], but the mechanism here is yet unclear. In warmer parts of Europe, it is associated with colony depopulation and collapse [10]. For example, in Spain [11], and Greece [12], it is correlated with high summer and winter colony mortality. It was also thought to be one of the top causes of winter colony mortality in Poland [13,14]. In Italy, beekeepers reported that the spread of *N. ceranae* was favored by high temperatures and high humidity during the summer, leading to a reduction in colony development and wintering of smaller colonies [15] Northern Europe does not seem to have this problem (but the pathogen is present). This is likewise in both North and South America [16–19]. A study from the United States also shows that *N. ceranae* in American bees may not be as virulent and dangerous as described in other studies [20].

However, in Spain (subtropical climate) it is considered as the main factor causing major colony losses [2], whereas in Sweden (polar climate), even though the parasite is seen,

it is not a major threat to bees [21]. As Poland is located in a temperate climatic zone, it is "in-between" those two extremes, which may have an influence on *N. ceranae* pathogenicity and host-pathogen, as well as pathogen–pathogen interactions. To date, there is not much comprehensive research on *N. ceranae* in Poland.

There are three viral infections associated with nosemosis, among which black queen cell virus (BQCV) seems to pose the biggest threat to bees [22]. It is thought that *Nosema* opens the infection route for BQCV by damaging the epithelial cells of the midgut [23]. BQCV and *N. ceranae* have been shown to interact synergistically in honeybee workers, which significantly decreased their survival. It is classified as a *Dicistrovirus* (*Dicistroviridae*) and was originally found in dead queen larvae and prepupae [24], but more recent studies have also revealed its presence in pollen and honey [25], and it is known to aggravate the course of *Nosema* infection without showing any distinct symptoms in adult bees [22,26].

BQCV is a very common infection found in Europe [27–32], however it is unevenly distributed. For instance, in Denmark this virus is scarce [33], whereas in France it is found very frequently [28].

In this study, we try to unravel interactions of *N. ceranae* with *N. apis* and BQCV in Poland, as well as investigate differences in both *Nosema* species on bee mortality.

2. Material and Methods

All experiments were conducted in wooden hoarding cages (Figure 1), kept in incubators in 34 °C and 80% RH, and 60% sucrose solution, distilled water and protein supplement (FeedBee™) were available for bees ad libitum.

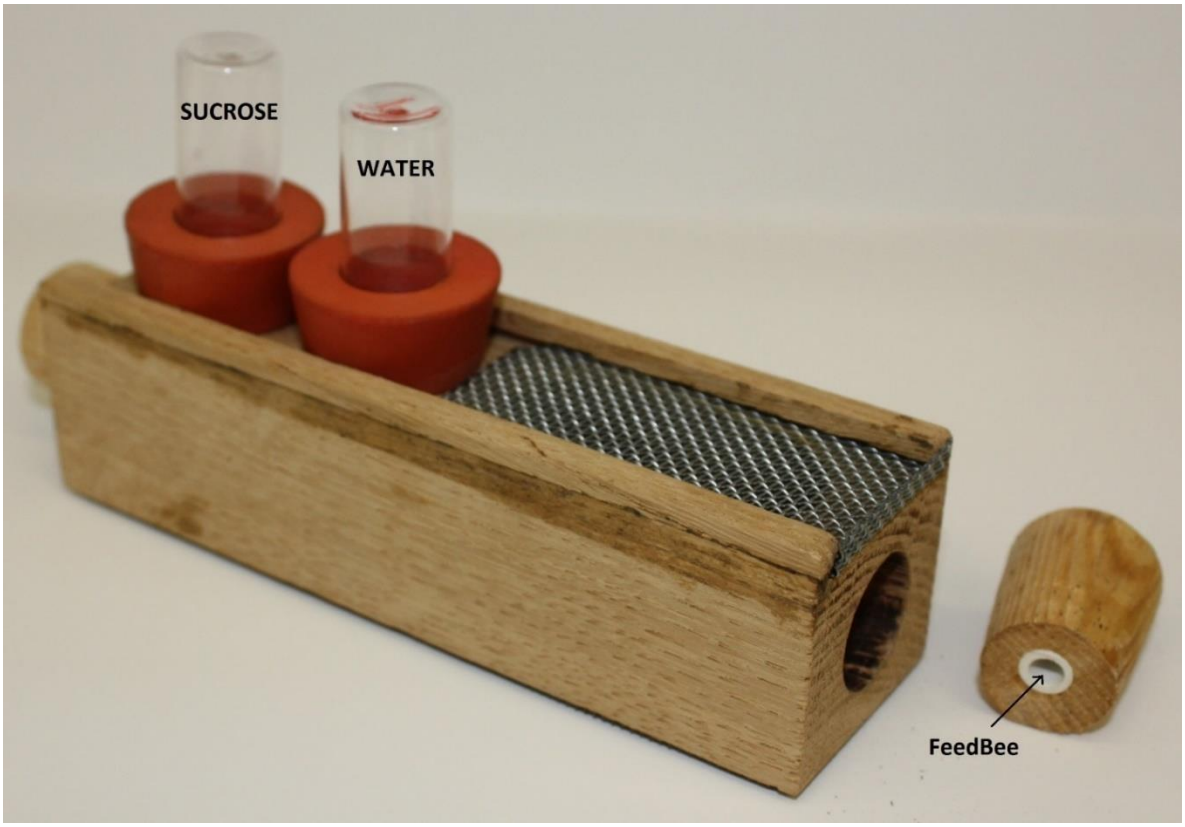


Figure 1. Hoarding cage used in both experiments.

Each cage was given a commercially available strip with artificial queen pheromone, to ensure conditions were as close to natural as possible.

2.1. The Origin of Test Subjects

Bees, spores and the virus were all obtained from Polish apiaries.

2.2. The Bees

Bees used in experiments were obtained at emergence from three colonies with no *Nosema*, BQCV, ABPV, DWV, SBV, and CBPV infections in August. They were hand-picked immediately after emergence from the cell and evenly distributed in cages.

During both experiments, the cages were inspected daily, and dead bees were removed and frozen for further examination.

2.3. Material for Infection

N. ceranae spores were obtained and purified from foragers from heavily infected colonies which tested positive for pure *N. ceranae* infection.

N. apis spores were obtained from field samples of frozen dead bees and then cultivated in pathogen free bees in hoarding cages to ensure viability and freshness of spores for infections in both experiments.

Spore suspensions were prepared at the day of infection from the digestive tracts of infected bees after grinding them up in distilled water, filtering and centrifuging five times in 8000 g to ensure pureness of the spores. The resulting pure spore pellet was suspended in distilled water and the number of spores was calculated using the haemocytometric method, using Fuchs-Rosenthal chamber.

To each 10 mL of spore solution, 1 mL of an anti- BQCV serum (1:32 titre) was added in order to avoid unwanted BQCV infections, since this virus is very common in colonies with nosemosis.

BQCV was obtained pre-experiment from dead queen cells, using the high speed centrifugation method [22], and frozen (for 2 days) until the day of infection. The number of virus copies was determined (during that 2 day period) using a quantitative real-time RT-PCR (as described later, in Section 2.5).

2.4. Experiment 1: Competition between Nosema ceranae and Nosema apis in Mixed Infection

2.4.1. The Infection Procedure

At the 5th day after emergence bees were starved for 2 h and then individually fed with 10 µL of 60% sucrose solution containing spores (or clean sucrose solution in case of the control group). No sedation method was used, bees were placed under a Petri dish and picked one by one by both wings. Experimental groups were created as shown in Table 1. Each group consisted of three cages of 20 bees.

Table 1. The setup of experimental groups for experiment on competition between *Nosema ceranae* and *Nosema apis* in mixed infection.

Group	Spores Per Bee
N. apis	10^6
N. ceranae	10^6
N. apis + *N. ceranae*	10^6 + 10^6
Control	0 (sucrose solution)

Dead bees were collected daily and kept at a temperature of −20 °C before further examination.

2.4.2. Nosema DNA Extraction and Quantification

Abdomens of individual bees were macerated in 1 ml of distilled water and filtered in extraction bags and then transferred to 1.5 mL Eppendorf tubes and centrifuged to obtain spore pellets. Pellets were crushed in liquid nitrogen and *Nosema* DNA was extracted according to OIE guidelines [34].

Quantitative real-time PCR to determine *Nosema* DNA copy numbers was carried out as described in [35], using MX 3005P thermal cycler (Stratagene).

2.5. Experiment 2: Influence of BQCV on the Course of Nosema Infection

2.5.1. The Infection Procedure

The first infection step (infections with *Nosema* spp.) was performed as described above in Experiment 1.

The second step was the infection with BQCV; 10 µL of 60% sucrose solution containing the virus particles was fed to respective groups on the 8th day post emergence (3rd day after *Nosema* infection). All control groups were individually fed sucrose solution two times, as the infected groups received spores and the virus.

Each group consisted of three cages of 20 bees. Experimental groups were created as shown in Table 2.

Table 2. The setup of experimental groups for experiment on influence of BQCV on the course of *Nosema* infection.

***Nosema* sp.**	**Spores Per Bee**	**Virus Copies Per Bee**
N. apis + *BQCV*	10^6	10^{10}
N. ceranae + *BQCV*	10^6	10^{10}
N. apis	10^6	0 (sucrose solution)
N. ceranae	10^6	0 (sucrose solution)
Control	0 (sucrose solution)	0 (sucrose solution)

Dead bees were collected daily and kept at a temperature of −20 °C before further examination.

2.5.2. BQCV RNA Extraction and Nosema Spore Count Assessment

Abdomens of individual bees infected with each *Nosema* spp. and the virus were pulverized in liquid nitrogen and further submitted to RNA isolation using Total RNA Mini Kit (A & A Biotechnology, Gdańsk, Poland). According to manufacturer's protocol, the powder was suspended in phenosol, but 20 µL of the suspension was transferred to a new tube, and the phenosol was evaporated. Next, the resulting spore pellet was resuspended in 20 µL of distilled water and the spores were counted in Fuchs-Rosenthal haemocytometer. The RNA extraction from the rest of the sample was carried out normally, right after transferring the 20 µL to a new tube.

2.5.3. RT qPCR for BQCV Detection and Quantification

The reverse transcription of obtained RNA was carried out using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's protocol.

Resulting cDNA was quantified using a quantitative real-time PCR. The template BQCV fragments for creating standard curves were obtained from SLU University Uppsala. Standard curve was prepared by using serial dilutions of target cDNA fragments, ranging from 10^2 to10^{12} as quantification standards in every run.

The quantitative real-time PCR reactions (all in duplicates) were performed in 20 µL volumes using 10 µL of iQ™ SYBR® Green Super mix (Bio-Rad Laboratories, Warsaw, Poland), 0.4 µM of each primer (BQCV-qF7893, BQCV-qBB8150), 2 µL of template cDNA and the final reaction volume adjusted to 20 µL with nuclease-free water. A reaction containing water instead of the DNA template was set up as a negative control. In a positive control reaction, a cDNA previously tested positive for BQCV was used. The amplification and data collection were performed using MX 3005P thermal cycler (Stratagene) under the following conditions: initial activation step in 95 °C for 5 min, and PCR cycling consisting of 40 cycles of 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s, including data collection. The amplified product was confirmed using melting curve analysis. To create the melting curve the reaction was incubated by raising the incubation temperature from 55 to 95 °C in 0.5 °C increments with a hold of 1 s at each increment. The specificity of amplicons was determined based on the melting temperature of amplified products. Reported results are an average of the duplicate reactions (standards, unknowns, controls).

2.5.4. Statistical Analysis

All statistical calculations were performed using Statistica 10 (StatSoft Inc., Tulsa, OK, USA).

Survival in both experiments was analyzed using Kaplan–Meier test.

A multivariate scatter Graph was used to determine the nature of change in *N. apis* and *N. ceranae* copy numbers. Single variation scatter Graphs were also used to compare the overtime change between bees with single *N. ceranae* and *N. apis* infections, but also to compare the overtime differences in cDNA copy number changes of BQCV in bees with *N. ceranae* and *N. apis*.

A Mann–Whitney U Test was applied to determine the differences in DNA copy numbers of *N. ceranae* and *N. apis* spores in bees with and without BQCV.

To determine differences between the number of DNA copies of *N. ceranae* and *N. apis* in mixed infection, a T-student test was used, and to determine statistical differences between DNA copy numbers in groups with *N. ceranae*, *N. apis* and a group with both species, a Kruskal–Wallis test was used.

To determine the differences in cDNA copy numbers of BQCV between bees with *N. apis* and *N. ceranae* a Brown–Forsyth test was used.

In experiments on influence of BQCV on the course of *Nosema* infection, a post hoc analysis (Gehan–Wilcoxon test) Bonferroni corrected was used to determine the differences in survival rates in bees infected and not infected with BQCV.

3. Results

3.1. Experiment 1: Competition between Nosema ceranae and Nosema apis in Mixed Infection

Bees in all infected groups started dying after the 14th day post infection (Figure 2). All bees from the control group remained alive through the entire experiment, so until the 24th day when all the bees that survived were frozen until further examination. There was no statistically significant difference (Kaplan–Meier test) between the survival rate of bees infected with *N. apis*, *N. ceranae* and both *N. apis* and *N. ceranae* (in equal amounts). However, the Kaplan–Meier test showed a clear tendency for the bees infected with *N. apis* to survive longer than bees infected with *N. ceranae* and bees infected with both species.

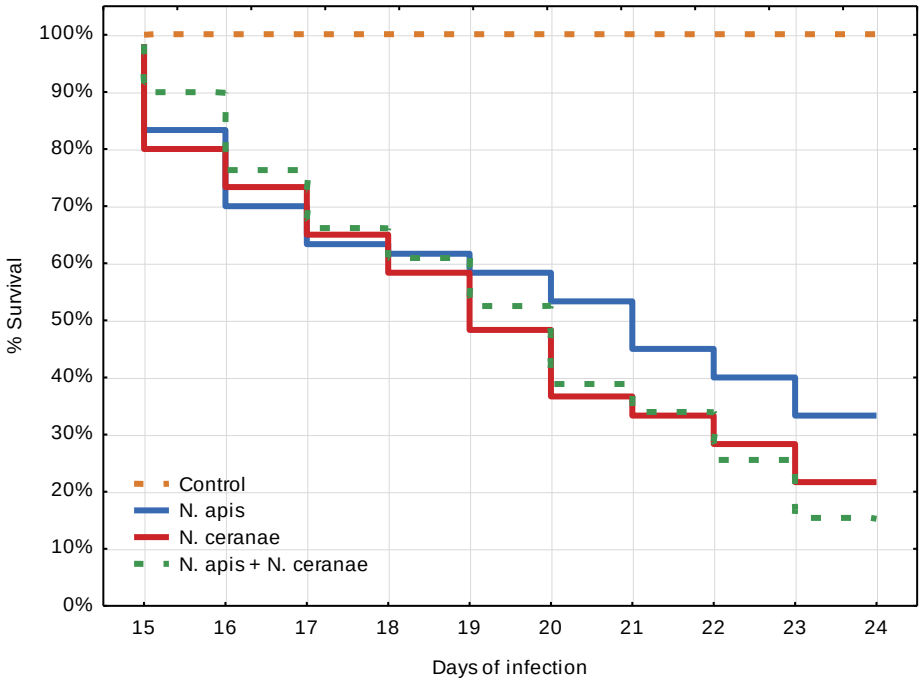


Figure 2. Survival rate of bees infected with *N. apis*, between *N. ceranae* and both species at the same time.

In both groups of bees infected with only one *Nosema* species, the DNA copy numbers of both species were growing overtime, but in the case of *N. apis*, the numbers were significantly lower (Figure 3).

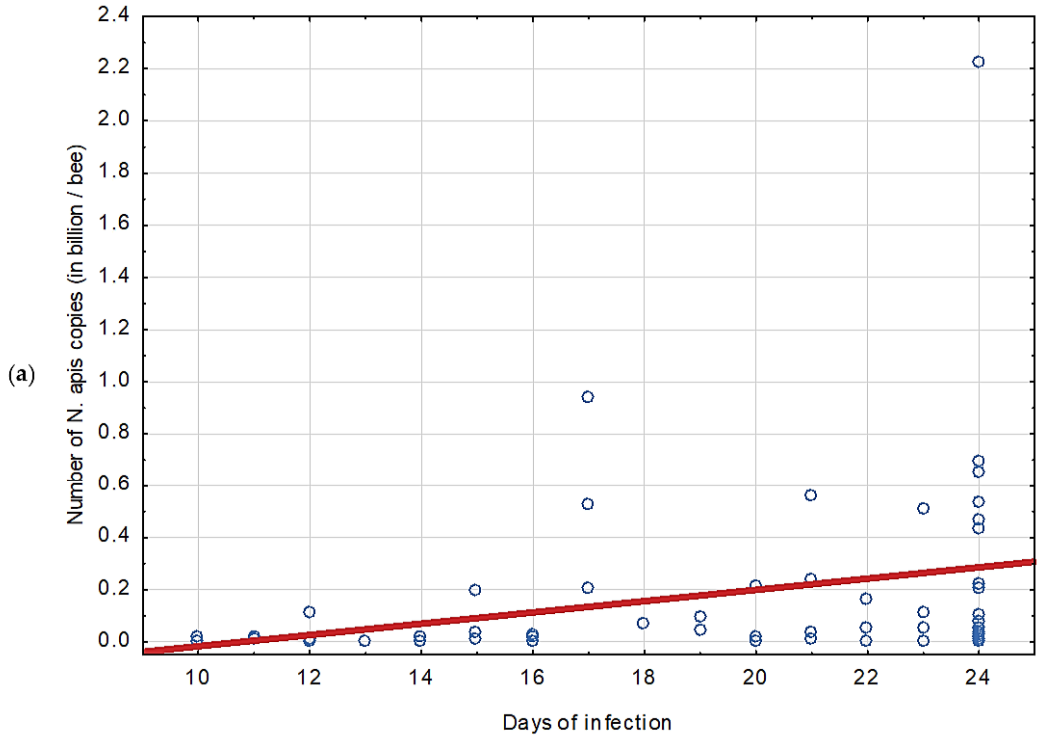


Figure 3. *Cont*.

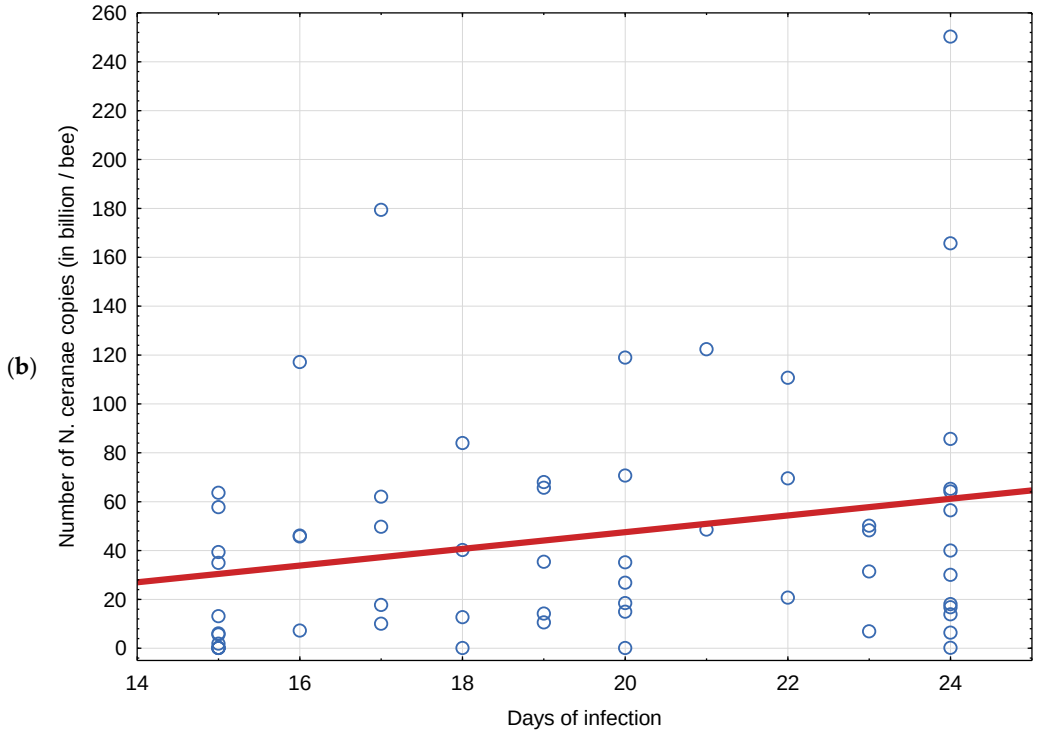


Figure 3. Overtime change in DNA copy numbers in bees infected with one species of *Nosema* (**a**) of *N. apis* (**b**) of *N. ceranae*.

In bees infected with both *Nosema* species, DNA copy numbers of *N. apis* were growing minimally, whereas DNA copy numbers of *N. ceranae* were growing significantly more over time (Figure 4).

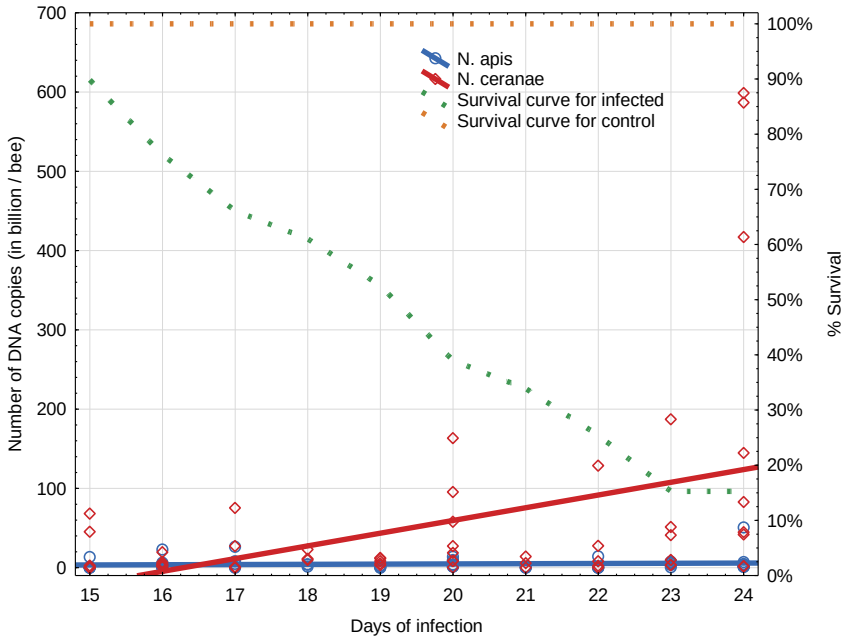


Figure 4. Change in *N. apis* and *N. ceranae* DNA copy numbers overtime in bees infected with both species (*N. ceranae* and *N. apis*) (showing survival rate curves for bees infected and non-infected bees).

In general, in bees infected with both species in a mixed infection, DNA copy numbers of *N. apis* were significantly lower (Kruskal-Wallis test, $p < 0.0001$) than DNA copy numbers of *N. ceranae* in all bees independently from the day of death (Figure 5).

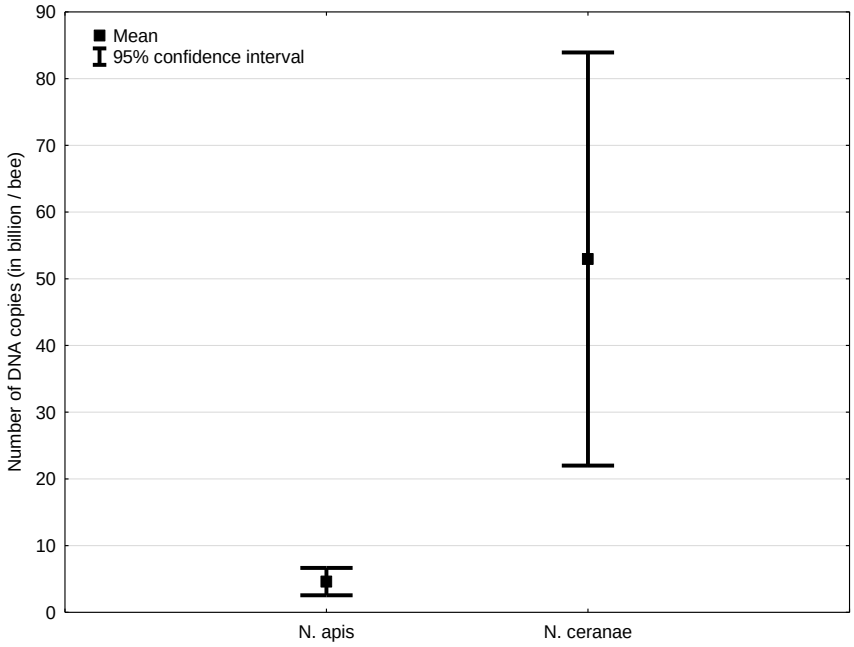


Figure 5. Differences in *N. apis* and *N. ceranae* DNA copy numbers in bees infected with both species (*N. ceranae* and *N. apis*). Paired Student's t-test $p = 0.003$.

3.2. Experiment 2: Influence of BQCV on the Course of Nosema Infection

All bees from (non-infected) control group survived to the end of experiment, that is to the 30th day after infection with *Nosema* spores. The experiment ended on day 30, when all bees that were still alive were frozen. This was due to the fact that the group infected with the virus had no remaining living bee. The lowest survival rate was in bees infected with *N. ceranae* and BQCV. Survival of bees infected with only *N. ceranae* was also significantly lower (Kaplan–Meyer Test) than of those infected only with *N. apis* and even with *N. apis* and BQCV (Table 3 and Figure 6). The highest survival rate was observed in the group infected only with *N. apis*. The last bees infected with *N. ceranae* and BQCV died five days before the end of the experiment, while the last bees infected with only *N. ceranae* died one day before the end of the experiment. In general, bees infected with *Nosema* spp. and BQCV had a significantly lower survival rate than bees infected only with *Nosema* spp. (Figure 6).

Table 3. Post hoc analysis (Gehan-Wilcoxon test) Bonferroni corrected of differences in survival rates in bees infected with BQCV and *N. apis*, BQCV and *N. ceranae*, *N. apis* and *N. ceranae*.

Group	***N. apis* + BQCV**	***N. ceranae* + BQCV**	***N. apis***	***N. ceranae***
N. apis + BCQV	XXXX			
N. ceranae + BCQV	0.08	XXXX		
N. apis	0.04	<0.0001	XXXX	
N. ceranae	0.15	0.22	<0.0001	XXXX

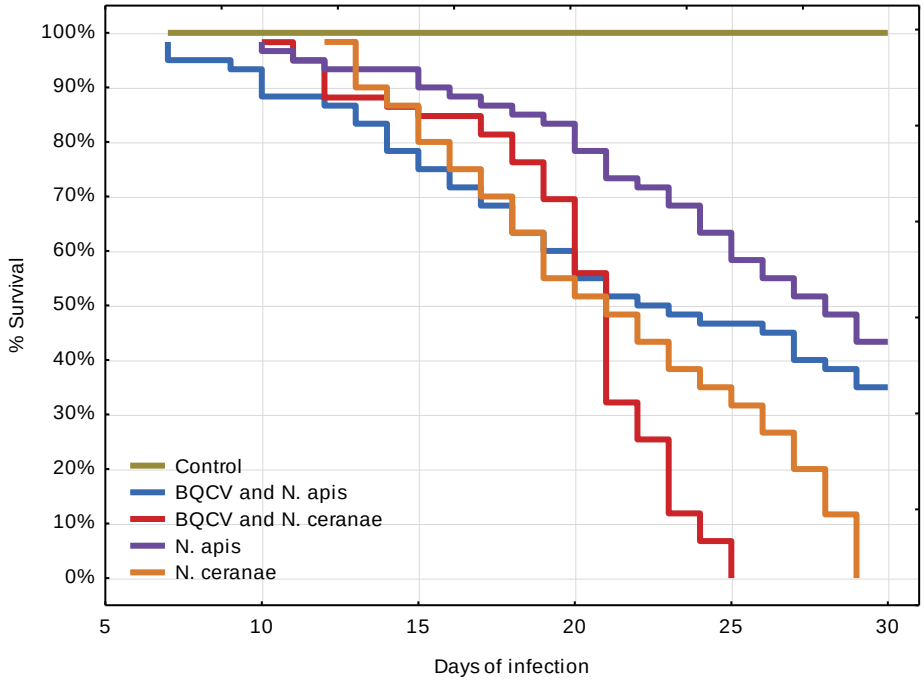


Figure 6. Survival rate in bees infected with *N. apis*, *N. ceranae*, BQCV + *N. apis* and BQCV + *N. ceranae*.

BQCV cDNA copy numbers in bees infected with *N. ceranae* and BQCV grew significantly more overtime than cDNA copy numbers in bees infected with *N. apis* and BQCV (Figure 7).

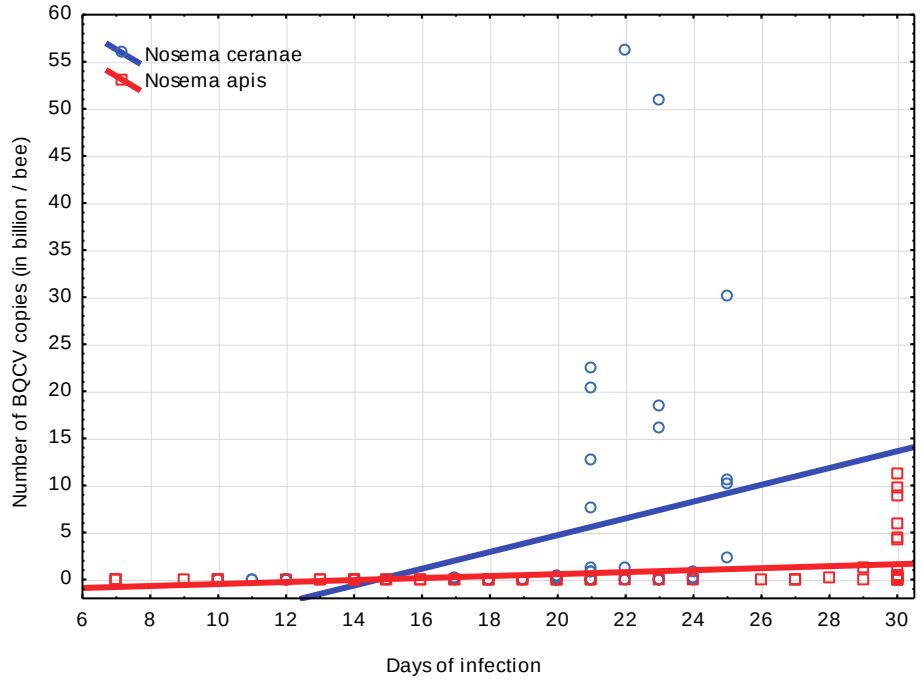
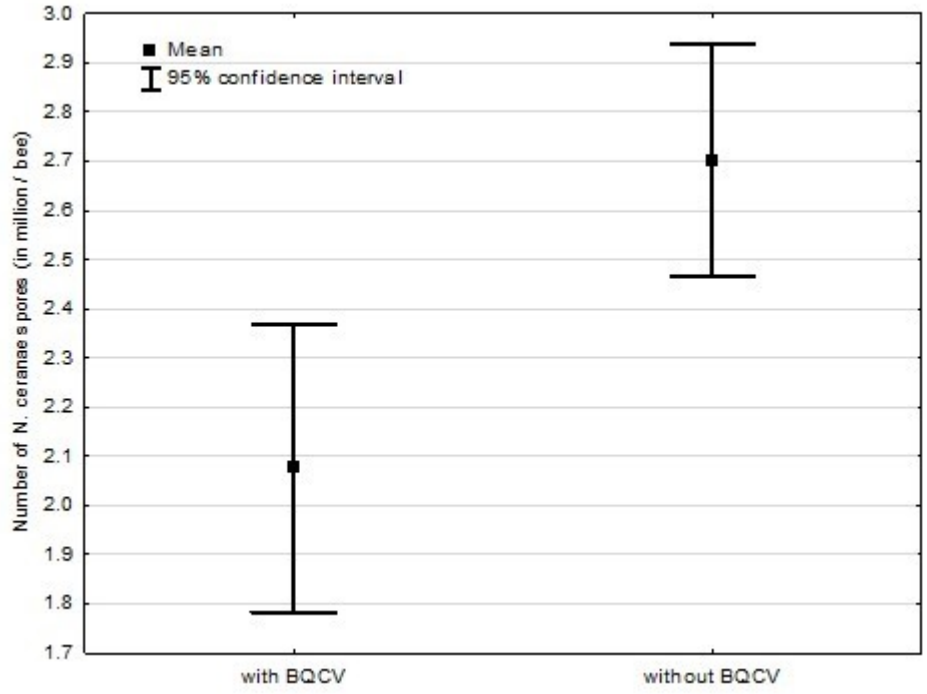


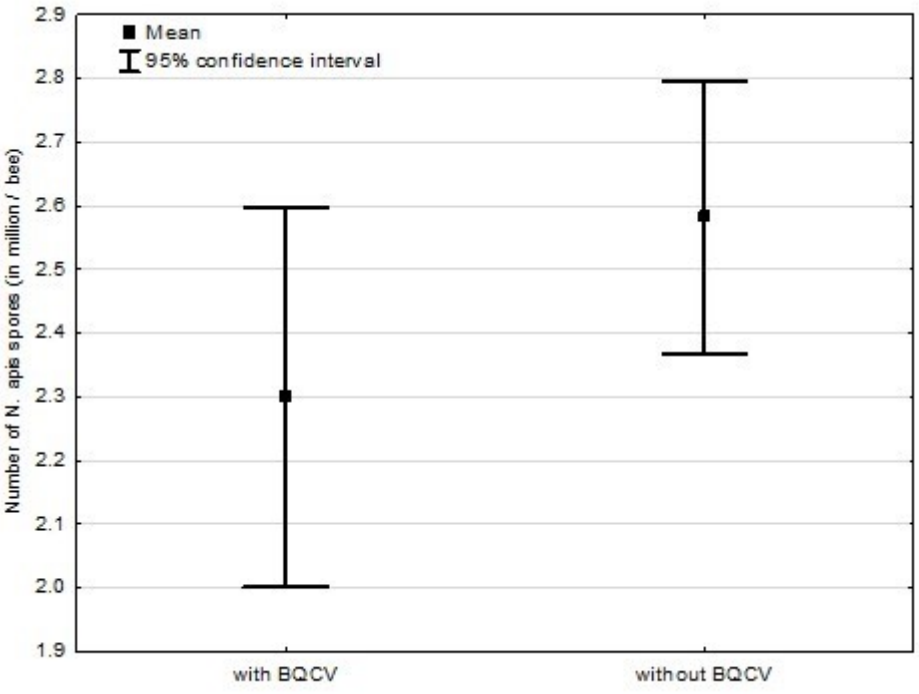
Figure 7. Overtime BQCV cDNA copy numbers in bees infected with *N. ceranae* and BQCV (shown as a blue line) and *N. apis* and BQCV (shown as a red line) Pearson's product-moment correlation coefficient (r). For *N. ceranae* r = 0.41, $p = 0.001$. For *N. apis* r = 0.10, $p = 0.443$.

Interestingly, spore counts in bees infected with *N. ceranae* and BQCV were significantly lower than in case of bees not infected with BQCV. Additionally, in the case of bees infected with *N. apis* and BQCV, the same trend was observed, although it was not as clear as in

the group infected with *N. ceranae* and BQCV (Figure 8). In general, BQCV cDNA copy numbers were significantly higher in bees infected with *N. ceranae* and BQCV in comparison to bees infected with *N. apis* and BQCV (Figure 9).



(**a**)



(**b**)

Figure 8. *Nosema* spp. spore counts in bees infected and not infected with BQCV and (**a**) *N. ceranae*, Mann-Whitney U test $p = 0.001$ (**b**) *N. apis* Unpaired Student's *t*-test $p = 0.135$.

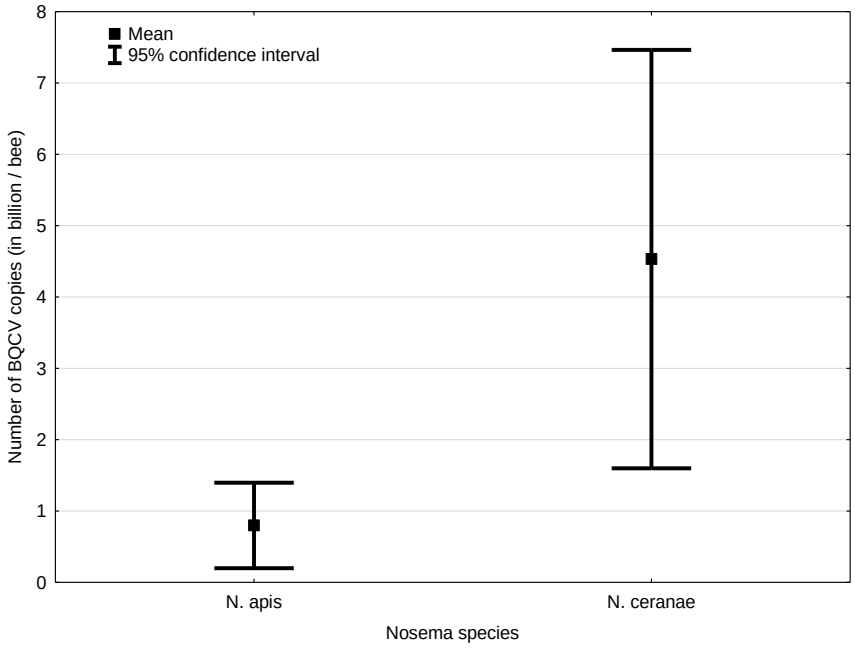


Figure 9. BQCV cDNA copy numbers in group of bees infected with *N. ceranae* and BQCV and *N. apis* and BQCV Unpaired Student's *t*-test $p = 0.017$.

4. Discussion and Conclusions

4.1. Experiment 1: Competition between Nosema ceranae and Nosema apis in Mixed Infection

Our results suggest that *N. ceranae* has a negative effect on the longevity of infected bees. The effect seems to be more clearly visible than in case of *N. apis*, although in the first experiment (when the bees that survived were frozen at 24th day post infection) only the tendency (a clear one nevertheless) was observed. However, during the second experiment the difference was significant, which confirms the findings of a Canadian study [36]. However, this contradicts with findings from Sweden and Germany [35,37], where such a difference was not found.

This may suggest a geographical/climatic component of the virulence level of *N. ceranae* compared to *N. apis*. As mentioned before, in the warmer parts of Europe it causes high colony losses, and in the colder ones it seems to be quite a mild infection.

There is also the experimental design component, which may significantly impact the results. We infected five-day old bees and mentioned authors who infected, respectively, two-week old bees, 48 h old bees and three-day old bees. Younger bees may be more susceptible to *N. ceranae* infection since their perytrophic membrane is slightly thinner and may be more easily penetrated by the spores and the shorter life cycle of *N. ceranae* than *N. apis* (three days, and five days respectively) [5] enabling faster multiplication. It would also explain why we found higher *N. ceranae* DNA copy numbers in mixed infection (*N. apis* and *N. ceranae*), but also singular infections with either *N. apis* or *N. ceranae*. Young bees may also be more prone to energetic stress [7], which is more evident during the infection caused by *N. ceranae* than *N. apis*. It was proven [38] that five-day old bees (or slightly older) are the most susceptible to infection. However, the two-week old bees may just be too old already, and their peritrophic membrane too thick to accommodate *Nosema* as well as in the case of younger bees. On the other hand, the newly emerged bees are known to be more resistant to *Nosema* infection (the reasons of this phenomenon are still unclear), therefore newly emerged bees are always free from infection, even after they chew on infected wax or eat infected food [38]. Our findings confirm that bees infected with *N. ceranae* had higher cumulative mortality than those infected with *N. apis* [39]. The results from our experiment also correspond with the research showing higher virulence of *N. ceranae* than *N. apis* [16]. Discrepancies between results may be also due to different temperatures in which bees were kept, namely bees in the Swedish experiment [35], which

were kept in 30 °C, when bees in our experiment were kept in 34 °C, quite similarly to a 2009 study [40] with which we received comparable results.

We think that infecting young bees and keeping them at a temperature of 34 °C more accurately mimics natural conditions, where bees can be infected during the first days after emergence. During the following days, they mainly stay in warmer parts of the hive because of the tasks performed in the colony, allowing the spores time to multiply. This is why, in field conditions (apart from constant ingestion of spores with food overtime), the numbers of spores in older bees are higher.

In our experiment, infection of individual bees at the 5th day post emergence with *N. ceranae* resulted in the first deaths after the 14th day post infection, similar to the case of bees infected with *N. apis* (in the second experiment it was 12 and 10 days, respectively), and with the mixture of both species. These findings are similar to the results of another study researching the virulence of both *Nosema* species in individual bees [35]. But another study [31] found that the first deaths were observed at the 6th day post infection, and all infected bees were dead at the 8th day post infection. The difference between results might have been caused by using bees from different times of the season, namely, in the contradicting paper, late spring bees were used, and in our experiment we used August bees, which are practically winter bees already and have different body constitutions and, in general, live much longer than spring bees. We also did not use CO_2 when handling the bees, since it was proven to shorten their life significantly [41,42]. In addition, more recent studies clearly show, that CO_2 treatment influence was so considerable, that the influence of *N. ceranae* on the longevity of bees could no longer be observed [43].

It is also highly possible that bees (either used for the experiment or used to extract spores from) in experiments of most of the contradicting studies, might have been infected with BQCV (partly or entirely), which was not investigated. The results might be due to presence of this virus in the group of bees infected with *N. apis* and absence in the group infected with *N. ceranae* [38] and its presence in the *N. apis* spore solution [20]. Adding the BQCV antiserum to *Nosema* spore solutions eliminated this possibility in our experiments.

Our finding, that *N. ceranae* is highly competitive against *N. apis* in mixed infections and reaches much higher spore counts contradicts other results [20], in which *N. apis* was the one with higher (or similar to *N. ceranae*) spore counts. This might suggest that there may be several *N. ceranae* strains exhibiting different virulence rates, but this was proven untrue [44], so, in this case, a conclusion that different bee subspecies undergo nosemosis differently seems logical, which is supported by the Danish case [45] and other studies [39,46,47]. In one of the studies [38], *A. m. ligustica* was used, whereas in others it was Buckfast bees [36] and *A. m. carnica* [37].

4.2. Experiment 2: Influence of BQCV on the Course of Nosema Infection

In the 1980s [22], it was proven that bees infected with *N. apis* and BQCV lived shorter than bees infected with *N. apis* alone. In our study, in the case of *N. ceranae*, this effect was expressed even better. This can also be correlated to the higher number of virus copies in bees with *N. ceranae*, which, because of a very quick multiplication and causing much more damage to epithelial cells than *N. apis*, opened more entrance sites for BQCV. What is more, greater (than with *N. apis*) energetic stress caused by *N. ceranae* can explain why BQCV would multiply better in *N. ceranae* infected bees.

The fact that *Nosema* and BQCV act synergistically in adult honeybees and induce high and rapid mortality is in agreement with several other findings [36,48] and seems to be universally true.

Occurrence and mean peptide count of viral pathogens and Nosema in honeybee colonies sampled from widespread locations in USA CCD colonies showed, instead, a high frequency of iridescent virus and a low frequency of BQCV [49].

Bees infected with either species of *Nosema* and BQCV showed lower spore counts than the ones infected with only *Nosema*. Since dead bees were investigated, this result was probably due to the synergistic effect of the virus and *Microsporidium*, which caused faster death of bees, hence the spores could not multiply further. As the effect was much stronger in the case of *N. ceranae*, as expected, this dissonance is much clearer between bees with *N. ceranae*, together with BQCV, and bees with only *N. ceranae*, than in respective groups with *N. apis*.

Since this experiment was conducted in 2013, we based the design on available publications on this topic [22,50], which state that there is no virus propagation, or it is very minor, without the presence of *Nosema* sp. However, a more recent study [48] proved that the virus can multiply on its own, so a design with a control group with only BQCV infected bees is needed.

Further experiments are needed since it would also be desirable to compare the above results with spore and virus counts from live bees, as well as through the experiment. Studies on temperature influence on the course of virus/*Nosema* infection are also needed.

It has also become more apparent that a thorough comparison of *Nosema* infection course in different bee subspecies and, simultaneously, different climatic conditions, is needed. Those two factors together may have a greater influence on the course of this infection than previously thought.

This study shows the effects of interaction between *N. ceranae*, *N. apis* and black queen cell virus, as well as the effect of both *Nosema* species on the longevity of bees. All organisms (bees, *Nosema*), as well as the virus, were of Polish origin and show only the interaction of actors of said origin. As shown above, further multi-direction research is needed to say with certainty what influences the pathogenicity/virulence of *N. ceranae*, which, in time, may open new paths to controlling *Nosema* infections and preventing related colony losses.

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Appendix A. Pre-Testing Colonies for the Presence of Viruses and *Nosema* spp.

To test the presence of abovementioned viruses at least 20 emerged bees from each comb were pulverized in liquid nitrogen. RNA extraction was carried out using Total RNA Mini (A&A Biotechnology) according to manufacturer's protocol.

RNA was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) also according to protocol enclosed by the manufacturer.

To detect viruses specific primer pairs were used as shown in Table A1.

Table A1. Primers used to detect specific virus sequences.

Virus	Primer Pairs	Source
BQCV	BQCV76f, BQCV299r	(Bakonyi, 2002) [51]
ABPV	ABPV1f, ABPV2r	(Bakonyi, 2002) [51]
CBPV	CBPV1f, CBPV2r	(Ribiere et al., 2002) [52]
SBV	SBV1f, SBV2r	(Ghosh et al., 1997) [53]
DWV	DWV2345f, DWV2779r	(Lanzi et al., 2006) [54]

All PCRs were performed in 50 µL reactions, each containing 0.3 µM of each (forward and reverse) primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.5 U of Taq Polymerase and 2 µL of query cDNA. A positive and negative (no template) controls were included for every virus.

All reactions were carried out in MiniCycler 25 (MJ Research). Thermal protocol for BQCV, ABPV, CBPV and SBV consisted of a single initial activation step in 94 °C for 3 min followed by 40 PCR cycles (denaturation: 94 °C for 1 min, annealing: see Table A2, elongation: 72 °C for 1 min). The reaction was closed with a final elongation step in 72 °C for 2 min.

Table A2. Annealing temperatures for specific primer pairs.

Primer Pairs	Annealing Temperatures and Times
BQCV76f, BQCV299r	50 °C for 1 min
ABPV1f, ABPV2r	55 °C for 1 min
CBPV1f, CBPV2r	50 °C for 1 min
SBV1f, SBV2r	50 °C for 1 min

Thermal protocol for DWV consisted of an initial activation step in 94 °C for 3 min followed by 35 PCR cycles (denaturation: 94 °C for 30 s, annealing: 53 °C for 1 min, elongation: 72 °C for 30 min). The reaction was closed with a final elongation step in 72 °C for 10 min.

The size of PCR product for each virus is presented in Table A3.

Table A3. The size of PCR product for each virus.

Virus	PCR Product Size (bp)
BQCV	224
ABPV	398
CBPV	455
SBV	646
DWV	435

Products were separated electrophoretically in 1.5% agarose gel. Visualization under UV light and documentation of obtained results was carried out using GelDoc-It Imaging System (UVP).

The absence of *Nosema* spores was first assessed microscopically, and confirmed with a PCR according to the OIE protocol (2008) [34].

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