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Effects of *Varroa destructor* on Hemolymph Sugars and Secondary Infections in Honeybees (*Apis mellifera*)

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Featured Application: Featured Application: Diagnostic techniques have been adapted to better understand the health status of honeybees exposed to *Varroa destructor* over time. These techniques and the results presented herein could help beekeepers to prevent winter losses and to better manage the beehives exposed to life-threatening diseases. The concept of comparative medicine, which has a great importance in veterinary medicine, is put forward in this project.

Abstract: The European honeybee contributes to the agriculture by its pollination; however, the overwintering loss rate over the last decades is worrisome. *Varroa destructor* is considered one of the most important causes of bee colony declines. This project aims to correlate the infestation by varroa to the hemolymph sugar concentrations and bacterial and viral coinfections. Six highly infested and six control hives were compared over time. Pooled hemolymph samples from honeybees were collected for sugar concentration measurements using a previously validated portable glucometer. The hemolymph samples were submitted for bacteriology. Multiplex RT-PCR analysis was performed on honeybees for six viruses: DWV-A, DWV-B, BQCV, ABPV, KBV, and IAPV. There was also no predominance of pathogenic bacteria. In September, sugar concentrations in hemolymph were significantly lower in highly infested hives than in control hives. Infested hives showed markedly higher viral loads except for ABPV. DWV-A and BQCV viral loads from highly infested hives were significantly higher in September compared to July. A continued and severe exposure to varroa leads to increased viral charges and decreased sugar concentrations, suggesting alterations in immunity, metabolism, and reserve mobilization. These parameters contribute to the weakening and mortality of the colonies.

Keywords: honeybee; disease; pathogen; DWV; BQCV; ABPV; KBV; IAPV; sugar; hemolymph

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1. Introduction

Honeybees are an important and fascinating species for humans, with regards to their complex social structure, honey production, and pollination of cultivated crops. Honeybees in temperate climates gain specific physiologic characteristics throughout the seasons to adapt for overwintering [1–4]. Overwintering honeybees show an increased life span [5,6] as well as a larger fat body for better storage of nutrients, such as lipids and proteins, required to respond to the great energy expenditure throughout the winter, from the vibration of their wings to regulating the temperature within the hive [1,7–10].

The increased honeybee mortality rate has become a worrying issue in the last three decades for both the scientific and general population. A Colony Losses Monitoring (COLOSS) survey showed a winter beehive loss rate around 20–25% in Europe [11]. In

Canada, the beehive loss rate even reached 45.5% in the winter of 2022, corresponding to the highest recorded loss since 2007 [12]. This phenomenon is reported to be caused by several factors, most particularly by the parasite *V. destructor* [12,13]. Varroa is thus a great threat for *Apis mellifera* in temperate areas [14].

Varroa has a life cycle that follows that of the honeybee's [15]: the female varroa produces eggs within the honeycomb where the bee pupae grow, causing a weakening of the emerging honeybee [16]. The varroa mother and daughters continue to feed on the adult honeybee, further weakening the bearer [16]. Varroa can also transfer viruses to developing and adult bees when feeding, thus spreading and intensifying viral loads [15–18]. Evidence of mite contribution in viral transmission (vector role) is clearly demonstrated for deformed wing virus (DWV), acute bee paralysis virus (ABPV), cashmere honeybee virus (KBV), and Israeli acute paralysis virus (IAPV) [15–18] (Table A1).

Varroa feeds on the hemolymph and the fat body of the honeybees [19], an organ playing a key role in honeybee overwintering [1] and in metabolic functions such as growth, neural control, immunity, detoxification, and nutrient storage [20,21].

Since varroa infestations lead to altered metabolism and reserve mobilization, sugar concentrations in hemolymph might fluctuate directly from parasite consumption or indirectly from parasitic stress and co-infections [22]. Glucose and trehalose, which are produced in the fat body, are the main energy substrates for honeybees during their flight and their thermoregulation [20,23].

Varroa is also known to affect the immune system of the honeybee by decreasing expression of immune genes such as *apidaecin* [16,24,25] and by decreasing hemocyte counts and melanin synthesis, both playing key roles in immunity [16,26]. Therefore, the susceptibility to opportunistic agents is likely to increase in Varroa-infested honeybees. As a matter of fact, some researchers showed that *Serratia marcescens*, a commensal bacterium from the gut, can induce bacteremia with varroasis [27]. Burritt et al. even isolated a cytotoxic strain (Ss1) of *S. marcescens* in the hemolymph of dying, infested, and overwintering honeybees as well as in varroa mites [27,28].

Diagnostic techniques are currently lacking to evaluate the physiologic and biological impacts of varroa on honeybees. An innovative way to improve knowledge would be to adapt diagnostic techniques commonly used in veterinary medicine (i.e., biochemistry, glucometer, RT-PCR, bacteriology) to monitor the health status of honeybees and, consequently, help beekeepers prevent winter losses and better manage the beehives.

The objective of this study was to determine the impact of varroa infestation on the sugar concentrations in honeybee hemolymph over time and on the presence of concomitant viral and bacterial infections.

2. Materials and Methods

2.1. Cohort Study

A cohort study was planned using 12 hives, including 6 highly infected by varroa mites (Nos. 505, 593, 503, 522, 345, and 427) and 6 control hives (Nos. 465, 208, 258, 328, 277, and 321) from the *Centre de recherche en sciences animales de Deschambault* (CRSAD, Deschambault, QC, Canada). Highly infested and control hives were located 34.8 km apart.

Highly infested hives were one year old and originally maintained for research purposes. The high level of mite infestation was achieved by avoiding any miticidal treatment in the fall preceding the study, adding drone brood and operculated brood, and exchanging worker honeybees between colonies. No varroa mite was directly introduced in the hives. Highly infested hives had 5 to 7 frames at the beginning of the experimentation.

Control hives were two years old and originally dedicated to queen breeding. Control hives had two honey supers throughout the experimentation. The control hives were treated with oxalic acid and strips (amitraz) in the preceding fall.

All hives shared similar genetics derived from sister queens of lineage 416 developed by the CRSAD. Queens from the highly infested hives were one year old at the time of sampling, and those from the control hives were two years old.

2.2. Parasitic Load

The parasitic load of *Varroa destructor* within each hive was monitored using a sticky board on the bottom floor of each beehive. Daily mite falls were recorded over periods of approximately 5 to 7 days (Table A2).

2.3. Honeybee Sampling

Although insects are not covered by the Canadian Council on Animal Care guidelines, all procedures were performed while respecting ethical considerations. Samples of worker honeybees were collected at different time points, as specified in the following sections. On each occasion, worker honeybees were captured from the honey super of each examined hive and placed in a jar with a net and a plastic lid. Samples (n = 250 worker honeybees/time/hive) were transported to the laboratory of the *Faculté de médecine vétérinaire*, *Université de Montréal* (Saint-Hyacinthe, QC, Canada) by car. Upon reception, samples were immediately processed for hemolymph extraction. Additional worker honeybees (n = 10/time/hive) were stored at −80 °C for further viral analysis.

2.4. Hemolymph Sample

At three different time points (on 19 June, 2 August, and 18 September 2019), 40 worker honeybees were used. Honeybees were anesthetized with dry ice (CO_2) and their hemolymph was collected as per Borsuk and al [29]: the antenna was pulled out with clean tweezers, and finger pressure on the thorax created a hemolymph bulla, which was aspirated with a micropipette [29]. Hemolymph samples were pooled in an Eppendorf tube maintained at 0 °C (iced water) to limit melanization [30]. Pooled hemolymph samples from each hive were used for glucose and trehalose measurement and bacteriological culture.

2.5. Sugar Concentrations

Sugar concentrations in hemolymph were assessed at three time points (on 19 June, 2 August, and 18 September 2019), where 10 µL of pooled hemolymph was mixed with saline solution (0.9% NaCl) (1:4 dilution). The glucose concentration in hemolymph was measured five times with a human Accu-Chek glucometer, previously validated for honeybee hemolymph [31]. Trehalose concentrations were indirectly measured five times by the glucometer following trehalase hydrolyzation, which consisted of adding commercial trehalase (Millipore Sigma, T8778, Burlington, MA, USA) to samples, followed by 3 h incubation at 30 °C [31].

2.6. Bacteriology

Hemolymph samples from each hive and time point (on 19 June, 2 August, and 18 September, 2019) were submitted for aerobic bacterial culture: 2 µL per sample of pooled, diluted 1:9 (0.9% NaCl) hemolymph was used to inoculate a Columbia agar medium with 5% sheep blood (CBA) (BD Difco, Fisher Scientific, Ottawa, ON, Canada) and a brain heart infusion (BHI) broth (BD Difco, Fisher Scientific, Ottawa, ON, Canada). Media were incubated at 35 °C ± 2 °C for 48 h. When turbid, the BHI broth was plated on CBA and MacConkey agar (BD Difco, Fisher Scientific, Ottawa, ON, Canada) and incubated at 35 °C ± 2 °C for 24 h. Bacteria were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany). A qualitative approach was used to quantify each bacterial isolate on Columbia Blood Agar (Excel File S1). Bacteria recovered only in BHI broth were recorded as present or absent.

2.7. Virology

Viral analyses on honeybees from the cohort study were conducted to assess the presence of specific viruses (DWV-A, DWV-B, BQCV, ABPV, KBV and IAPV) with RT-qPCR on honeybees collected on 18 July and 26–27 September 2019. Each sample consisted of a pool of 10 worker honeybees originating from each hive at each time point.

Frozen (−80 °C), pooled honeybee samples (n = 10) were placed in stainless metal tubes (P000952-LYSK0-A.0, Bertin Corp, Rockville, MD, USA) and lysed by bead beating (ceramic beads Bertin Corp, P000931-LYSK0-A.0, Bertin Corp, Rockville, MD, USA) with liquid nitrogen. Stainless tubes were transferred and homogenized during 10 s at 8800 rpm and 0 °C (Combo Precellys Evolution + Cryolys, K002198-PEVO0-A.0, Bertin Corp, Rockville, MD, USA). Each resulting homogenized and powdered sample (50 ± 5 mg) was transferred to a 1.5 mL Eppendorf tube on dry ice. RNA was isolated using TriZol (Invitrogen, Waltham, MA, USA) and purified with a Direct-zol kit RNA miniprep (Zymo, Irvine, CA, USA) according to the manufacturer's instructions. Quantification and purity of extracted RNA were evaluated using the Infinite M200 Pro device (Tecan, Männedorf, Switzerland). Extracted RNA samples were stored at −80 °C.

For reverse transcription (RT-PCR), extracted RNA samples were used to generate complementary DNA (cDNA) by using qScript cDNA SuperMix (Quantabio, Berverly, MA, USA) as per manufacturer's instructions; cDNA master mix was stored at −20 °C and used for PCR assays.

For PCR assays, two multiplex PCR reactions were carried out using pairs of primers as described in Table S3. Primers were designed from viral genomic sequences in the GenBank database. For each multiplex PCR assay, 18 µL multiplex mix (complete formula in Table S3) and 2 µL of DNA were used for each reaction. The first multiplex reaction (IAPV, BQCV, KBV, and actin) required one cycle of denaturation and enzymatic activation at 95 °C for 3 min, followed by 40 cycles at 95 °C of 5 s and 60 °C of 45 s. The second multiplex reaction (DWV-A, DWV-B, ABPV, and actin) consisted of one cycle of denaturation and enzymatic activation at 95 °C for 3 min, followed by 40 cycles at 95 °C of 5 s and 60 °C of 20 s. Quantification of expressed genes from PCR was assessed with TaqMan qRT-PCR using the AriaMx software (Agilent, Santa Clara, CA, USA). Data from AriaMx software was exported into a Microsoft Excel file. Standard curves were prepared by 10-fold serial dilutions (103 to 109) of DNA segments that included target and referenced amplicons (gBlock, IDT, Coralville, IA, USA). The number of copies was reported for each amplification.

2.8. Statistical Analysis

All analyses were performed within the R statistical environment. The Shapiro–Wilk test was used to determine if data or residuals were normally distributed (except for sugar concentration, where the Anderson–Darling test was used). A Levene test was used to assess the variance in the homogeneity of data. If data followed a normal distribution, and variance was homogeneous, *t*-test, lineal model, or linear mixed models (LMM) were used. Otherwise, nonparametric methods (Mann–Whitney or Wilcoxon tests for paired data) were used.

Natural varroa mite fall in each group (Excel File S2) was compared over determined time periods. The average of natural varroa mite fall in each hive over each period was compared with a LMM with the group (control vs. varroa), periods, and the interaction between treatment and period as fixed factors. Hive identity was used as a random factor considering auto-replication. A posteriori comparison (post hoc Tukey test) was performed combined with a downward adjustment of the alpha level with the Bonferroni correction. Post hoc Tukey test helped to elucidate which specific period showed significant differences between group means.

For sugar (glucose + trehalose) concentration analysis, the average of the five replicates of combined sugar concentrations for each hive at each time point was compared using a LMM with the group (control vs. varroa), time (19 June, 2 August, or 18 September 2019), and the interaction between treatment and time as fixed factors and treatment nested within the hive as a random factor. A priori contrasts between pairs of means were performed combined with a downward adjustment of the alpha level with the Benjamini–Hochberg procedure.

Several statistical tests were used for the six studied viruses. A Mann–Whitney test was performed between ratios of KBV, ABPV, DWV-A, and DWV-B/actin in each group

(control vs. varroa). A Wilcoxon test was performed for paired data (KBV, ABPV, DWV-A, and DWV-B/actin) to assess the effect of time. A linear model was used to determine the relationship between the ratios of BQCV/actin and both group and time. Mean ratios of IAPV/actin were compared between groups (control vs. varroa) with a *t*-test. Data from the highly infested hive No. 345 was removed from the analysis since a varroa mite was mistakenly included in the pooled honeybee sample.

3. Results

3.1. Parasitic Load

The natural daily varroa mite fall was significantly higher in infested hives (averaging 67–230 fallen varroa mites/day) compared to control hives (averaging 4.30–12.56 fallen varroa mites/day) from mid-July to late September ($p < 0.0001$), excluding June ($p = 1.00$, post hoc Tuckey test in LMM) (Figure 1). The number of fallen varroa at the bottom of the hives in the highly infested group significantly increased over time ($p < 0.0001$, post hoc Tuckey test in LMM) (Figure 1) from July to late September, with the highest count recorded in September with 230 fallen varroa mites/day.

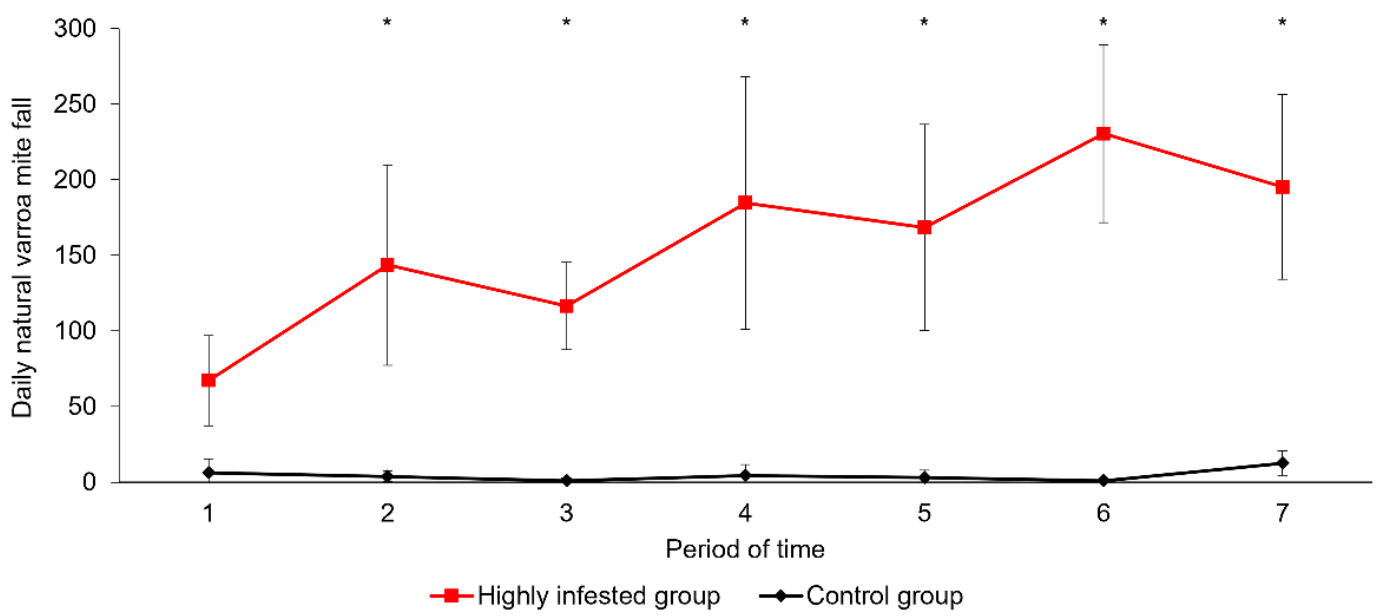


Figure 1. Daily varroa mite fall in highly infested and control hives over time. Average data (+/−SD) over determined periods for each group are shown in the figure. Daily varroa mite fall was significantly higher in infested hives compared to control hives for most periods (*, $p < 0.0001$, post hoc Tukey test in LMM) except for the first period ($p = 1.00$, post hoc Tukey test in LMM).

3.2. Sugar Concentrations in Honeybee Hemolymph

Sugar (glucose and trehalose) concentrations at the first two time points (19 June and 2 August) were similar between groups and stayed relatively constant. At the third time point (18 September), highly infested hives showed a significantly lower concentration of sugars in hemolymph (mean concentration = 289.44 ± 46.39 mmol/L) compared to control hives (mean concentration = 390.11 ± 61.03 mmol/L) ($p < 0.001$, a priori contrasts in LMM) (Figure 2). Combined sugar concentration in hemolymph was significantly higher in the control hive group on 18 September compared to the first collection time points (19 June and 2 August) ($p \leq 0.0001$, a priori contrast in LMM) (Figure 2).

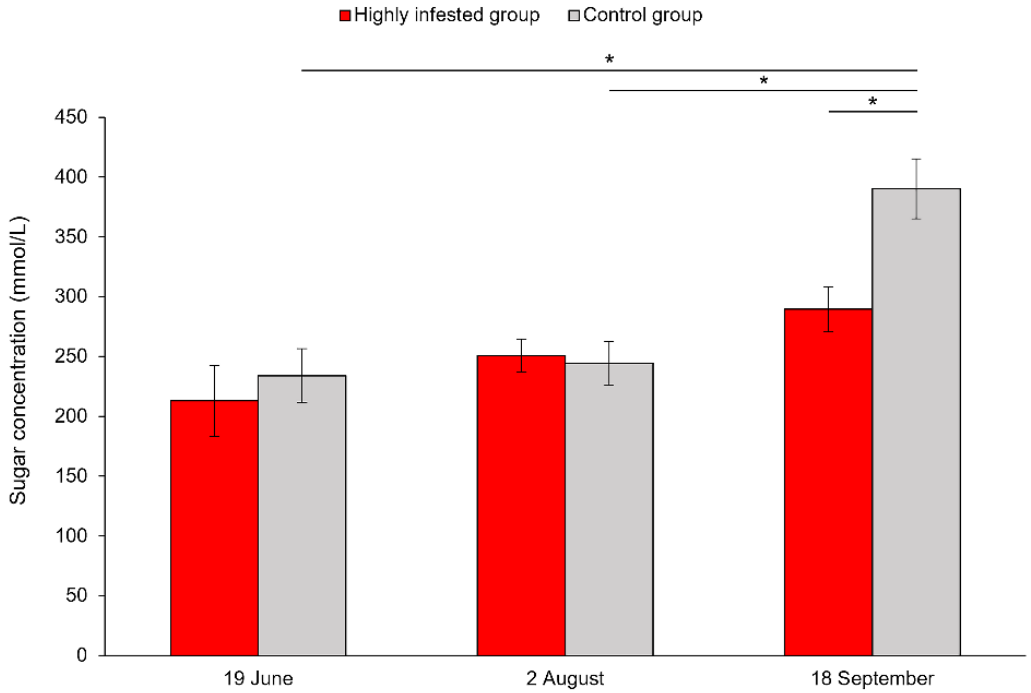


Figure 2. Sugar (glucose and trehalose) concentrations in hemolymph through time. On 18 September 2019, highly infested hives showed a significantly lower concentration of combined sugars in hemolymph compared to control hives (*, $p < 0.001$, a priori contrasts in LMM).

3.3. Bacteriology

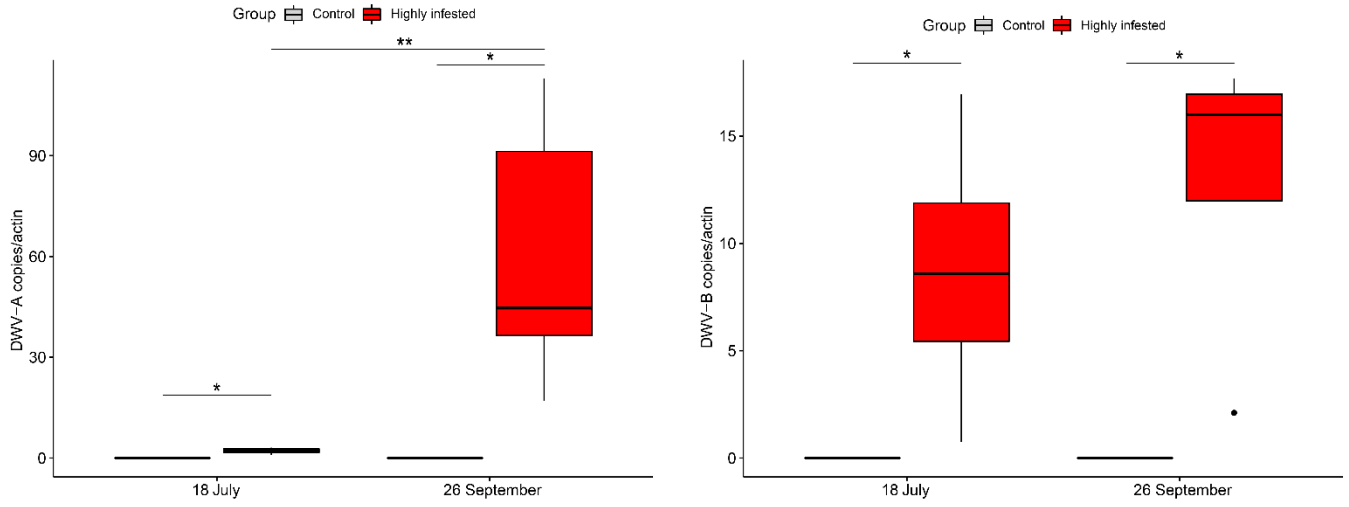
There were no predominant bacteria detected in either highly infected or in control hives aside from nonpathogenic bacteria: *Lactobacillus* spp., *Staphylococcus epidermidis*, *Staph. aureus*, *Klebsiella* spp., or *Enterobacteriaceae*. *Serratia marcescens* was only identified in one control hive (No. 328) and one infested hive (No. 522) (Excel File S1).

3.4. Virology

Multiplex PCR results show viral co-infections (DWV-A, DWV-B, BQCV, ABPV, KBV, and IAPV with higher viral loads when honeybees were highly infested by varroa. A significant difference in viral loads (copies/actin) between infested and control groups was observed at each sampling time ($p = 0.013$, 0.036, 0.004, and <0.0001; *t*-test, Mann–Whitney and linear model) except for ABPV (Figures 3–5). Viral loads were significantly higher ($p < 0.05$) in late September than in late July for DWV-A and BQCV (Figures 3a and 4a). There was also a trend toward an increased viral load throughout time for KBV and ABPV (Figures 4b and 5a) but not for DWV-B and IAPV.

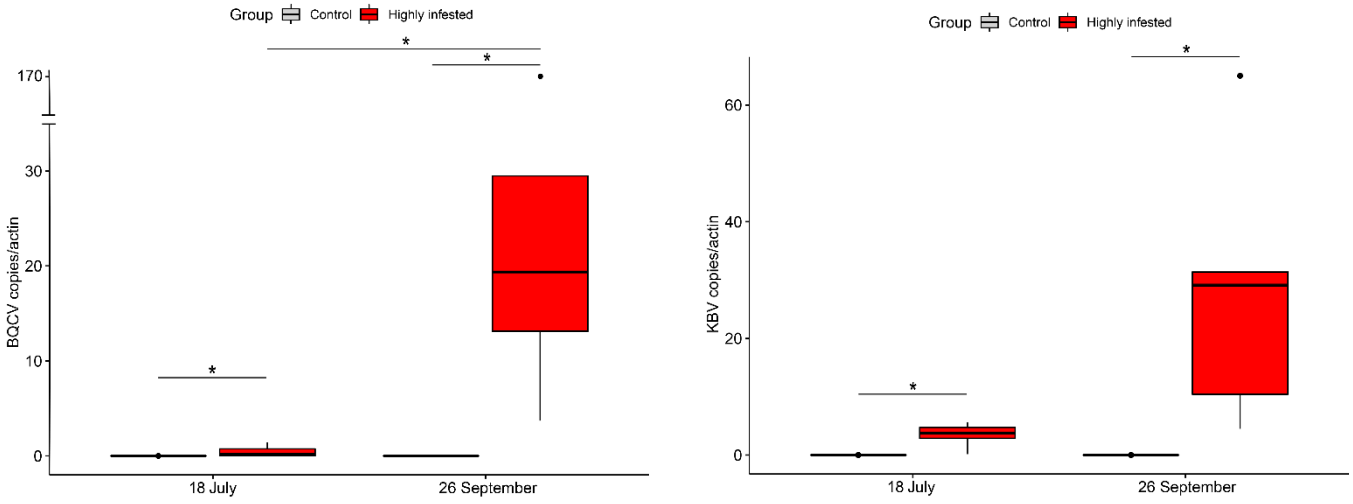
The ratios (copies/actin) of DWV-A, DWV-B, BQCV, ABPV, KBV, and IAPV in control hives were near zero or non-detectable over time (from late July to late September) (Table S4).

DWV-A loads were significantly higher in infested hives at each time point ($p < 0.05$, Mann–Whitney test) than in the controls, with a significant increase ($p = 0.001$, Wilcoxon test) over time (from late July to late September) (Figure 3a). In July, most infested hives had DWV-A loads that were near zero, except for hives No. 345 and No. 593, with ratios of 2.33 and 3.11, respectively (Table S4). In late September, DWV-A ratios varied from 16.99 to 112.90 in the infested hive group (Table S4).



(**a**) (**b**)

Figure 3. DWV loads on July and September in honeybees of highly infested and control hives: (**a**) DWV-A loads (copies/actin); (**b**) DWV-B loads (copies/actin). Overall, a predominance of DWV variant A is seen. DWV-A and DWV-B copies/actin were significantly higher in infested hives than in controls (*, $p < 0.05$, Mann–Whitney test), with a significant increase over time (**, $p = 0.001$, Wilcoxon test) for DWV-A.



(**a**) (**b**)

Figure 4. Viral loads in in honeybees of highly infested and control hives over time: (**a**) BQCV loads (copies/actin); (**b**) KBV loads (copies/actin). Viral copies/actin for BQCV and KBV were significantly different between the two studied groups (*, $p < 0.001$, linear model and Mann–Whitney test) at each period of the cohort study. There was a great increase of BQCV copies/actin in highly infested hives over time (*, $p < 0.001$, linear model).

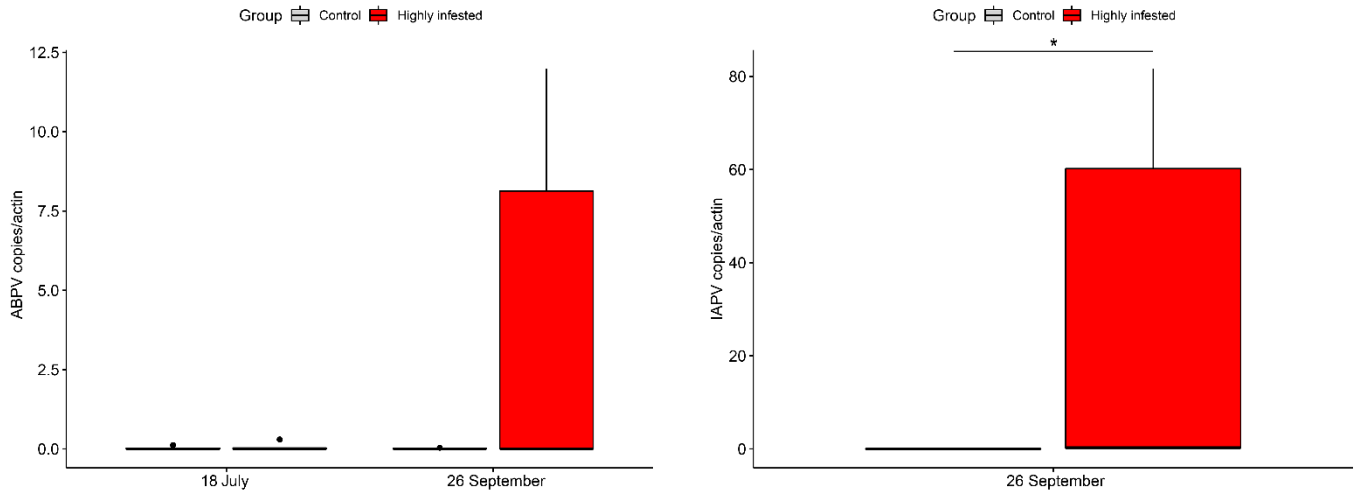


Figure 5. Viral loads in in honeybees of highly infested and control hives over time: (**a**) ABPV loads (copies/actin); (**b**) IAPV loads (copies/actin). Infested hives Nos. 522 and 505 showed a high ratio of viral copies in September for both ABPV and IAPV. A significant difference (*, $p < 0.05$, *t*-test) between studied groups was seen for IAPV.

For each sampling time, DWV-B loads were significantly higher in the infested group than in the control ($p < 0.05$, Mann–Whitney test) (Figure 3b). Throughout the experiment (from late July to late September), the DWV-B loads in the infested group remained unchanged ($p > 0.10$, Wilcoxon test) (Figure 3b), with ratios (copies/actin) from 0.76 to 17.65 (Table S4).

BQCV loads was significantly different between the two studied groups ($p < 0.001$, linear model) (Figure 4a). From late July to late September, there was a great increase of BQCV loads in highly infested hives ($p < 0.001$, linear model) (Figure 4a), with ratios (copies/actin) varying from 3.70 to 29.11 and reaching up to 170.36 for the hive No. 522 (Table S4).

KBV loads from the hives with severe parasitic loads were significantly higher than the controls ($p < 0.001$, Mann–Whitney test) and without an increase over time ($p > 0.05$, Wilcoxon test) (Figure 4b). In late July, KBV ratios were relatively low, from 2.66 to 5.63 in highly infested hives (Table S4). In late September, ratios remained similar to the first time point for most hives: ratios were from 4.49 to 31.38 but were much higher in the hive No. 522, with a ratio of 65.02 (Table S4).

In late September, ABPV ratios were 8.13 and 11.98 in infested hives No. 522 and No. 505 (Table S4). There were no significant differences between the two studied hive groups at each time point ($p > 0.10$, Mann–Whitney test) nor a significant increase in time ($p = 0.27$, Wilcoxon test) (Figure 5a).

In late September, IAPV loads were significantly higher in infested hives than in control hives ($p = 0.013$, *t*-test) (Figure 5b), with high ratios of 60 and 80 in the infested hives No. 522 and No. 505, respectively (Table S4).

However, ABPV and IAPV ratios were near zero or non-detectable in the other hives of both groups throughout the experimentation (Table S4).

4. Discussion

4.1. General Discussion

This study, including hives exposed to extreme loads of varroa mites, helps to understand the progression of viral infections and sugar concentrations in hemolymph throughout the season in correlation with the degree of infestation.

During the summertime (June and July), honeybees were able to maintain their homeostasis despite the parasitism, which was reflected by the stability of sugar concentrations in hemolymph. In the fall (late September), viral loads increased significantly, and the combination of a high parasitism and viral infections led to an alteration of their metabolism, demonstrated by the decreased sugar concentrations in hemolymph. This cohort study was initially planned to continue throughout overwintering. However, three weeks after the last time point (late September), four highly infested hives were dead, and the two others were very weak, containing only a handful of live bees.

The particularity of this study is its use of sugar concentrations in hemolymph as a predictive indicator of honeybee winter loss. However, further studies need to be performed to confirm this hypothesis.

4.2. Parasitic Load Monitoring

The infested beehives had an extremely high infection rate, with a mean drop of 145 mites/day (Figure 1), which greatly exceeds the maximal threshold of 15 fallen mites per day (end of June and early August) recommended to establish an immediate miticide treatment (*Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec*—MAPAQ). The fall rate of mites from the control hives was generally below 10 over the observational period, indicating a good miticide control as per the MAPAQ monitoring chart. As this cohort study included a group of hives that were exposed to extreme parasitic conditions, the comparison between the two groups and the evaluation of the effects of varroa on honeybees are also enhanced.

This study stresses the importance of controlling mite infestation by mid-August as per the modeling of mite population elaborated by DeGrandi-Hoffman et al. [15,32]. The difference in varroa parasitic load between the studied groups was insignificant in the first week of the experimentation (June), supporting the importance of an early miticide control to prevent an excessive growth of mite populations later in the summer and fall.

The sticky board method for monitoring of varroa parasitic load is frequently used in Quebec since it is easy to install and has a high sensitivity. However, this technique shows a great inter-day variation and does not give a direct estimate of mites per bee within the hive since it represents only the dead parasites and does not consider varroas in their reproductive stages [15]. Therefore, the exact number of varroa mites within the hive is much higher.

4.3. Sugar Concentrations in Hemolymph

According to Božič and Woodring, trehalose concentrations are higher in the fall than in the spring since foraging activity is less important later [33]. Similar observations were seen in this study. Concentrations in the control group were higher in late September than in late July and early August and higher than the infected bees in late September (Figure 2). As trehalose is a major part of the total sugar concentration, this could explain the higher concentrations observed in the control group in late September (Figure 2).

Regulation of the relationship between trehalose and appetite and sugar mobilization in fat bodies might be disrupted when honeybees are parasitized, especially in association with a high viral charge leading to decreased trehalose concentrations [34,35]. On the other hand, insects under high stress can better resist when sugar concentrations are higher [36], which might explain the weakness and death of highly infested hives.

4.4. Bacteriology

Most of the hemolymph samples contained scarce bacteria from the normal honeybee flora, likely from contamination, such as *Lactobacillus* spp., *Staphylococcus epidermidis*, *Staph. aureus*, *Klebsiella* spp., or *Enterobacteriacae* [37–39]. These bacteria come from contamination from the bees' gut or from the non-sterile manipulation of the honeybee during hemolymph collection from antenna.

Serratia marcescens was isolated in one highly infested hive (No. 522) as well as one control hive (No. 328). Based on the data obtained herein, our study does not support the transmission of *S. marcescens* in the hemolymph by varroa.

4.5. Virology

A strong correlation between varroa parasitism and viral loads was observed in this study. There were also simultaneous viral infections (DWV-A, DWV-B, BQCV, ABPV, KBV, and IAPV) with charges that were more important when honeybees were heavily infested by varroa mites (Figures 3–5). Viral loads were significantly higher in highly infested hives than in the controls for most studied viruses (Figures 3–5) except for ABPV (Figure 5a). Consequences of these high viral loads are also more evident in highly infested hives, as demonstrated by the death of colonies in the fall.

Fall is an optimal period for the increase of viral charges, where the number of phoretic varroa mites is much higher in the colony, combined with a great number of honeybees infected by viruses that are available to feed on [40,41]. In other words, the higher density of varroa mites increased the transmission of viruses [42]. According to Francis et al. [43], viral titers for ABPV, KBV, IAPV, and DWV increase from spring to fall, as observed in our study for DWV and KBV. In this study, the increase of KBV loads until fall was not statistically significant, as previously seen in other studies [44,45]. European studies also present increased DWV charges in the fall [46] as well as for ABPV, showing seasonal variation in the viral prevalence and charges [43,47,48].

Fall is also a critical season for colony weakening and mortality since honeybee reproduction decreases. Overall, exposure to varroa [49,50] and higher DWV and ABPV titers in the fall are correlated with overwintering mortality [43,50,51]. Further studies need to be performed to establish a correlation between viral charges and overwintering mortality in Canadian territory.

The dissemination of viruses in the colony is intensified by the presence of *Varroa destructor*. As demonstrated by many studies, *V. destructor* is a major contributor of horizontal transmission of viruses, including DWV, ABPV, KBV and IAPV [15,18,44,48,52–57]. Phoretic and reproductive varroa mites ingest viral particles when feeding on an infected honeybee and can subsequently inject saliva containing viral particles into another healthy bee [58]. This inoculation directly into the hemolymph increases the pathogenicity of most viruses [45,48,52,55,59–63].

Among those viruses, it is mostly accepted that DWV also replicates in varroa mites [57,64,65] (Table A1). It has been suggested that other viruses, including KBV and IAPV, can also replicate within mites [48,53,66] (Table A1).

RT-PCR analysis demonstrates that honeybees can be constantly exposed to small viral charges even with a controlled parasitic load. Indeed, viruses were detected throughout the experiment, albeit with small ratios. As seen in the control hives, a good management of varroa controls viral infections and subsequent mortality [67], supporting the importance of varroa treatment to limit the viral loads within the colony [15,43].

The results show a strong correlation between DWV loads and varroa infestation (Figure 3a,b), as also demonstrated by other researchers [47,49,57,68,69] and for which a synergistic interaction has been previously demonstrated [64].

This study helps to evaluate, chronologically, the genetic dynamic of two DWV genotypes (DWV-A and DWV-B) in the honeybee colonies of a specific area, i.e., Quebec, Canada. This study shows a decreased DWV genotype diversity under severe varroa infestation. The two DWV genetic variants co-exist with an exposure to a high parasitic load, with a predominance of DWV-A in September. Varroa might be involved in the selection of some genotypes that are better replicated [70–72], which thus diminishes genetic diversity [69]. The impact of this genotype shift on colony mortality would need further studies [73], and a closer evaluation of the DWV genotype variation in Quebec apiaries would be useful.

Data from this study show a significant increase of BQCV loads in highly infested hives over time (Figure 4a), as observed in a previous Spanish study [74]. BQCV might be an

opportunistic infectious agent, as seen in immunosuppressed honeybees [41,74]. Although a clear correlation between BQCV and varroa infestation has not been established [18,52,64], a recent study suggests its role as a mechanical vector [66]. Our study also suggests that varroa infestation loads influence BQCV loads in honeybees.

5. Conclusions

In conclusion, this study demonstrates that honeybees exposed to high loads of *Varroa destructor* have a higher load of different viruses, with charges increasing through time but without increasing the risk of bacterial infections. This long exposure to combined parasitism and viral infections leads to decreased sugar concentrations in hemolymph and colony mortality. These life-threatening parameters, particularly in the fall, might be potential predictors of overwintering colony losses in Canada.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122211630/s1, Excel file S1: Bacteriological results; Excel file S2: Varroa mite monitoring; Table S3: Primers and probes used for viral analysis; Table S4: Summary of the viral infection results between highly infested and control hives over time. References [75–80] are cited in the supplementary materials.

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Appendix A

Table A1. Review of the vector role of varroa in transmission of honeybee viruses.

Analyzed Virus	Vectorial Role of Varroa *	
	Mechanical	Multiplicative
Deformed wing virus (DWV)	Yes [a–g]	Demonstrated for • DWV-A [h] • DWV-B [g, i–k]
Acute bee paralysis virus (ABPV)	Yes [d, l–n]	No [d]
Cashmere bee virus (KBV)	Yes [d, p, q]	Suggested [o]
Israeli acute paralysis virus (IAPV)	Yes [r, s]	Suggested [r]

Table A1. *Cont.*

Analyzed Virus	Vectorial Role of Varroa *	
	Mechanical	Multiplicative
Bee queen cell virus (BQCV)	No [t, u] Possible [o]	Uncertain [o]

References (author, date) * Complete references are available in the main article. [a]. Bowen-Walker et al., 1999 [65]; [b]. Erban et al., 2005 [81]; [c]. Nordström, 2003 [82]; [d]. Ribière, 2008 [48]; [e]. Santillán et al., 2008 [58]; [f]. Tentcheva et al., 2004 [47]; [g]. Yue and Genersh, 2005 [71]; [h]. Annoscia et al., 2019 [83]; [i]. Gisder and Genersh, 2020 [84]; [j]. Gisder et al., 2009 [72]; [k]. Ongus et al., 2004 [85]; [l]. Ball, 1983 [86]; [m]. De Miranda et al., 2010 [44]; [n]. Wiegers, 1988 [56]; [o]. Lester et al., 2022 [66]; [p]. Shen et al., 2005 (1) [54]; [q]. Shen et al., 2005 (2) [55]; [r]. Di Prisco, 2011 [53]; [s]. McMenamin and Genersh, 2015 [87]; [t]. Amiri et al., 2021 [52]; [u]. Vidal-Naquet, 2015 [18].

Table A2. Timeframes used for the comparison of daily varroa mite fall per group.

Timepoint	Highly Infested Group	Control Group
June	18–28 June	18–28 June
Mid-July	11–17 July	11–17 July
Late July	24–29 July	July 29–8 August
Late August	21–26 August	23–26 August
Early September	26 August–5 September	28 August–5 September
Mid-September	5–17 September	5–20 September
Late September	17–26 September	20–30 September

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