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Treatment of waxborne honey bee (Hymenoptera: Apidae) viruses using time, temperature, and electron-beam irradiation

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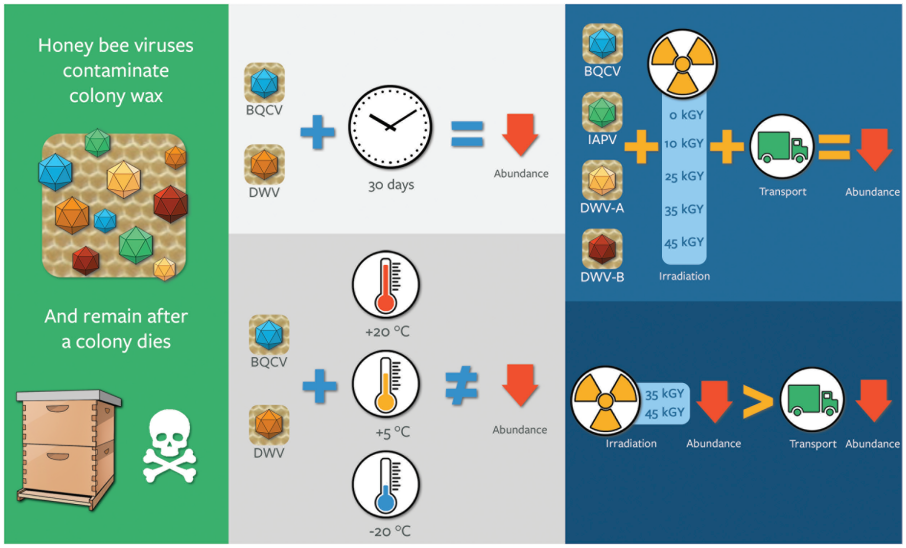
Viruses are one of many serious threats to honey bee (*Apis mellifera* L.) health. There are many transmission routes for honey bee viruses, and there is potential for wax comb to act as a reservoir for transmission of viruses. Some work has been done on treating viruses on wax, focusing on irradiation as a potential treatment. However, irradiation is not universally available or economically viable for beekeepers in many regions. With increased colony deaths over winter beekeepers potentially risk further loss from reusing contaminated equipment from dead colonies. Here we explored the use of storage time and temperature on the reduction of waxborne virus levels from winter loss colony wax over 30 days and at –20, 5, and 20 °C. Furthermore, because irradiation has previously worked against waxborne viruses, we performed a dosage experiment with electron-beam irradiation. Winter loss wax was again used, and exposed to 10, 25, 35, and 45 kGy irradiation, including a nonirradiated transport control. Storage time decreased abundance of black queen cell virus and deformed wing virus at times equal or greater than 30 days but temperatures had no significant effect on virus levels. All irradiation doses decreased virus abundance and prevalence, yet only 35 and 45 kGy did so at a greater rate than the effect of transport alone.

Key words: honey bee virus, irradiation, black queen cell virus, deformed wing virus, Israeli acute paralysis virus

Graphical Abstract



Introduction

When honey bee (*Apis mellifera* L.; Hymenoptera: Apidae) colonies die over winter, beekeepers incur the economic cost of replacing the bees themselves and are also left with potentially pathogen-contaminated hive equipment. Hive equipment is costly to replace, and if the comb is replaced with wax foundation the bees have to expend further energy to draw out the wax, potentially further reducing productivity. A major factor affecting winter survival in honey bees is their parasite and pathogen load, with the ectoparasitic mite *Varroa destructor* Anderson and Trueman (Acari: Varroidae) and various honey bee viruses as key elements affecting honey bee health (Guzmán-Novoa et al. 2010, Nguyen et al. 2011, Berthoud et al. 2015, Carreck et al. 2015, Steinmann et al. 2015, Kulhanek et al. 2021). A potential risk of reusing equipment from colonies that have died over winter is that viable viruses may remain on wax, which could serve as a fomite or passive transmission route of the many viruses present in colonies. Unfortunately, little is known about the risk of reusing comb contaminated by viruses.

One of the most common honey bee viruses is deformed wing virus (DWV). Overt infections of DWV cause pupal deaths and wing deformation coupled with significantly reduced lifespans in emerged bees (Allen and Ball 1996, Yang and Cox-Foster 2007, de Miranda and Genersch 2010). DWV has a particularly close association with varroa (Bowen-Walker et al. 1999, Gisder et al. 2009, de Miranda and Genersch 2010, Martin et al. 2012, Brettell et al. 2017), which serves as a mechanical vector for the DWV-A strain (Posada-Florez et al. 2019) and a biological vector for the virulent strain DWV-B (McMahon et al. 2016). In addition, varroa promotes selection of DWV-B within infested colonies (Moore et al. 2011, Martin et al. 2012, Ryabov et al. 2014, Wilfert et al. 2016, Gisder and Genersch 2020). DWV, and especially DWV-B, is a major factor in honey bee winter losses (Highfield et al. 2009, Dainat et al. 2012, Nazzi et al. 2012, McMahon et al. 2016, Natsopoulou et al. 2017). Contradictory evidence from the UK shows that DWV-A is more virulent and possibly excludes DWV-B; however, McMenamin and Flenniken (2018) suggest this may be due to selective breeding and resistance in a geographic area rather than a true difference in virulence (Mordecai et al. 2016).

Black queen cell virus (BQCV), as implied by its name, affects developing queens and causes mortality in young queens. There has been recent evidence of BQCV affecting worker honey bees, whereby workers drifting from their home colony had significantly higher levels of BQCV (Retschnig et al. 2019). BQCV does not have the same relationship with varroa as DWV, and whereas there is much evidence linking DWV with wintering losses, there is less evidence that BQCV is strongly correlated with it (Natsopoulou et al. 2017, Borba et al. 2022).

Israeli acute paralysis virus (IAPV) produces overt signs of disease in honey bees and has been implicated in colony losses and lower spring population sizes (Cox-Foster et al. 2007, Maori et al. 2007, Chen et al. 2014, Desai and Currie 2016). In overt infections it can cause trembling, paralysis, and death in individual bees (Maori et al. 2007). IAPV can be vectored by varroa (Di Prisco et al. 2011), though is typically less prevalent than DWV and BQCV (Desai et al. 2015, Fahey et al. 2017).

Honey bee virus transmission may be more covert than once believed, as viruses have recently been detected on wax (Colwell et al. 2017, Colwell 2022a). DWV-A inoculated on wax is infective to honey bees and negatively affects honey bee health (Schittny et al. 2020). Similarly, the DWV-B strain appears to be transmitted on wax as it is higher in bees reared on DWV-B-contaminated wax compared to controls (de Guzman et al. 2019). Whereas there are established chemical controls for *V. destructor*, there are no such registered control methods for honey bee viruses (Rosenkranz et al. 2010, Beyer et al. 2018). Although there have been interesting advancements in using RNAi technology (Maori et al. 2009, Hunter et al. 2010, Desai et al. 2012), fungal extracts (Stamets et al. 2018), and plant phytochemicals (Palmer-Young et al. 2017, Hsieh et al. 2020) to control virus levels in honey bees, none have become commonplace nor are registered for use in beekeeping. Additionally, treating only adult honey bees for varroa in colonies overlooks the potential role virus-contaminated equipment may play in reinfecting bees that are introduced onto comb reused from colonies that have died from high virus levels.

Storage using temperature as a mitigation treatment has proven an effective management tool for several honey bee parasites and pathogens that contaminate hive equipment. For example, viability of *Nosema ceranae* Fries spores on beeswax was significantly reduced by 7 days at −20, −12, and 33 °C compared to 20 °C (MacInnis et al. 2020). Furthermore, storage time and temperature are known to have a substantial impact on the stability of honey bee viruses in samples. Gene copies of DWV were significantly higher in samples of bees stored at −80 °C than those at −20 °C for the same length of time (Robson-Hyska 2017). Total RNA extracted from honey bee samples was also significantly reduced when stored at 4 °C relative to −20 °C (Chen et al. 2015). Additionally, other researchers have reported interactive effects between storage time and temperature on the levels of DWV and BQCV in single workers (Dainat et al. 2011). As such, controlled-temperature storage has potential as a method to reduce waxborne viruses, especially as the only special equipment required to maintain commonly used temperatures, such as −20 °C in freezers, 4–5 °C in cold rooms or room temperature, are potentially less expensive than other infrastructure.

The use of irradiation to control honey bee pests and pathogens is not a new concept (Thomas et al. 1981, Shimanuki et al. 1984). Whereas gamma irradiation has commonly been used in honey bee pathogen sterilization experiments, electron-beam (e-beam) irradiation has dose equivalency to gamma (Byun et al. 2019) and shows promise as an alternative irradiation technique. E-beam is also the most accessible form of irradiation treatment available to Canadian beekeepers and in other regions of North America. Both gamma and e-beam irradiation have been used to control waxborne pathogens such as: *Paenibacillus larvae* (White), the causative agent of American foulbrood (Katznelson and Robb 1962, Melathopoulos et al. 2004, Baggio et al. 2005); *Melissococcus plutonius* (White), the causative agent of European foulbrood (Pankiw et al. 1970, Hornitzky 1994, Pharo 2006); *Ascosphaera apis* (Maasen ex Claussen), the causative agent of chalkbrood (Melathopoulos et al. 2004, Aronstein and Murray 2010, Simone-Finstrom et al. 2018), and *N. ceranae* (Simone-Finstrom et al. 2018). There is also evidence that irradiation can be effective in reducing viral loads on hive equipment. Workers reared on 25 kGy gamma irradiated wax comb have reduced levels of DWV early in the season compared to those reared on nonirradiated wax (de Guzman et al. 2017). Similarly, stock solutions of viruses injected into pupae showed the eradication of DWV and a significant reduction of BQCV after irradiation (Simone-Finstrom et al. 2018). Irradiation can also control pathogens in food stored in comb, whereby mortality caused by IAPV in pollen was significantly reduced by 16.9 kGy of gamma irradiation (Meeus et al. 2014). Gamma irradiation doses of 10–25 kGy also produce no detectable physico-chemical changes to beeswax (Baggio et al. 2005).

There is a need to explore the potential control of waxborne honey bee viruses from the perspective of beekeepers. The objectives of this study are to determine if the prevalence and abundance of

waxborne viruses can be decreased by time spent with storage, storage temperature, or e-beam irradiation at different doses. For our storage time and temperature experiment, we focus on the 2 most prevalent honey bee viruses: BQCV and DWV (Beaurepaire et al. 2020), and easily sustainable temperatures. Our e-beam dosage experiment focuses on BQCV, IAPV, and 2 DWV strains, DWV-A and DWV-B, and employs a wide range of irradiation levels in conjunction with a transport control. We hypothesize that longer storage times and exposure to warmer temperatures will accelerate degradation of viruses and that higher doses of e-beam irradiation will suppress or eliminate them.

Materials and Methods

Experimental Design and Sampling

Storage time and temperature.

The effect of storage conditions on waxborne viruses was tested using 3 temperature treatments and 5 time points. Wax foundation (Mann Lake Ltd., Hackensack, MN) was experimentally contaminated with waxborne viruses by exposure to worker bees from colonies managed without varroa treatment. Five full-depth 5-frame nucleus colonies (nucs) were made up from these nontreated colonies (mean mite infestation of 3.28% in July 2017 in source colonies) in the mite rearing apiary on the campus of the University of Manitoba, Winnipeg (49°48′33.0″N, 97°07′35.2″W). Every nuc was queenless and had 2 brood frames covered in adult worker bees from 1 of the 5 source colonies, a single-frame feeder filled with 4 L of light sugar syrup (1:1 v/v sucrose and water), and 1 experimental frame. Supplying bees with sugar syrup promotes wax construction (Whitcomb 1946). Sheets of wax foundation were placed in new wooden frames and a strip of TempQueen (infused with queen mandibular pheromone, QMP; Mann Lake Ltd., Hackensack, MN) was attached to each frame to promote wax building and prevent egg laying. Each experimental foundation frame was inserted between the 2 brood frames in each nucleus colony, and were left for 7 days (10–16 August 2017) to allow the bees to draw out wax comb.

Experimental frames were then removed from the nucleus colonies and 13 subsamples of ~1 cm^2 of drawn wax were cut from each of the 5 frames with a sterilized scalpel (washed with 95% ethanol between samples). Of the 13 subsamples, 1 was used as the initial time (day 0) sample, and the remaining 12 were randomly assigned to the 3 temperature groups and placed in fresh 90 mm polystyrene petri dishes, with 4 samples per dish to facilitate subsequent sampling. Petri dishes were then stored in a conventional chest freezer (−20 °C; Frigidaire Chest Freezer, 14.8 cu. ft. capacity), a refrigerated chamber (5 °C; Thermo Scientific Precision 818 Low Temperature Incubator ± 1.5 °C), or an incubator (20 °C; Percival I-35L ± 0.75 °C). Storage started on 16 August 2017, and samples were subsequently taken at day 1 (17 August), day 7 (23 August), day 15 (31 August), and day 30 (15 September). Upon sampling, each piece of wax was removed from its dish using sterile forceps, placed into a fresh 2.0 ml microcentrifuge tube, and kept at −80 °C until sample processing for virus quantification.

E-beam irradiation.

The effect of e-beam irradiation on waxborne viruses was tested using wax comb from 10 winter loss colonies. These colonies died between December 2017 and April 2018 in the University of Manitoba's indoor wintering facility. Ten dead colonies were selected based on the highest fall 2017 varroa infestation levels (mean 2.61%, range 1.21–6.13) for which there were sufficient numbers of empty brood frames available to sample. We selected empty frames for 2 reasons: to ensure there was no honey in frames which would reduce the efficacy of e-beam penetration (Melathopoulos et al. 2004), and to avoid microbial growth over the time required for transportation and processing. Five empty brood frames selected from each colony were randomly assigned to 1 of 5 treatments: 10, 25, 35, 45 kGy, and a transport control that was not irradiated. A subsample of ~1.0 ml of wax was taken to evaluate pretreatment levels from every frame the day before shipment. Frames were individually wrapped in clear plastic bags and packed in wooden Langstroth boxes for ground transport and shipped on 22 May 2018 to Iotron Industries Canada Inc. (Sterigenics as of 2020) in Port Coquitlam, BC, from Winnipeg, MB, where they were received 3 days later. Frames were processed individually, passing through a nominal dose twice to receive a cumulative dose approximating the treatment values. A cumulative dosimeter recorded the actual exposure levels: 9.6 kGy for 10 kGy treatment, 24.6 kGy for 25 kGy treatment, 35.9 kGy for 35 kGy treatment, and 44.6 kGy for 45 kGy treatment. The transport control frames were not manipulated or exposed to radiation. Frames were re-packed and shipped back via ground transport, and were received and sampled for posttreatment levels on 13 June 2018. Samples for posttreatment were taken from the same area of the frame as pretreatment samples. There were 23 days between pre and posttreatment sampling times. All wax samples were stored at −80 °C until processing.

RNA and cDNA

A modified TRIzol (Life Technologies, Carlsbad, CA) wash was used to detect honey bee virus levels directly from wax (Aparicio et al. 1999, Singh et al. 2010). A ~100 mg wax subsample was placed in a 2.0 ml microcentrifuge tube with sterilized forceps. Wax samples included the base of and sides of drawn out cells. A 1 ml volume of TRIzol (Life Technologies, Carlsbad, CA) was added to the tube with the wax, vortexed for 30 s, and then incubated at room temperature for 5 min. Then, 200 µl of chloroform was added and the tube shaken by hand for 15 s and incubated for 3 min at room temperature. The sample was then centrifuged at 16,099 RCF for 15 min at 4 °C. After careful removal from the centrifuge, the clear RNA containing supernatant was removed by pipette (~350 µl). stored in a fresh 1.5 ml tube, and kept at −80 °C until RNA extraction.

Winter loss wax and e-beam experiments used a double wash with TRizol on the same wax subsample to obtain more RNA from comb samples. The additional RNA was used to test for more viruses per sample. RNA was extracted with PureLink RNA Mini Kits (Qiagen, Germantown, MD) and cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Due to scarcity of RNA, 100 ng of RNA was used to synthesize cDNA with a reaction volume of 20 µl.

RT-qPCR

Samples were quantified using Touch Deep Well Real-Time PCR Detection Systems (storage time and temperature on a CFX96, e-beam irradiation on a CFX 384 Bio-Rad) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The run protocol included a single heat cycle (3 min at 95 °C) followed by 40 cycles (15 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C), with a plate read for each cycle. Viruses were quantified using serial dilutions of gBlock gene fragment (Integrated DNA Technologies) for standard curves.

Wax from the storage of experiment was tested for BQCV and DWV (generic primer). Samples were run in technical duplicate with 4 µl of undiluted cDNA per well. Standard curves were based on

concentrations from 10^2 to 10^8 with an efficiency range of 86.0–86.2% and R^2 = 0.937–0.983 for BQCV and 10^3–10^9 with an efficiency range of 91.8–93.0% and R^2 = 0.991–0.996 for DWV. Plates were run with a nontemplate control.

Wax from the e-beam experiment was tested for BQCV, DWV-A, DWV-B, and IAPV. Samples were run in technical duplicate with 3 µl 1:4 dilution of cDNA per well. Standard curves were based on concentrations from 10^2 to 10^9 for all viruses. BQCV efficiency was 102% and R^2 = 0.999, DWV-A efficiency was 96% and R^2 = 0.998, DWV-B efficiency was 93% and R^2 = 0.998, and IAPV efficiency was 89% and R^2 = 0.998. Plates were run with nontemplate and no reverse-transcription controls. Details of quantification for each primer pair were reported in Supplementary Table S1 to meet MIQE requirements (Bustin et al. 2009).

All primers used in this study can be found in Supplementary Table S1; for gBlock sequences see Supplementary Table S2. To verify PCR products, excised amplicons were purified using NucleoSpin Gel and PCR clean-up kit (Macherey Nagel, Duren, Germany). Elutes were sent for Sanger sequencing (Psomagen, ML). Obtained sequences were trimmed and compared to nucleotide sequences in the NCBI database by BLAST (percent identities: BQCV 100%, DWV 89.04%, IAPV 98.35%, DWV-A 98.99 %, DWV-B 94.62% S3).

Statistical Analysis

Statistical analyses were performed in SAS software version 9.4 (SAS Institute, Cary NC). We used prevalence (the number of wax samples that tested positive for viral gene copies divided by the number of samples tested) and mean abundance (the number of viral gene copies from a wax sample divided by the number of samples tested) (Bush et al. 1997). Prevalence of viruses was analyzed using logistic analyses (PROC CATMOD, SAS 9.4) and differences among means were compared using maximum likelihood with Bonferroni corrections, except where performing only preplanned comparisons against controls. Standard errors for prevalence in figures were calculated as the standard error of the sample proportion. All figures display data based on maximum likelihood estimates for prevalence and least squares means for abundance.

Absolute viral gene copy data were log + 1-transformed (Pirk et al. 2015) prior to analyses. Data that did not meet the assumptions related to homogeneity of variance (Levene's test) were adjusted using Kenward-Rogers degrees of freedom approximation during analysis (SAS 9.4). Repeated measures ANOVA was used to analyze mean abundance data in all experiments (PROC MIXED, SAS 9.4). Temperature treatment was the main effect factor and colonies were treated as subjects with repeated measures over time. Changes in mean abundance of waxborne viruses after transport and e-beam irradiation were also tested using PROC MIXED with dose as the main effect factor, sample as the subject with repeated measures over time. All mixed models were analyzed with a compound symmetry covariance structure. Differences among means were compared using Pdiff with Tukey corrected contrasts, except where performing only preplanned comparisons with controls. Covariance structures for each variable were chosen based on lowest AIC values and nonsignificant higher level interactions were dropped from models where present (Kincaid 2005).

Results

Storage Time and Temperature

We tested drawn wax foundation to determine if storage time and temperature conditions during storage could reduce waxborne virus levels. We tested wax samples from the initial time period (day 0, n = 5), day 1 (n = 15), day 7 (n = 15), day 15 (n = 15), and day 30 (n = 15) for BQCV and DWV (generic primer). For each time period after initial samples were taken, wax was exposed to 1 of 3 temperatures (5 replicates each). Prevalence of BQCV was similar across samples at all time periods and temperatures, with positive detections on 100% of day 0 wax samples, 93.3% of day 1 samples, 93.3% of day 7 samples, 100% of day 15 samples, and 80% of day 30 samples (χ^2 = 2.69, df = 4, P = 0.61). Prevalence of DWV over time was also similar, with 100% positive detections in all time point samples (χ^2 = 0.60, df = 4, P = 0.96).

We assessed changes in pooled virus abundance over time and after exposure to different temperature treatments. Storage time significantly reduced gene copies of both viruses ($F_{(4,16)}$ = 10.83, P < 0.001), whereas temperature ($F_{(2,8)}$ = 2.27, P = 0.17) had no effect (Fig. 1; see Supplementary Fig. S1 for a breakdown by temperature and Supplementary Fig. S2 for log + 1 transformed data). Virus levels on foundation were significantly reduced by day 30; BQCV was reduced by 51% from day 0 to day 30, and DWV was reduced by 17% over the same time period. Viruses were pooled for analysis by time because there was no significant interaction between virus type and time ($F_{(4,16)}$ = 0.60, P = 0.66). There were significantly lower levels of BQCV compared to DWV ($F_{(1,4)}$ = 747.52, P < 0.0001).

E-Beam Irradiation

We tested winter loss wax to determine if varying dosages of e-beam irradiation could reduce waxborne virus levels. We tested wax samples for viruses (BQCV, IAPV, DWV-A, and DWV-B) before and after e-beam treatment at 4 doses (10, 25, 35, and 45 kGy) and a transport control. The prevalence of virus types on wax (pooled by time and treatment) differed significantly (χ^2 = 58.17, df = 3, P < 0.0001; Fig. 2; see Supplementary Fig. S3 for raw prevalence data). IAPV was the least commonly detected virus at 12%, significantly below detections of BQCV at 57% (χ^2 = 39.03, df = 1, P < 0.0001), DWV-A at 50% (χ^2 = 29.34, df = 1, P < 0.0001) and DWV-B at 66% (χ^2 = 49.22, df = 1, P < 0.0001). Overall prevalence also varied over time (all viruses and treatments pooled), with more detections pretreatment than posttreatment (χ^2 = 39.03, df = 1, P < 0.0001).

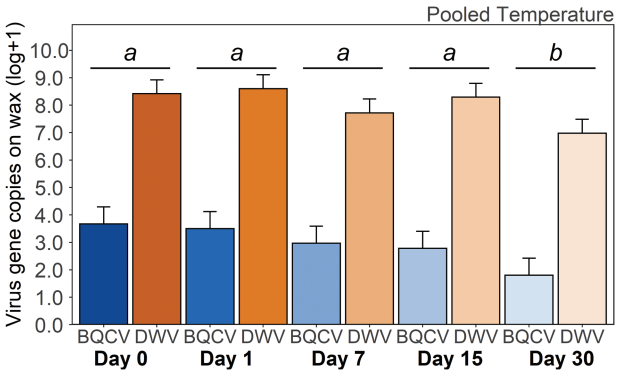


Fig. 1. Effect of storage time on the levels of virus gene copies (log + 1) per 10 ng of cDNA on drawn wax foundation. Mean abundance ± SE of BQCV (black queen cell virus) and DWV (deformed wing virus, generic primer) on stored wax foundation at each time point (day 0 n = 5, day 1 n = 15, day 7 n = 15, day 15 n = 15, day 30 n = 15, per bar), pooled over all temperature treatments (–20, 5, and 20 °C). Plotted data are based on least squares means. Time points with the same letters are not significantly different (P > 0.05: Tukey adjusted).

Treatment group affected prevalence of viruses pooled by virus and time ($\chi^2 = 20.37$, df = 4, $P = 0.0004$; Fig. 3; see Supplementary Fig. S4 for raw prevalence data). Wax in the paired samples that made up the 45 kGy treatment had more detections than wax in the paired samples that made up the control group ($\chi^2 = 9.97$, df = 1, $P = 0.0016$).

We tested differences in mean abundance to assess the effect of our treatments on the reduction of the different virus types, owing to some variation in initial virus concentration among samples assigned to different treatments. The analysis revealed a significant interaction between virus type and time ($F_{(3,339)} = 3.73$, $P = 0.012$). Pretreatment levels for DWV-B, were greater than DWV-A and IAPV, and both BQCV and DWV-A higher than IAPV (Fig. 4; see Supplementary Fig. S5 for log + 1 transformed data). In posttreatment groups, DWV-B was still the most abundant and at higher concentrations than all other virus types. BQCV and DWV-A were still at higher levels than IAPV. Each virus type was significantly reduced from pretreatment levels at the posttreatment time point. We used orthogonal contrasts to compare the slope of change over time for each virus to see if the reduction in virus levels decreased from pre to posttreatment at the same rate. BQCV decreased at a greater rate relative to IAPV ($F_{(1,339)} = 10.13$, $P = 0.0016$; Fig. 4); there were no other significant differences in rate of change among viruses.

There was also an interaction between time and treatment group ($F_{(4,339)} = 3.63$, $P = 0.0066$) for pooled virus type. We partitioned the interaction effects individually for each treatment level; there were significant decreases between pre and posttreatment time for all of the dosage levels and the control (Fig. 5; see Supplementary Fig. S6 for log + 1 transformed data). As was the case with prevalence, differences were found among treatment groups prior to exposure ($F_{(4,60.6)} = 3.61$, $P = 0.01$). We used orthogonal contrasts to compare the degree of change of e-beam dosage levels relative to

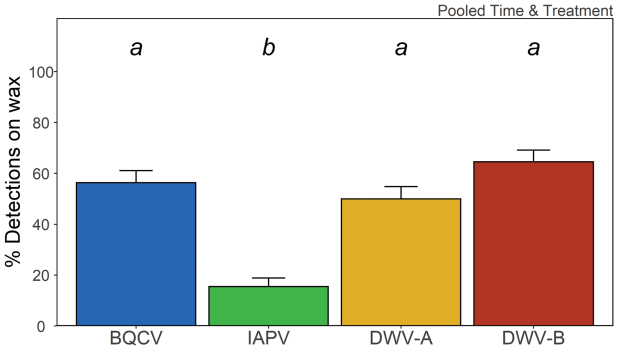


Fig. 2. Prevalence ± SE of positive detections per 10 ng of cDNA of BQCV (black queen cell virus), IAPV (Israeli acute paralysis virus), DWV-A (deformed wing virus, strain A), and DWV-B (deformed wing virus, strain B) on wax pooled by time (pre and posttreatment) and e-beam treatment levels (control and 4 dosage levels; all bars $n = 100$). Virus types by time with the same letters above bars are not significantly different ($P > 0.05$: Tukey adjusted).

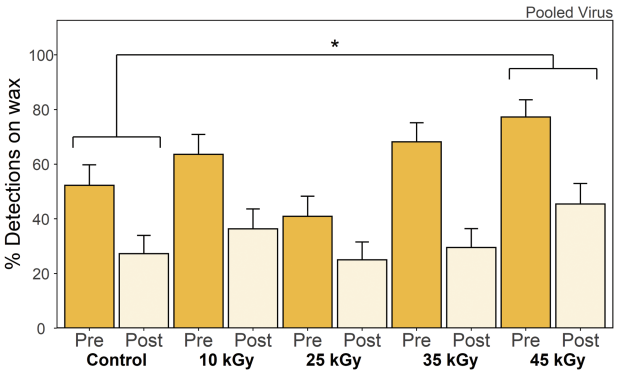


Fig. 3. Effect of e-beam treatment dose on prevalence of pooled viruses per 10 ng of cDNA on winter loss wax comb. Prevalence ± SE of positive detections of treatment levels (control and 4 dosage levels) paired by time (pretreatment (Pre) and posttreatment (Post) for pooled virus types on e-beam irradiated wax (all bars $n = 10$). Plotted data are based on maximum likelihood estimates. Standard error was calculated by SE of binomial proportions. Overall, there were more virus detections in pooled pretreatment samples vs. pooled posttreatment samples ($\chi^2 = 39.03$, df = 1, $P < 0.0001$). Bracket spanning different treatment levels topped by an asterisk denote a significant difference from the control, with prevalence pooled by virus type and time ($P < 0.05$).

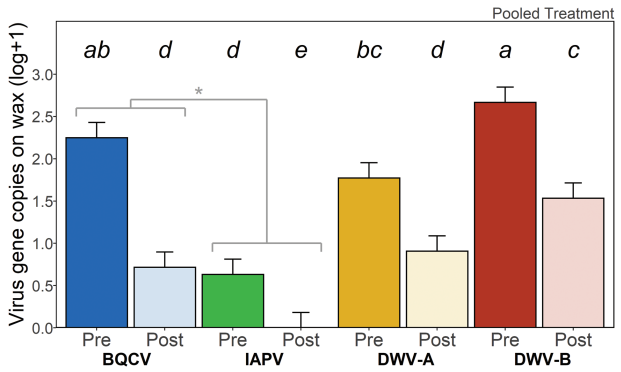


Fig. 4. Effect of e-beam treatment on gene copies (log + 1) per 10 ng of cDNA of different virus types on winter loss wax comb. Mean abundance ± SE of BQCV (black queen cell virus), IAPV (Israeli acute paralysis virus), DWV-A (deformed wing virus, strain A), and DWV-B (deformed wing virus, strain B) on wax in paired bars for pretreatment (Pre) and posttreatment (Post) wax comb pooled by e-beam treatment levels (control and 4 dosage levels; all bars $n = 50$). Plotted data are based on least squares means. Virus types by time with different letters above bars are significantly different ($P < 0.05$; Tukey adjusted). Gray brackets spanning different virus types topped by an asterisk represent a significantly different slope of change pre to posttreatment relative to the other bracketed virus ($P < 0.0083$: orthogonal contrasts, Bonferroni corrected).

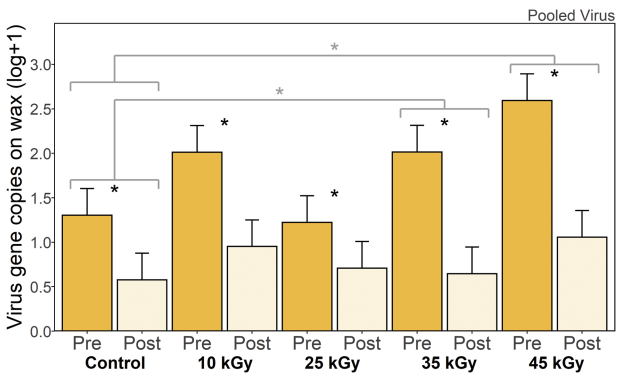


Fig. 5. Effect of e-beam treatment dose on pooled gene copies (log + 1) per 10 ng of cDNA on winter loss wax comb. Mean abundance ± SE of pooled virus type for paired pre and posttreatment for the control and each e-beam dosage treatment level (all bars $n = 10$). Plotted data are based on least squares means. An asterisk (*) between paired treatment bars shows a significant difference in abundance between time points ($P < 0.01$; protected LSD). Gray brackets spanning different treatment levels topped by an asterisk represent a significantly different slope of change pre to posttreatment relative to the control ($P < 0.05$: orthogonal contrasts).

change in the control. Both the 35 kGy ($F_{(1,339)} = 4.09$, $P = 0.044$) and 45 kGy ($F_{(1,339)} = 6.51$, $P = 0.011$) doses produced greater decreases in virus abundance than the control (Fig. 5), although there were no differences found among posttreatment virus levels regardless of dose ($F_{(4,60.6)} = 0.47$, $P = 0.75$).

Discussion

In this paper, we examined 2 possible treatment options for the mitigation of waxborne viruses that are reasonably available to beekeepers. To our knowledge, we are the first to show that storage time, regardless of temperature or e-beam dosage, was sufficient to reduce waxborne virus levels on drawn foundation and wax comb. All doses of e-beam irradiation used in our study reduced the abundance of viruses, however reductions were also seen in our control treatment. Nevertheless, our results showed the doses of 35 and 45 kGy had greater rates and magnitudes of virus reductions when directly compared with the controls. This suggests that irradiation levels greater than 35 kGy could present a sterilization option for honey bee viruses on wax comb.

Our storage experiment showed intervals of 15 days or less result in no reduction in virus level relative to controls and that storage temperatures between −20 and 20 °C did not affect the amount of degradation of virus on drawn foundation. Future work is required on the potential infectivity of waxborne viruses following storage and/or irradiation treatments before firm recommendations can be made. However, this work suggests understanding differential sensitivity to time and irradiation is important if viruses on wax need to be controlled to reduce their impact on colony health. Given the high costs of shipping and treating comb and regional availability, e-beam irradiation may not always be a universally viable option.

Storage Time and Temperature

Impacts of wax storage time on honey bee virus levels were investigated in several contexts in this study. Only DWV and BQCV were tested in this experiment because they were the only primers optimized for wax samples at the time. On drawn foundation, the 2 viruses tested, BQCV or DWV (generic primer), both decreased over time. Though prevalence of these 2 viruses was unaffected by storage time, abundance decreased after 30 days. Surprisingly, there were no differences among our temperature treatments, which included a broad range under which comb would commonly be stored under temperate management conditions, including −20, 5, and 20 °C. These results suggest that any benefit of reductions in viral load achieved by delaying the reintroduction of comb into use by producers will not depend on the storage temperature conditions within this range. This may be a benefit as no special equipment would be required to cool or heat during storage. Our storage times were relatively short, ending at 30 days, compared to durations a beekeeper may keep winter loss hive equipment stored; nevertheless, some winter loss colonies fail in spring and these could be reused with reference to our evaluation timeframe. Though there was a reduction in viruses by 30 days, we recommend extending time used in future work to determine when viruses could be brought to a level where they do not cause economic damage.

While the impact of storage time on virus degradation on comb has not been well studied, the impacts of time and temperature on sample storage to prevent degradation of bee RNA before testing has been examined. RNA in dry whole bee samples is significantly degraded after 1 wk of storage at 4 °C, whereas dry whole bees stored at −80 °C and at −20 °C have much higher quality RNA compared to 4 °C (Chen et al. 2015). We did not measure RNA integrity in the same way, but it is interesting to note that we did not see a difference in prevalence or abundance of virus levels over a similar time frame and range of temperatures. In our storage experiment, initial samples were taken within hours of last exposure to living bees. It is likely that the viruses introduced by bees onto a dry substrate such as wax do not function the same way as those inside bees, which would be subjected to a variety of other factors that would enhance degradation of RNA. Bee storage experiments also suggest differences in the degradation of different viruses. In the same study by Chen et al. (2015), both BQCV and DWV were detected more reliably after storage treatments than Kashmir bee virus (KBV) and sacbrood virus (SBV); this may be analogous to our findings in which BQCV and DWV remained at high prevalence over the 30 day storage treatment.

E-Beam Irradiation

Following e-beam irradiation, the prevalence of detections for viruses decreased, which differed from the storage experiment where prevalence was not reduced over 30 days. The experiments differed in the length of time, with the e-beam experiment storage time being shorter by 7 days (30 days for the storage experiment and 23 days for the e-beam experiment). The significant difference was detected when samples were pooled by virus type and treatment, suggesting e-beam could be playing a role in the observed difference in prevalence. However, we cannot discount the possibility that time and transport conditions contributed to the reduction in prevalence as there was a trend toward reduced prevalence in the untreated controls in the post treatment sampling period. There was no interaction between treatment and time for prevalence suggesting that prevalence of viruses in the controls decreased at a similar rate as in treated colonies.

Mean abundance for all viruses, pooled over e-beam doses, also decreased over time. Similarly, mean abundance for all treatment levels and the control, as pooled by virus types, decreased significantly over time. However, the 35 and 45 kGy dosage levels of e-beam were associated with a greater rate of reduction in virus abundance than the control. This suggests that e-beam irradiation at high dosage is a more effective treatment than storage alone. One caveat for this result is that bees did not naturally contaminate the wax to where 100% prevalence was achieved for viruses in the pretreatment; ideally we could compare the difference between pre and posttreatment with higher certainty with uniformly contaminated wax. However, we obtained field-realistic virus contamination for our experiments, making our results relevant to real world hive conditions. Moreover, it could be possible that manual application of purified virus would not accurately represent natural contamination by bees, omitting components such as bee saliva that may play a role in colony epidemiology. Although there were differences in pooled virus abundance among treatment groups pretreatment, there were no differences among treatments posttreatment. This suggests there may be little to no advantage to using e-beam irradiation to sterilize comb at least when long intervals occur between the time of treatment and reintroduction of comb into colonies.

An assumption held here is that detectable viruses are indicative of whole and viable virus on wax. The regions of viruses amplified by primers here range from ~100 bp (DWV and strains), to ~300 bp (BQCV), to nearly 600 bp (IAPV). It is conceivable that IAPV, with its relatively larger genome section for primer detection, was not detected posttreatment because it was more likely to be degraded compared to the smaller regions for BQCV and DWV. Whereas it is possible that we are only detecting those regions of virus that are still intact, other studies have shown waxborne viruses are still infective

to bees (de Guzman et al. 2017, Schittny et al. 2020), though those studies did not examine storage time as a factor.

Levels of waxborne viruses similar to those found in this paper can differentially affect the virus levels of bees reared on it. Adult bees reared on wax contaminated with high detectable levels of virus gene copies had higher viral levels compared to adults reared on wax with no detectable levels of waxborne viruses (Colwell 2022b). Thus, the amount of viruses identified in this study have biological significance to honey bees. Furthermore, this validates our categorization of virus levels below our limit of detection limit effectively as zeroes, as they are of little biological relevance.

Ground transport from the University of Manitoba to the e-beam irradiation facility in British Columbia is approximately 2,300 km each way. Future work of a similar nature would ideally include a temperature and humidity recording device to track the conditions during transport and at the facility, as well as a nontransport control group.

We found fairly high variability in virus levels on comb assigned to different treatments. Due to the time required to conduct viral analyses we were not able to block samples by virus level before assigning them to treatments. The assignment of comb to treatments may have resulted in some variation among pretreatment levels for some doses. For example, the 45 kGy treatment had higher prevalence of pooled virus detections than the control, but this is likely due to the higher prevalence from pretreatment samples. Differences in prevalence and abundance of viruses in pretreatment samples is likely due to random chance; therefore the overall statistical difference between the control and 45 kGy dosage is unlikely to be biologically significant.

Interestingly, there were no detections of IAPV in our posttreatment samples. Its relatively low prevalence compared to the other viruses may be why it was not detected posttreatment; however, it could be possible that IAPV is more susceptible to degradation than the other viruses that were still detected posttreatment. The rate of decrease in IAPV was significantly less than for BQCV, though this difference may be attributable to the low starting abundance of IAPV on pretreatment wax.

Gamma and e-beam irradiation do inactivate the honey bee pathogens *P. larvae*, *A. apis*, *N. ceranae*, and viruses (Hornitzky 1994, Melathopoulos et al. 2004, Simone-Finstrom et al. 2018, Strange et al. 2023). As treatment for American foulbrood commonly uses a dose of 10 kGy, there is an opportunity to increase that dose to 35 kGy to achieve control of viruses; however, the potential benefits and costs to colony health should be better studied before recommendations are made. Moreover, the type of sample treated by irradiation could influence the efficacy against viruses, as DWV-contaminated pollen treated with ~30 kGy gamma irradiation did not have lower DWV levels posttreatment compared to untreated pollen (Strange et al. 2023).

Doses equivalent to what we used (25 kGy) inactivate DWV, but do not fully inactivate BQCV when injected into pupae (Simone-Finstrom et al. 2018). Although we were not measuring the ability of the virus to successfully infect bees, the disparate inactivation between DWV and BQCV appears contrary to our storage and e-beam experiments, in which detectable gene copies of DWV and BQCV (presumably "intact" virus particles) were reduced at the same rate over time when pooled by e-beam treatment levels. A major difference between the methods of these studies in addition to the source of irradiation, was that the current study irradiated waxborne viruses and quantified reductions with direct measurement of detectable virus whereas Simone-Finstrom et al. (2018) irradiated stock solutions that were then injected into bees. It may be possible that BQCV and DWV are affected by irradiation in a different manner while on wax compared to when they are suspended in solution, or that there is a biological difference in their infectivity to bees not addressed in our study. Virus particles that are severely degraded through irradiation would not likely be detected by RT-qPCR, however, that does not mean that relatively intact viral particles would remain viable. A study on animal viruses showed that the larger the particle size and genome, the higher the inactivation rate due to irradiation (Nims et al. 2011); the viruses tested in our study range from ~8,500 to 10,000 nucleotides, which are rather small genome sizes (Lanzi et al. 2006, Maori et al. 2007, Abou Kubaa et al. 2020). Additional work needs to be done to connect the abundance of viruses that can be detected on beeswax to their potential biological impact on bees.

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Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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