



# Gamma irradiation inactivates honey bee fungal, microsporidian, and viral pathogens and parasites

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## ABSTRACT

Managed honey bee (*Apis mellifera*) populations are currently facing unsustainable losses due to a variety of factors. Colonies are challenged with brood pathogens, such as the fungal agent of chalkbrood disease, the microsporidian gut parasite *Nosema* spp., and several viruses. These pathogens may be transmitted horizontally from worker to worker, vertically from queen to egg and via vectors like the parasitic mite, *Varroa destructor*. Despite the fact that these pathogens are widespread and often harbored in wax comb that is reused from year to year and transferred across beekeeping operations, few, if any, universal treatments exist for their control. In order to mitigate some of these biological threats to honey bees and to allow for more sustainable reuse of equipment, investigations into techniques for the sterilization of hive equipment and comb are of particular significance. Here, we investigated the potential of gamma irradiation for inactivation of the fungal pathogen *Ascosphaera apis*, the microsporidian *Nosema ceranae* and three honey bee viruses (*Deformed wing virus* [DWV], *Black queen cell virus* [BQCV], and *Chronic bee paralysis virus* [CBPV]), focusing on the infectivity of these pathogens post-irradiation. Results indicate that gamma irradiation can effectively inactivate *A. apis*, *N. ceranae*, and DWV. Partial inactivation was noted for BQCV and CBPV, but this did not reduce effects on mortality at the tested, relatively high doses. These findings highlight the importance of studying infection rate and symptom development post-treatment and not simply rate or quantity detected. These findings suggest that gamma irradiation may function as a broad treatment to help mitigate colony losses and the spread of pathogens through the exchange of comb across colonies, but raises the question why some viruses appear to be unaffected. These results provide the basis for subsequent studies on benefits of irradiation of used comb for colony health and productivity.

## 1. Introduction

In recent years, honey bee populations have been under a near constant threat of various pathogens, parasites, pests and anthropogenic stressors (Cox-Foster et al., 2007; vanEngelsdorp and Meixner, 2010). While treatments currently exist for some of these pathogens and parasites, there is the continual risk of the development of resistance to chemical treatments and contamination of hive products. For example, the causative bacterial agent of the larval disease American foulbrood (AFB; *Paenibacillus larvae*) exhibits widespread resistance to antibiotic treatment (Evans, 2003) and treatment residues persist in honey, which is a human health concern (reviewed in Genersch (2010)). In addition, currently registered antibiotic treatments for some pathogens are only moderately effective (Williams et al., 2008) or can possibly make the infection worse, as seen in the case of the microsporidian gut parasite

*Nosema ceranae* (Huang et al., 2013).

Complicating the fact that reliable and safe treatments do not currently exist for the treatment of many honey bee diseases, is the continual reuse and exchange or transfer of old equipment within and across beekeeping operations, which can lead to the spread of various pathogens and parasites (Cox-Foster et al., 2007; Fries and Camazine, 2001; Pettis et al., 2007; vanEngelsdorp et al., 2009a; vanEngelsdorp and Meixner, 2010). The fungal agent of the larval disease chalkbrood (CB; *Ascosphaera apis*) produces spores that can remain viable in wax comb and can be spread throughout a colony and among colonies when adult bees clean the comb and transfer spores to developing larvae (Aronstein and Murray, 2010; Flores et al., 2005). The same is true for spores of *Nosema* spp. (Fries, 1988; Higes et al., 2010) and AFB (Hansen and Brødsgaard, 1999; Haseman, 1961). Additionally, recent evidence suggests that at least some viruses can be found in wax comb ((Colwell

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et al., 2017); Simone-Finstrom, Rinkevich and de Guzman, preliminary data), which could potentially serve as a point of infection. Investigations regarding treatments that better facilitate the use of older comb have been of significant interest, particularly with respect to AFB-infected comb as the current standard practice is to burn contaminated equipment (Hansen and Brødsgaard, 1999). Therefore, treatments that work against multiple pathogens and parasites, especially viruses, where there are no treatments available for honey bees, can have a significant positive impact on the health and productivity of honey bee colonies and thus, profitability for beekeeping operations.

One method that has been explored over the years is the use of gamma irradiation for sterilization of hive components (Hornitzky, 1994). Gamma irradiation is commonly used throughout the agricultural and human health industries to sterilize equipment and food products (ISO#13409, 2002; Sommers, 2012). Much of the previous work on sterilization of bee equipment has focused on its effectiveness against AFB-contaminated comb. Several studies have shown that a dose of 10 kGy is an effective treatment to inactivate AFB spores for the reuse of equipment (Baggio et al., 2005; De Guzman et al., 2011; Gochbauer and Hamilton, 1970; Gosselin and Charbonneau, 1990). More recent work has provided some evidence that gamma irradiation may prevent colony losses when bees are hived on old comb that has been treated (Cox-Foster et al., 2007; Pettis et al., 2007). Other research has focused on the use of gamma irradiation to sterilize bee-collected pollen before feeding it to commercially reared bumblebees to limit the spread of pathogens. These studies have shown that gamma irradiation can inactivate viruses (Meeus et al., 2014) and reduce the frequency of detecting various pathogen and parasite DNA/RNA in pollen (including DWV, BQCV, and *Nosema*; Graystock et al., 2016). However, gamma irradiation does not eliminate or completely reduce infectivity of all pathogens, including *N. ceranae* and the parasitic protozoan *Apicystis bombi*, in particular (Graystock et al., 2016). Incomplete inactivation has also been noted for the bacterial agent that causes the larval disease European foulbrood (Pankiw et al., 1970). Conditions of irradiation, including sample preparation, can also impact the ability of gamma irradiation to inactivate certain types of viruses (Hume et al., 2016; Thomas et al., 1981). With this in mind, assays that examine infectivity post-irradiation are of critical importance to understand the relevance of such a treatment.

The aim of the current study was to investigate the potential of gamma irradiation to inactivate multiple honey bee pathogens that can be found in hive products. Since much of the previous work regarding honey bees have focused on bacterial pathogens, here we examined the infectivity of CB (a fungal pathogen), *N. ceranae* (a microsporidian gut parasite), the two most prevalent bee viruses—*Deformed wing virus* (DWV) and *Black queen cell virus* (BQCV) (Chen and Siede, 2007; Traynor et al., 2016), and a potential emerging viral threat—*Chronic bee paralysis virus* (CBPV) (Ribière et al., 2010; Traynor et al., 2016). These pathogens impact different honey bee life stages and represent a larger swath of the potential infections that honey bees face. Understanding how gamma irradiation may reduce the infectivity of this suite of pathogens provides supporting evidence for the use of gamma irradiation to sterilize bee equipment.

## 2. Materials and methods

Three batches of each of the following pathogen/parasite solutions were prepared for the following treatments: (1) untreated stock solution, not transported nor irradiated but stored at 4 °C until use (stock); (2) stock solution transported to the irradiation facility without being irradiated (transported); and (3) stock solution transported to the irradiation facility for irradiation (irradiated). The transportation and process of irradiation were completed over 5 days in Spring 2016 and over 3 days in Summer 2017.

The irradiation treatment was conducted at an USDA/APHIS certified phytosanitary treatment facility (Gateway America, Gulfport, MS).

Stock solutions were contained in 1.5 mL microcentrifuge tubes in cardboard boxes (transported) or in ziploc bags stapled to the inside wall of a commercial, wooden Langstroth hive box (irradiated). The transported stock solutions remained in the same warehouse where the irradiated solutions were kept and treated and were maintained at 28 °C. Thereafter, the transported solutions were brought to the laboratory at the same time as the irradiated group. The irradiated stock solutions were exposed to 25 kGy of gamma irradiation delivered over 9 h 45 min, which is a standard dose used for sanitization purposes (ISO#13409, 2002).

### 2.1. Chalkbrood (CB)

Chalkbrood (*Ascosphaera apis*) spores were collected following well-established methods (Aronstein et al., 2015; Aronstein and Murray, 2010). Briefly, an *A. apis* strain (BBR) isolated from previous work (Aronstein and Murray, 2010) was grown on YGPSA (1% yeast extract, 1% glucose, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, 1% soluble starch, and 2% agar) solid culture medium with 100 µg/mL ampicillin sodium salt and 6 µg/mL streptomycin sulfate. Plates were incubated at 33 °C for 7 days and then at room temperature for an additional 3 days. Ascospores, the sexual stage of ascomycetes fungi, were harvested from culture plates and stored at 4 °C.

#### 2.1.1. Growth assessment

After the vials containing CB returned from the irradiation facility, spores were tested for viability by plating ~10<sup>4</sup> spores following the same protocol used to culture the spores. Three plates were prepared for each of the three treatments (stock, transported, and irradiated) and monitored for growth and sporulation for 2 weeks at 33 °C.

#### 2.1.2. Infectivity of CB spores in *in vitro*-reared larvae

To examine infectivity, individual larvae were inoculated *in vitro* with CB spores. *In vitro* rearing of larvae followed modifications of standard protocols (Aupinel et al., 2005; Crailsheim et al., 2013). First instar larvae were grafted to plastic queen cell cups and mass provisioned with 190 mg of larval rearing diet (50% royal jelly, 6% glucose, 6% fructose, 1% yeast extract). Preliminary work has found 190 mg to be the optimum amount of diet needed by a first instar larva to complete its development through eclosion to an adult worker bee (Simone-Finstrom, unpublished data). Larvae were treated topically near the mouthparts with 10<sup>4</sup> CB spores in 5 µL water just after grafting. Control larvae were treated with water only. There were a total of four treatments: control (n = 24), stock (n = 16), transported (n = 16), and irradiated (n = 32). Larvae were maintained at 34 °C and 85% relative humidity and monitored daily for survival and development of symptoms until pupation.

Differences in survival were compared between the 4 treatment groups using the Kaplan-Meier survival analysis (JMP Pro 12), followed by post hoc pair-wise analysis when there was a significant effect from treatment.

### 2.2. Black queen cell virus (BQCV)

Ten symptomatic queen larvae collected in early Spring 2016 and stored at –80 °C were homogenized in 10 mL sterilized phosphate buffered saline (PBS). The viral solution was semi-purified (de Miranda et al., 2013) by centrifugation in a 15 mL tube at 4700g for 20 min at 4 °C. The supernatant was filtered with 0.22 µm syringe filter, and divided into thirds and stored at 4 °C until use.

Identification and quantification of virus in the solution was confirmed by qPCR following standard methods (Boncristiani et al., 2013; de Miranda et al., 2013). The region encoding a capsid protein of BQCV was amplified using Forward—TTT AGA GCG AAT TCG GAA ACA and Reverse—GGC GTA CCG ATA AAG ATG GA primers [(vanEngelsdorp et al., 2009b); (HQ655494.1)].

### 2.2.1. Infectivity of BQCV in *in vitro*-reared larvae

Infectivity of BQCV was first examined in larvae reared *in vitro*, which is the main stage of development affected by this virus. Larvae were reared as described above (Section 2.1.2), with one exception. In this case, larvae were mass provisioned with 235 mg of larval diet, an amount previously found to produce bees that are queen-like under *in vitro* conditions (Simone-Finstrom, unpublished data). The viral solutions (stock, transported and irradiated) were diluted 1:125 in sterile PBS, which corresponded to  $10^{10}$  viral copies, and 5  $\mu$ L was topically applied near the mouthparts of each larva. Control larvae were treated with sterile PBS. Twenty larvae per treatment (control, stock, transported, and irradiated) were monitored daily for mortality and presentation of symptoms for 12 days. Survival data were analyzed as above (Section 2.1.2).

### 2.2.2. Virus injection assay

To examine infectivity and the ability of BQCV to replicate, white-eyed worker pupae from a single source colony were injected with the viral solutions from the different treatment groups. Pupae were injected with 3  $\mu$ L of  $10^{10}$  BQCV copies ( $n = 35$  per treatment) or  $10^8$  BQCV copies ( $n = 15$  per treatment) for each of the three treatment solutions (stock, transported, or irradiated) using a Hamilton syringe fitted with a 30G needle at an infusion rate of 1  $\mu$ L/s using a Micro4™ Microsyringe Pump Controller adapted from standard methods (Boncristiani et al., 2013; de Miranda et al., 2013). PBS injections (control) were completed on another 35 pupae. Pupae were maintained on filter paper in petri dishes at 34 °C and 80% relative humidity. Mortality was assessed daily for 3 days, after which all pupae were frozen at  $-80$  °C for analysis of viral replication by qPCR.

Eight pupae per treatment (control, stock, transported, and irradiated) were assessed for viral loads 3-days post-injection in 2016. RNA extractions were completed using the Maxwell® 16 LEV simplyRNA system (Promega) and cDNA template was generated from 2  $\mu$ g of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. qPCR was performed in triplicate with a QuantStudio™ 6 Flex System (Applied Biosystems) using 2  $\mu$ L of cDNA template per 20  $\mu$ L reaction containing 1X PowerUP™ SYBER® Green (ThermoFisher Scientific). The thermal profile was as follows: 2 min at 50 °C then 2 min at 95 °C, followed by 45 cycles of 95 °C for 15 sec, 53.5 °C for 20 sec, and 72 °C for 30 s. A melt-curve dissociation analysis completed each run.

Black queen cell virus titers were determined using standard curves generated from plasmid standards containing the sequence listed above (generated by GeneArt, Invitrogen). Linearized plasmid standards containing  $10^5$  to  $10^{12}$  copies per reaction were used as templates to assess primer efficiency and quantify the amount of virus following standard practices (Cavigli et al., 2016; Hou et al., 2010). Linear standard equations were generated using the  $\log_{10}$  of the initial plasmid copy number. Differences in copy number across treatments were then expressed as the ratio of BQCV in virus-injected samples to the average level of BQCV in PBS controls, log-transformed and analyzed via one-way ANOVA (JMP Pro 12).

A second trial in 2017 was conducted to confirm mortality results. As described above, pupae from two unrelated colonies were injected with  $10^{10}$  BQCV copies and monitored for 7 days after injection.

### 2.3. Deformed wing virus (DWV)

Deformed wing virus was obtained using a similar protocol that was used to isolate BQCV. Twenty bees symptomatic for DWV were homogenized in 20 mL sterile PBS. Deformed wing virus was semi-purified as in Section 2.2 and the identity of the virus was confirmed via qPCR and maintained at 4 °C until use.

In 2016, DWV infectivity and replication was examined using methods and analysis as described above (Section 2.2.2). The region of DWV amplified was based on the following primers: Forward—GAG

ATT GAA GCG CAT GAA CA; Reverse—TGA ATT CAG TGT CGC CCA TA [(vanEngelsdorp et al., 2009b); (AY292384.1)]. Briefly, pupae were injected with  $10^9$  ( $n = 35$  per treatment) or  $10^7$  viral copies ( $n = 15$  per treatment) of viral solution or sterile PBS only ( $n = 35$ ). Differences in copy number across treatments were expressed as the ratio of DWV in virus-injected samples to the average of DWV in PBS-injected controls, log transformed and analyzed by Welch's ANOVA due to unequal variances across treatment groups.

Mortality results were confirmed in 2017 by injecting pupae from two unrelated colonies with  $10^{10}$  DWV copies or PBS alone, and monitored for 7 days post-injection for presentation of symptoms at emergence.

### 2.4. Chronic bee paralysis virus (CBPV)

Chronic bee paralysis virus was also obtained as previously described (Section 2.2). In 2017, 20 bees symptomatic for CBPV were homogenized in 20 mL sterile PBS. Chronic bee paralysis virus was semi-purified and the identity of the virus was confirmed via qPCR and maintained at 4 °C until use. A region of the CBPV genome was amplified using the following primers: Forward—CGC AAG TAC GCC TTG ATA AAG AAC; Reverse—ACT ACT AGA AAC TCG TCG CTT CG [(Blanchard et al., 2007); (EU122229.1)]. To examine infectivity and replication, 30 pupae per treatment (stock, transported, and irradiated) were injected with a solution containing  $10^7$  CBPV copies. Test pupae were obtained from two colonies. All but 4 of the 180 CBPV-injected pupae were necrotic 24 h post-injection. Pupae were frozen at  $-80$  °C for analysis of viral replication. Fifteen pupae from each of two colonies per treatment were analyzed for viral load. Differences in copy number across treatments were expressed as the ratio of CBPV in the virus-injected samples to the average level of CBPV in PBS-injected controls, log-transformed, and analyzed via one-way ANOVA with colony source included as a random effect.

### 2.5. *Nosema ceranae*

Midguts from 40 foragers were collected from a local colony with a high level of *N. ceranae* infection. Midguts were macerated in 5 mL sterile water producing a solution that contained  $\sim 2.9 \times 10^4$  spores/ $\mu$ L. The spore solution was maintained at 4 °C. The identity of *N. ceranae* spores was confirmed by PCR following standard methods (Fries et al., 2013).

#### 2.5.1. Infectivity of *Nosema ceranae* in caged bees

To examine the infectivity of *N. ceranae* spores, an analysis of spore reproduction in newly emerged bees was conducted. Each of the spore solutions was diluted 1:1 in 50% sucrose solution. Concentrations of spore solutions were reconfirmed prior to inoculation and the following doses were given in 5  $\mu$ L to 30 newly emerged bees per treatment—stock: 42,800 spores; transported: 37,600 spores; irradiated: 38,200 spores; control (sugar syrup): 0 spores.

Bees were placed in cages and given sucrose syrup and water *ad libitum*. Another 20 untreated bees were paint-marked on the thorax and added to each cage to increase the population in the cages to a sustainable level. Bees were maintained at 30 °C with  $\sim 65\%$  relative humidity in a controlled incubator (Huang et al., 2014). After 7 days, the experiment was terminated to assess *Nosema* infectivity and reproduction. Bees were stored at  $-20$  °C until spore loads were determined using standard methods (Fries et al., 2013). Those performing the spore counts were blind to the treatment designations. Analysis of *Nosema* infection was performed by calculating the log ratio of the 7-day spore load to the original spore dose to account for the slight difference in the initial doses. The difference in the change in spore load due to treatment was analyzed by Welch ANOVA due to unequal variances across treatment groups (JMP Pro 12).

2.5.2. Germination assay

Solutions containing *N. ceranae* spores were shipped to the University of Minnesota, Twin Cities, to determine spore viability. Solutions were stored in the dark at room temperature for 6 weeks until the assay could be completed. To conduct the germination assay, the solutions were vortexed and 2 µL of each was placed onto a glass slide. This was replicated 6 times per solution. The slides were allowed to air-dry for 10 min at room temperature in a laminar flow hood. Then, 10 µL of a 0.5 M NaCl/0.5 NaHCO<sub>3</sub> (pH to 6.0 with phosphoric acid) germination solution was placed over the dried spot. The slides were cover-slipped and incubated at 37 °C and 90% relative humidity for 30 min. After 30 min, the number of germinated spores was counted by evidence of an extruded polar filament at 400× with phase contrast microscopy. Ten different fields of view were chosen at random, and 10 spores within each field were counted along a transect. A total of 100 spores were counted per slide with a total of 600 spores being counted per treatment. Counts were conducted blindly with respect to treatment. Differences due to treatment in percent germination of spores within the 10 fields of view were analyzed by Welch ANOVA due to unequal variances across treatment groups (JMP Pro 12).

3. Results

3.1. Chalkbrood

Chalkbrood spore development and normal growth of cultures of *A. apis* were observed for spores that were maintained at 4 °C (stock) and those that were transported, but not the irradiated spores (Fig. 1A). Similarly, larvae that were inoculated with irradiated spores had higher survival and did not develop symptoms of CB infection, unlike those fed stock or transported spores (Fig. 1B). Significant differences in lifespan were detected among larvae inoculated with stock or transported *A. apis* spores versus larvae fed spores that were irradiated or no spores (control) ( $\chi^2 = 67.68$ ,  $df = 3$ ,  $P < 0.0001$ ; Fig. 2).

3.2. Black queen cell virus

3.2.1. Infectivity of BQCV in *in vitro*-reared larvae

Larvae were inoculated with solutions containing stock, transported, and irradiated BQCV or a control solution without virus. Mortality and development of symptoms were assessed for 12 days. Most mortality was asymptomatic, though some treated larvae did exhibit typical BQCV symptoms (Simone-Finstrom, personal observations). Overall, control mortality was higher than was seen in the CB assays (Fig. 2), largely due to the increased amount of diet as has been

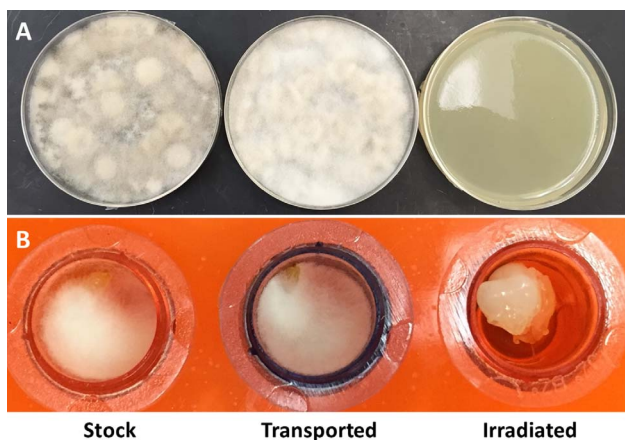


Fig. 1. Growth of *Ascosphaera apis* in cultures or in *in vitro*-reared larvae. (A) Growth of spores in cultures after 96 h. (B) Development of chalkbrood symptoms in a larva treated with either stock or transported spores versus a larva inoculated with irradiated spores. Control (inoculated without spores) not pictured.

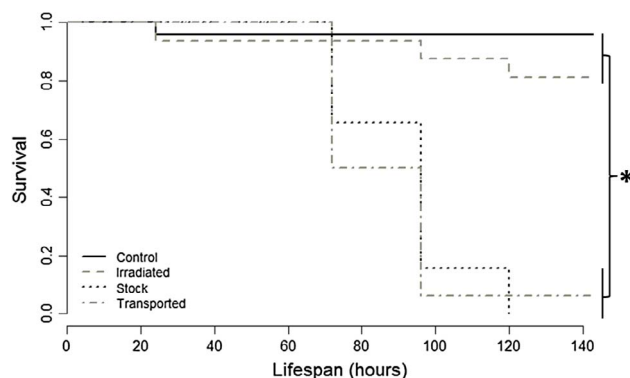


Fig. 2. Proportion of honey bee larvae surviving over time after inoculation with chalkbrood spores. Curves with an overlapping bar were not significantly different ( $P > 0.05$ ) based on pairwise post hoc analysis.

noted in previous research (Linksvayer et al., 2011; Simone-Finstrom, unpublished data). Significant differences in lifespan were seen among larvae inoculated with stock or transported solutions containing BQCV versus larvae inoculated with irradiated or control solutions ( $\chi^2 = 9.69$ ,  $df = 3$ ,  $P = 0.021$ ; Fig. 3). The mean time to death was  $213.6 \pm 21.5$  h for BQCV stock,  $235.2 \pm 17.1$  h for the transported solution,  $276 \pm 6.0$  h for the irradiated solution, and  $274.8 \pm 5.6$  h for the PBS control treated larvae.

3.2.2. Virus injection assay with BQCV

In 2016, pupae injected with stock, transported, or irradiated solutions containing BQCV exhibited varying levels of survival 3 d post-injection and each solution caused arrested development and acute mortality in some individuals (see Table 1 and Fig. 4A). The proportion of surviving pupae injected with the irradiated solution, however, was higher than that for pupae injected with stock or transported solutions, though less than pupae injected with PBS alone ( $\chi^2 = 78.18$ ,  $df = 6$ ,  $P < 0.0001$ ). Post-hoc analysis indicated that this difference was only for the higher dose ( $\chi^2 = 16.50$ ,  $df = 2$ ,  $P = 0.0003$ ), while at the lower dose all three BQCV solutions produced mortality in equivalent proportions ( $\chi^2 = 1.25$ ,  $df = 3$ ,  $P < 0.53$ ). In 2017, all pupae injected with the stock, transported, and irradiated solutions of BQCV were dead or had arrested development 7 days post-injection (Table 2). Therefore it is possible that if the mortality was assessed at 7 days post-injection in 2016 those pupae that appeared to be progressing in normal development might have actually been in an arrested state and potentially would have died by 7 days.

There was a significant difference in viral replication across treatments. The amount of BQCV 3 days post-injection, as analyzed in the 2016 samples, was significantly less for the irradiated treatment as

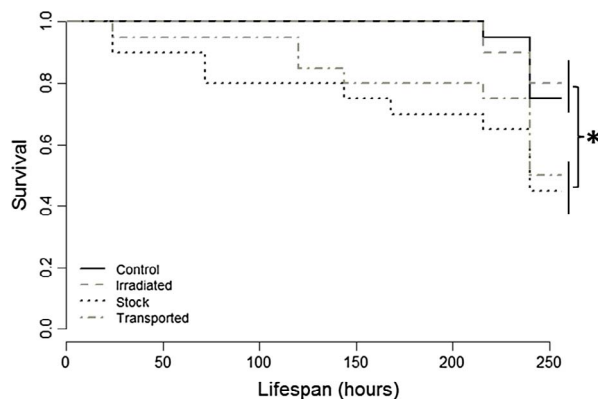
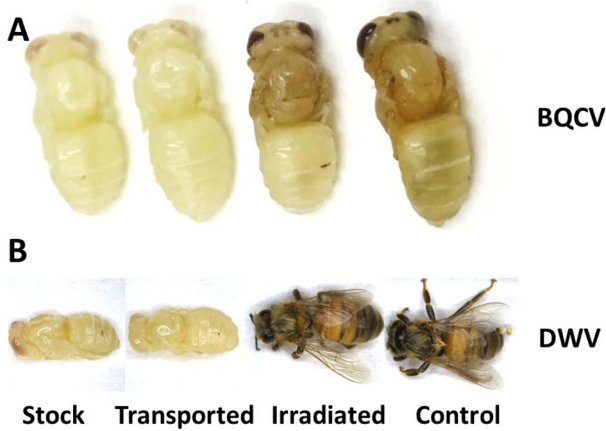


Fig. 3. Proportion of larvae surviving over time after challenge with BQCV. Curves with an overlapping bar were not significantly different ( $P > 0.05$ ) based on pairwise post hoc analysis.

**Table 1**

Pupal mortality 3 days post-injection for 2016 trials. Mortality includes pupae that were in a state of arrested development (based on lack of developmental progression or no increased eye pigmentation) or dead (development of necrotic tissue). Results for BQCV-, DWV-, and control-injected pupae are shown.

Virus	Dose	Treatment	N	% Mortality
None	PBS	Control	35	0%
BQCV	10 <sup>10</sup> copies	Stock	35	80%
	10 <sup>8</sup> copies	Stock	15	46.7%
	10 <sup>10</sup> copies	Transported	35	80%
	10 <sup>8</sup> copies	Transported	15	60%
	10 <sup>10</sup> copies	Irradiated	35	40%
	10 <sup>8</sup> copies	Irradiated	15	40%
DWV	10 <sup>9</sup> copies	Stock	35	100%
	10 <sup>7</sup> copies	Stock	15	100%
	10 <sup>9</sup> copies	Transported	35	100%
	10 <sup>7</sup> copies	Transported	15	100%
	10 <sup>9</sup> copies	Irradiated	35	0%
	10 <sup>7</sup> copies	Irradiated	15	0%

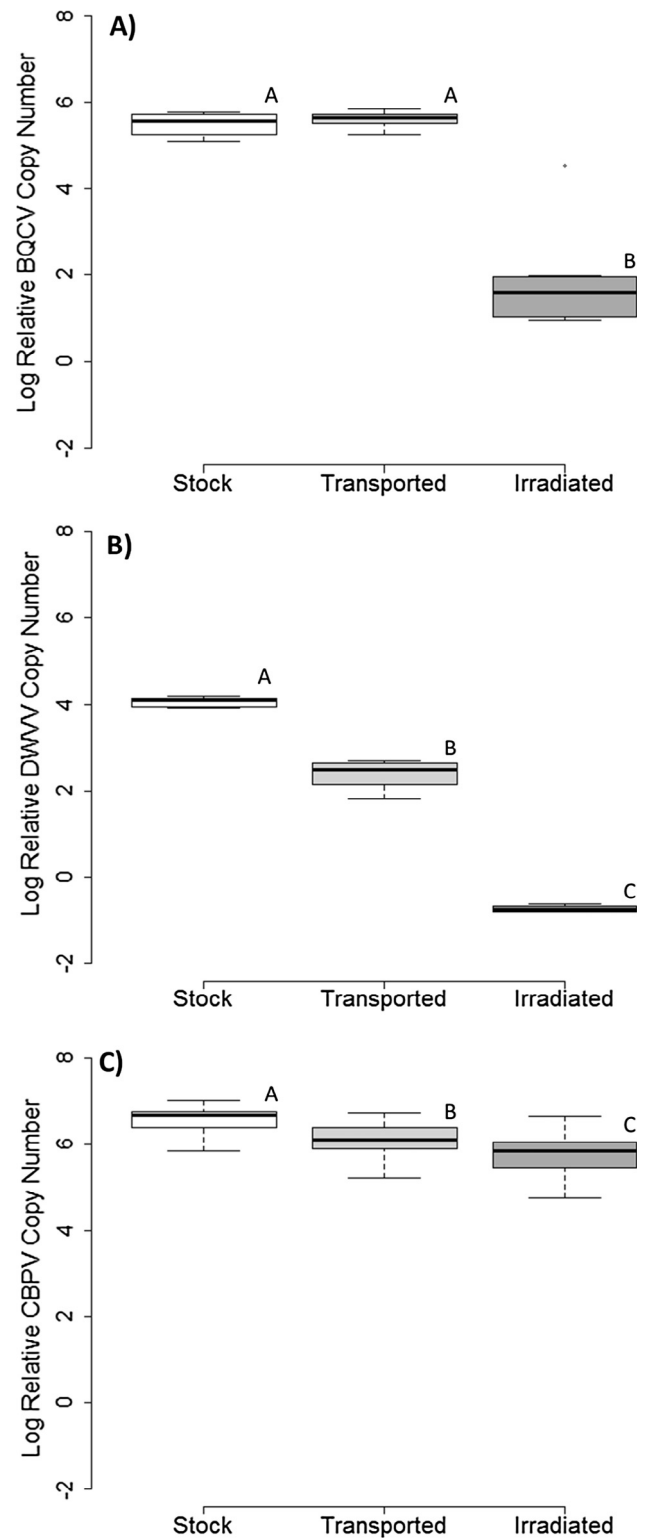


**Fig. 4.** Representative pupae injected with viral solutions. (A) Examples of the arrested development of pupae injected with stock and transported BQCV solutions 3 days post-injection versus typical development (e.g. darkening of eye pigmentation) seen in pupae injected with the irradiated and control solutions. (B) Injection of the DWV stock and transported solutions caused arrested development at 7 days post-injection, with little progression beyond the pink-eyed stage, while pupae injected with PBS control and irradiated DWV solutions exhibited normal development and emerged without overt expression of DWV symptoms.

**Table 2**

Pupal mortality 7 days post-injection for 2017 trials. Mortality includes pupae that were in a state of arrested development (based on lack of developmental progression or no increased eye pigmentation) or dead (development of necrotic tissue). Results for BQCV-, CBPV-, DWV- and control-injected pupae are shown combining pupae from both colonies tested as there were no differences seen across colonies. CBPV mortality was recorded 24 h post-injection.

Virus	Dose	Treatment	N	% Mortality
None	PBS	Control	27	11.1%
BQCV	10 <sup>10</sup> copies	Stock	27	100%
	10 <sup>10</sup> copies	Transported	28	100%
	10 <sup>10</sup> copies	Irradiated	27	100%
DWV	10 <sup>10</sup> copies	Stock	28	100%
	10 <sup>10</sup> copies	Transported	28	100%
	10 <sup>10</sup> copies	Irradiated	28	3.6%
CBPV*	10 <sup>7</sup> copies	Stock	60	96.7%
	10 <sup>7</sup> copies	Transported	60	98.3%
	10 <sup>7</sup> copies	Irradiated	60	98.3%



**Fig. 5.** Viral loads of bees 3 days post-injection with (A) BQCV or (B) DWV or (C) 24 h after injection with CBPV. Viral copy number is expressed as the log of the ratio to the average of the PBS-injected bees. Different letters indicate significant differences across treatment groups.

compared to the stock or transported treatments ( $F_{2,20} = 65.63$ ,  $P < 0.0001$ ; Fig. 5A). The mean copy number for each treatment was as follows: stock— $1.1 \times 10^{10}$  ( $n = 8$ ), transported— $1.3 \times 10^{10}$  ( $n = 8$ ), irradiated— $1.3 \times 10^8$  ( $n = 7$ ), and PBS— $2.9 \times 10^4$  ( $n = 8$ ).

### 3.3. Deformed wing virus

Pupae injected with the different DWV solutions exhibited clear differences with respect to mortality 3 days post-injection in 2016 (see Table 1; Fig. 4B) and at 7 days post-injection in 2017 (Table 2). All of the pupae injected with the stock and transported DWV solutions either experienced arrested development or acute mortality at both higher and lower concentrations. However, all of the pupae injected with the irradiated DWV solutions or the PBS control solution were alive and displayed the normal course of development after 3 days in 2016 and after 7 days in 2017. All of the adult bees injected with PBS and the irradiated DWV solution were asymptomatic.

In terms of viral replication, there were differences across treatments. The amount of DWV 3 days post-injection was the lowest for irradiated treatment, followed by the transported treatment and highest in the stock ( $F_{2,19} = 916.83$ ,  $P < 0.0001$ ; Fig. 5B). The mean copy number for each treatment group was as follows: Stock— $5.3 \times 10^9$  ( $n = 8$ ), Transported— $1.3 \times 10^8$  ( $n = 8$ ), Irradiated— $8.5 \times 10^4$  ( $n = 6$ ), and PBS— $4.5 \times 10^5$  ( $n = 8$ ).

### 3.4. Chronic bee paralysis virus

All but four pupae injected with the various CBPV solutions developed necrotic tissue within 24 h post-injection (Table 2). The amount of CBPV in injected pupae collected 7-days post-injection was lowest in the irradiation treatment, moderate in the transported treatment and highest in the stock ( $F_{2,70} = 22.86$ ,  $P < 0.0001$ ; see Fig. 5C). The mean copy number for each treatment group was as follows: Stock— $1.9 \times 10^{10}$  ( $n = 23$ ), Transported— $6.9 \times 10^9$  ( $n = 24$ ), Irradiated— $3.9 \times 10^9$  ( $n = 24$ ), and PBS— $5.5 \times 10^3$  ( $n = 16$ ).

### 3.5. *Nosema ceranae*

*N. ceranae* spore loads were assessed 7 days post-inoculation. The change in number of *N. ceranae* spores (ratio of spores at 7 days to inoculation dose) was significantly different due to treatment ( $F_{2,87} = 36.67$ ,  $P < 0.0001$ ; Fig. 6). Worker bees fed transported spores exhibited the highest increase in spore load (mean ratio  $\pm$  standard deviation:  $63.21 \pm 91.25$ ), followed by bees fed the stock solution ( $18.16 \pm 23.40$ ), with the lowest change in *Nosema* levels in the irradiated group ( $0.99 \pm 0.82$ ). Twenty un-inoculated bees were

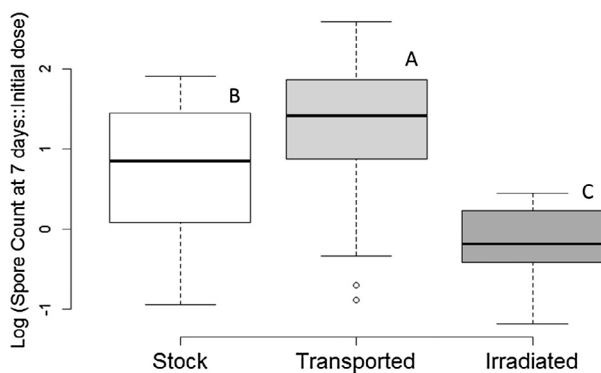


Fig. 6. Spore loads in *Nosema*-infected bees 7 days post-inoculation. Spore counts are presented as the log ratio of *Nosema* spores after 7 days compared to the original dose. A value above 0 indicates that *Nosema* increased in spore number in the honey bee gut, while a value of 0 indicates no change in spore number from the original inoculum. Different letters above each box plot indicate significant differences.

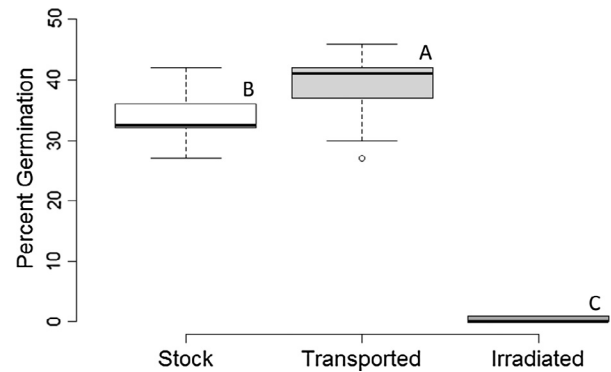


Fig. 7. Percent germination of *Nosema ceranae* spores that were irradiated, transported, or remained at 4 °C (stock). Different letters above each bar indicate significant differences.

also sampled from cages containing inoculated bees and were found to be free of *Nosema* infection (data not shown). Variation across individuals within treatments was likely, at least in part, due to inaccuracy during the inoculation procedure, though bees given stock and transported solutions containing *Nosema* largely saw increases with respect to spore levels, while those fed the irradiated solution saw little change in spore counts.

This finding was corroborated by the germination assay. Showing the same pattern as the inoculation assay, the percent germination was highest for the transported spores ( $39.1 \pm 6.2\%$ ), followed by the stock spores ( $33.8 \pm 4.3\%$ ), with the irradiated spores having the lowest ( $0.48 \pm 0.42\%$ ) ( $F_{2,27} = 464.6$ ,  $P < 0.0001$ ; Fig. 7).

## 4. Discussion

Previous work on the effectiveness of gamma irradiation against honey bee pathogens has focused mainly on the bacterial brood pathogen American foulbrood (Baggio et al., 2005; De Guzman et al., 2011; Gosselin and Charbonneau, 1990). Our results also showed that gamma irradiation effectively rendered the fungus *Ascosphaera apis* (CB), the microsporidian gut parasite *Nosema ceranae*, and *Deformed wing virus* (DWV) inactive and reduced infectivity of *Black queen cell virus* (BQCV). We found less of an effect of irradiation on *Chronic bee paralysis virus* (CBPV). These results suggest that gamma irradiation has a relatively broad effect against a suite of honey bee pathogens and highlights its potential as a treatment to reduce the impacts of pathogens harbored in older wax combs that are often exchanged among colonies and even beekeeping operations.

DWV showed a high level of inactivity in response to gamma irradiation. All pupae injected with the irradiated viral solution survived while none of the pupae injected with the stock or transported viral solutions survived at either a high or low dose. It is interesting to note that the same level of inactivation was not seen for BQCV. Despite the effect of slightly reducing viral replication, irradiation of the BQCV solution did not completely eliminate negative effects as some pupae died or experienced arrested development at 3 days post-injection in 2016 and all pupae injected with irradiated BQCV were dead or had arrested development at 7 days post-injection in the 2017 trials. Although there were clear effects for BQCV *in vitro* reared larvae, its effectiveness was not quite as strong as compared to DWV, CB or *N. ceranae*. Alternatively, irradiation had little to no effect against CBPV at the dose tested since nearly all of the injected pupae developed necrotic tissue within 24 h. While DWV and BQCV are both positive-sense single-stranded RNA viruses with icosahedral shape and similar size (de Miranda et al., 2013), they do have two different forms of genomic organization and are assigned to the genera *Ilfavirus* and *Cripavirus*, respectively. DWV has a single, large open reading frame, which could possibly make it more sensitive to irradiation, versus the fact that BQCV has two non-overlapping open reading frames. In addition, the genome

of DWV is monopartite monocistronic, while BQCV is monopartite bicistronic and this structural difference could result in BQCV being able to more efficiently translate proteins during cellular invasion and thus, infection (Chen and Siede, 2007), and therefore it may be more resilient against damage from irradiation. Chronic bee paralysis virus is unlike the other RNA honey bee viruses in that it is not isometric, has an unusual viral capsid, and has a unique genomic structure comprised of two major RNAs and seven putative open reading frames (Ribi re et al., 2010). These factors could play a role in the limited effectiveness of gamma irradiation against this virus at the relatively high dose tested. If anything, it raises interesting questions and makes the clear point that examining infectivity post-irradiation is key to understanding the usefulness of treatments like gamma irradiation for pathogen control. It is possible that the low intensity and amount of radiation (25 kGy delivered over 9h45m) was not adequate to inactivate these viruses. Future experiments to assess the effects of irradiation parameters will yield more insight into effective methods of virus inactivation.

Similarly, the gamma irradiation treatment did not completely inhibit *Nosema* germination as 3 out of the 600 counted spores had an extruded polar filament indicating that some irradiated spores may remain infectious after treatment. Nevertheless, this low level of germination did not translate into an increase in spores in inoculated bees, unlike what may be suggested by work with bumblebees fed irradiated or non-irradiated pollen (Graystock et al., 2016). Perhaps the biological relevance of inhibited infectivity of treated *Nosema* spores carries more significance than germination rate. Similar findings have been shown for effects of gamma irradiation on *N. apis* (Liu et al., 1990), which has largely been replaced by *N. ceranae* in most of the US in recent years (Chen et al., 2008; Klee et al., 2007). Regardless, the conditions of irradiation (e.g. sample preparation, length of treatment) and the structure of the microorganism are factors to consider in how effective gamma irradiation may be for sterilization, particularly in the case of viruses (Sullivan et al., 1971). Further studies are needed to test infectivity and perhaps even dose response curves to determine the optimum dose of irradiation.

Lack of available therapeutics against viruses, in particular, provides a strong impetus for the investigation of high dose gamma irradiation as treatment of honey bee equipment. This is especially true as viral infections are major drivers of colony loss (Amiri et al., 2015; Cornman et al., 2012; Cox-Foster et al., 2007) and wax comb can harbor viruses ((Colwell et al., 2017); Simone-Finstrom, Rinkevich and de Guzman, unpublished data). Although RNAi feed treatment has been tested against *Israeli acute bee paralysis virus* (Hunter et al., 2010; Maori et al., 2009), it has not developed into a product accessible to the apicultural community. The only current “treatment” per se for various hive-borne diseases is to rotate old comb out of operations (Berry and Delaplane, 2001; Fries, 1988); however this might not be economically feasible in some operations. Given this, there has been much interest in treatments that sterilize comb, in addition to gamma irradiation. Fumigation with ethylene oxide was a popular option in the 1970s and 1980s, as it was effective against CB (Gochnauer and Margetts, 1980) but had variable success against AFB (Gochnauer et al., 1979). Ozone has also shown to be effective against CB and AFB, but only when combined with high temperature exposure, which may limit its usefulness for wax comb (James, 2011). More recent work has investigated the possibility of using double inductively coupled low pressure plasma (DICLPP) as a veterinary approved method of honey bee comb sterilization (Priehn et al., 2016); however, this method of treatment is not 100% effective against bacterial spores and requires operators to use lower than optimal temperatures during the treatment process. Based on previous studies indicating broad effects of gamma irradiation against AFB (Hornitzky, 1994), pollen sterilization (Graystock et al., 2016; Meeus et al., 2014), and this study, the utility of gamma irradiation in beekeeping operations warrants further investigation. It is key to consider infectivity of post-irradiated materials, however, in all future studies. Reductions in detection can be noted post-irradiation,

but ultimately the functional infection rate and symptom development is still the most important factor for positive or negative effects regarding treatment, as seen with *Nosema* (small level of germination resulted in no infection) and the viruses CBPV and BQCV (reduced detection but still caused high mortality).

Subsequent studies aim to determine the potential long-term benefits that using gamma-irradiated comb in standard beekeeping practices can have on colony health and productivity. Since it has been suggested that placing colonies on irradiated comb may be beneficial (Cox-Foster et al., 2007; Pettis et al., 2007) and the work here has demonstrated clear, immediate effects against a suite of honey bee pathogens, the logical next step is to fully test the effects on actual honey bee colonies. Experiments are, in fact, underway to address this question.

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Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jip.2018.02.011>.

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