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# Phage Therapy is Effective in Protecting Honeybee Larvae from American Foulbrood Disease

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**ABSTRACT.** American foulbrood disease has a major impact on honeybees (*Apis melifera*) worldwide. It is caused by a Gram-positive, spore-forming bacterium, *Paenibacillus larvae*. The disease can only affect larval honeybees, and the bacterial endospores are the infective unit of the disease. Antibiotics are not sufficient to combat the disease due to increasing resistance among *P. larvae* strains. Because of the durability and virulence of *P. larvae* endospores, infections spread rapidly, and beekeepers are often forced to burn beehives and equipment. To date, very little information is available on the use of bacteriophage therapy in rescuing and preventing American foulbrood disease, therefore the goal of this study was to test the efficacy of phage therapy against *P. larvae* infection. Out of 32 previously isolated *P. larvae* phages, three designated F, WA, and XIII were tested on artificially reared honeybee larvae infected with *P. larvae* strain NRRL B-3650 spores. The presence of *P. larvae* DNA in dead larvae was confirmed by 16S rRNA gene-specific polymerase chain reaction amplification. Survival rates for phage-treated larvae were approximately the same as for larvae never infected with spores (84%), i.e., the phages had no deleterious effect on the larvae. Additionally, prophylactic treatment of larvae with phages before spore infection was more effective than administering phages after infection, although survival in both cases was higher than spores alone (45%). Further testing to determine the optimal combination and concentration of phages, and testing in actual hive conditions are needed.

**Key Words**: American foulbrood disease, bacteriophage, *Paenibacillus larvae*, honeybee

American foulbrood disease (AFB) is one of the most economically significant diseases of honeybees and is one of the many afflictions contributing to colony collapse disorder (Genersch 2010). Because of the important role of honeybees in pollination of crops (Klein et al. 2007), the control of diseases reducing their number, such as AFB, is of great importance (Genersch 2010). Common treatment methods for AFB include antibiotic therapy and, when ineffective, often complete destruction of infected hives by burning is required. Larvae are most susceptible to *P. larvae* infection 12–36 h after hatching (Genersch 2010). Once spores are ingested, they germinate in the midgut, and the vegetative bacteria propagate rapidly to disrupt the integrity of epithelial cells allowing them to reach the hemocoel. Dead larvae are degraded to a ropy mass which when dried creates scales (Genersch 2010). The scales contain millions of spores which can easily be transmitted across hives. What is more, spores are very resistant to extreme conditions such as heat and drying; they can maintain their infectivity up to 35 years (Haseman 1961). These factors have rendered the control of AFB very difficult.

Antibiotics are not sufficient to combat the disease due to increasing resistance among *P. larvae* strains (Evans 2003, Murray et al. 2007). Antibiotic resistance to oxytetracycline, which is commonly used to treat AFB, has also been reported (Miyagi et al. 2000, Tian et al. 2012). Residual antibiotics in honeybee products is also of concern (Ortelli et al. 2004), and this has resulted in many countries banning their use. Therefore, alternative therapeutic methods against *P. larvae* are of great interest. One such method is therapeutic use of bacteriophages.

Phages are self-propagating, host-specific viruses that are extremely abundant in environmental samples (Suttle 2005, Suttle and Fuhrman 2010). A few studies have investigated single phages isolated from *P. larvae* strains (Gochnauer 1970, Valerianov et al. 1976, Drobnikova and Ludvik 1982, Dingman et al. 1984, Bakheit and Stahly 1988, Campana et al. 1991, Sheflo et al. 2013) but not as a treatment for AFB.

*P. larvae*-specific phages (32 isolates) were previously isolated and characterized in our laboratory. These were obtained by screening *P. larvae* strains for induced lysogenic phage and isolation of lytic phages from soil, hive materials, and bee products (Yost 2014). Phages were tested in honeybee larvae cultivated under laboratory conditions to investigate their efficacy in protecting larvae infected with *P. larvae* spores.

## Materials and Methods

**Bacterial Strain and Bacteriophage Origin.** *P. larvae* strain NRRL B-3650, originally isolated from diseased honeybee larva in Australia (Agricultural Research Service [NRRL] Culture collection) was used in all experiments. Pure bacterial spores were kindly provided by Israel Alvarado. Three bacteriophages designated F, WA, and XIII previously isolated in our laboratory from wild *P. larvae* strain 2231, soil samples from underneath a hive (provided by Karen Bean, Brookfield Farm Bees and Honey), and an infected hive scale, respectively, were used in all experiments. The phages were selected based on susceptibility of *P. larvae* strain NRRL B-3650 to lysis. Presence of phages was determined by plaque formation in soft agar overlays of *P. larvae* (Hurst and Reynolds 2002, Yost 2014).

**Phage Amplification and Quantification.** Phage amplification and titer determination were performed by inoculating phage lysates into fresh overnight cultures of *P. larvae* (1:50 ml) at 37°C and 100 rpm Glucose modified Brain Heart Infusion (Gm-BHI) broth containing 38 g BHI (Difco) (Franklin Lakes, NJ), 1 mM each $CaCl_2$ and $MgCl_2$, and 0.4% Glucose (Sigma-Aldrich (St. Louis, MO)) per liter of $ddH_2O$. After overnight incubation, cells were pelleted by centrifugation at 4,000× *g* for 20 min followed by filtration of the supernatant through 0.45 µm cellulose acetate sealed filtration units (VWR (Visalia, CA)) to obtain cell free phage lysates. The three phages F, WA, and XIII were grown to a titer of $8.5 \times 10^9$, $4.75 \times 10^8$, and $1.64 \times 10^8$ pfu/ml, respectively, before use in treatments. Each phage (200 µl lysate) was mixed

**Table 1. Description of control and treatment groups in the study**

| | Experiment | No. of larvae | Description |
|---|---|---|---|
| Control | [1]Negative control | 30/treat | Basic larval diet (BLD) diluted in Gm-BHI Broth |
| | [2]F Phage control | 90 | F phage administered on day 0 |
| | [3]WA Phage control | 90 | WA phage administered on day 0 |
| | [4]XIII phage control | 90 | XIII phage administered on day 0 |
| | [5]Spores control | 90 | *P. larvae* NRRL B-3650 spores administered on day 1 |
| Treatment | [6]F→Spores | 90 | F phage administered on day 0 followed by spores administered on day 1 |
| | [7]WA→Spores | 90 | WA phage administered on day 0 followed by spores administered on day 1 |
| | [8]XIII→Spores | 90 | XIII phage administered on day 0 followed by spores administered on day 1 |
| | [9]Spores→F | 90 | Spores administered on day 0 followed by F phage administered on day 1 |
| | [10]Spores→WA | 90 | Spores administered on day 0 followed by WA phage administered on day 1 |
| | [11]Spores→XIII | 90 | Spores administered on day 0 followed by XIII phage administered on day 1 |

Each treatment group consisted of 30 larvae and was repeated in triplicate for a total of 90 larvae. A negative control (30 larvae) was included with each treatment group. Larvae were fed increasing amounts of food over time and were monitored from days 0 to 8. Treatments were administered on day 0 (grafting day) and day 1 (24 h following grafting). Superscripts refer to experimental results in Figs. 1–3.

with 800 µl of larval food (Crailsheim et al. 2012) immediately before use.

**Larval Experiments.** Table 1 summarizes control and treatment groups in the study. The final number of spores and phage fed to the larvae were calculated based on the phage and spore titer added to larvae food and the volume of food fed each day (Table 2). Bacterial spores were prepared by inducing sporulation, harvesting, and purifying spores as described by Alvarado et al. (2013). The spore suspension (200 µl) was mixed with 800 µl of food, and the spore count was adjusted by using a Petroff-Hauser counting chamber, so that 1,000 spores were fed to each larva. Each experiment was incubated for 8 d.

**Larvae Rearing.** Honeybee larvae were reared according to methods described by Crailsheim et al. (2012) and De Graaf et al. (2013). To control the location and timing of larvae production, the honeybee queen in each colony was confined on a suitable empty frame using specific queen excluder cages (self-made). If eggs were present the following day, the cage was removed, and the frame was placed back inside the hive until the eggs hatched. At 12–24 h post-hatching (first instar), the larvae were grafted into Petri dishes and reared in laboratory conditions simulating the beehive. The day of grafting was considered day 0 for experimental purposes. The Petri dishes were placed in a plastic container in an incubator at 33 ± 2°C and relative humidity of 90% for 8 d. Incubation humidity was maintained by placing 1 liter of 10% glycerol at the bottom of the plastic container, followed by a support system to avoid contact with the liquid, on top of which sat the Petri dishes containing larvae. Plastic containers were closed with loosely fitting lids. Metal trays filled with water were placed on the bottom of the incubator to maintain humidity inside the incubator. Larvae were fed daily with the amount of basic larval diet indicated in Table 2. For the negative control, larvae were fed the basic larval diet diluted in Gm-BHI broth (200 µl broth in 800 µl food). Each experimental treatment had a corresponding negative control ($n = 30$) prepared on the same day from the same bee frame. For phage or spores control groups, larvae were administered one dose of phages or spores on day 0 followed by the basic larval diet for the remainder of each experiment. Six treatment groups were tested as indicated in Table 2. The larvae were fed phage on day 0 followed by spores on day 1 or vice versa once, then basic larval diet for the remainder of the experiment.

Larvae were examined microscopically (Nikon SMZ-U dissecting microscope) for signs of death including lack of movement, change of color to gray or brown and engorgement followed by disintegration of body segments (Genersch et al. 2005). The number of surviving larvae was recorded daily. Dead larvae were collected and stored in −20°C for polymerase chain reaction (PCR) testing to determine presence of bacterial DNA.

**Basic Larval Diet Preparation.** Larval food was prepared according to Peng et al. (1992). The recipe consisted of 14.4 ml sterile distilled water, 4.2 g lyophilized royal jelly (Glory bee, Eugene OR), 0.6 g glucose (Difco), 0.6 g fructose (Difco), and 0.2 g yeast extract (Difco). Sugars and yeast extract were mixed with water and filter-sterilized using a 0.45 µm cellulose acetate membrane syringe filter (VWR). Royal jelly was aseptically added to the sugar water mixture and homogenized by vortexing. The food was prepared fresh daily.

**Table 2. Number of spores and phages (pfu) fed to each individual larva based on treatment group**

| Treatment | Day 0 | Day 1 |
|---|---|---|
| Spores-phage | | |
| No. of spores | 1,000 | 0 |
| F phage (pfu) | 0 | $1.7 \times 10^7$ |
| WA phage (pfu) | 0 | $9.5 \times 10^5$ |
| XIII phage (pfu) | 0 | $3.28 \times 10^6$ |
| Phage-Spores | | |
| No. of spores | 0 | 1,000 |
| F phage (pfu) | $1.7 \times 10^7$ | 0 |
| WA phage (pfu) | $9.5 \times 10^5$ | 0 |
| XIII phage (pfu) | $3.28 \times 10^6$ | 0 |

| Day | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| Basic larval diet (µl/larvae) | 10 | 10 | 20 | 30 | 40 | 50 | 50 | 60 | 0 |

Spores and phage were administered on either day 0 or 1. The bottom two rows indicate the amount of larval food fed to each larva on each day of the experiment.

***P. larvae* DNA PCR Amplification.** DNA from four dead larvae (stored at −20°C until use) was extracted using a DNeasy Blood and Tissue spin column kit according to manufacturer's instructions (Qiagen, Valencia CA). PCR was prepared to a 25 µl reaction volume containing 0.5 µM of each forward and reverse primers, 12.5 µl of 2xMidasMix with Taq DNA Polymerase (Monserate Biotechnology Group, San Diego, CA). The MidasMix contained 2 mM $MgCl_2$ and 0.22 mM dNTP; 50–100 ng of DNA template and enough nuclease-free water were added to reach 25 µl.

The primers were designed to amplify a 700-bp region of the *P. larvae* 16S rRNA gene (Piccini et al. 2002). The sequences of forward and reverse primers were as follows:

Forward primer (Pl5): 5'-CGAGCGGACCTTGTGTTTCC-3'
Reverse primer (Pl4): 5'-TCAGTTATAGGCCAGAAAGC-3'

The PCR conditions were 95°C (1 min), 30 cycles of 93°C (1 min), 55°C (30 s), 72°C (1 min),and one final cycle of 72°C (5 min) carried out on Perkin Elmer GenAmp PCR Thermocycler 2400. PCR product electrophoresis (120 V, VWR power source) was done on 0.8% (w/v) agarose gel (Amresco LLC, Solon, OH) with 1:10,000 (v/v) GelRed solution (Phoenix Research Products, Candler, NC). The gel was visualized on a UVP Biospectrum Imaging System (UVP LLC, Upland, CA). DNA extracted from bacterial strain 3650 was used as positive control.

**Statistical Analysis.** Kaplan–Meier survival analysis and log-rank test for pair-wise comparison between each treatment group was carried out using GraphPad Prism (GraphPad software, Inc. La Jolla, CA).

A $P$ value of less than 0.05 was considered significant.

## Results

Negative control, phage control, and spore-infected control survival rates are presented in Fig. 1. At day 8, the survival for the negative control was 84.4% (average of all negative controls). Survival rates with the F, WA, and XIII phage control larvae were 88.8%, 85.5%, and 86.6%, respectively. No statistically significant difference was observed between the survival rate of the negative control (basic larval diet diluted in Gm-BHI) and each of the phage control experiments. Spore-infected larvae began to die at day 2 but at a slow rate. The survival dropped to 70% by day 4, followed by a sharp decrease to 48.8% at day 5. The survival at day 8 for the spore-infected control larvae was 45.5%. The difference in survival of the negative control larvae and spore-infected larvae was significant ($P < 0.001$) demonstrating that the *P. larvae* spores were lethal to approximately half the larvae.

Figure 2 presents the percent survival of larvae when phages were administered before spores. The survival level at day 8 for larvae administered with F phage on day 0 (grafting day) and spores 24 h later on day 1 (F→ spores) was 82.2%. The same survival rate for WA phage on day 0 then spores on day 1 (WA→ spores) and XIII phage on day 0 then spores on day 1 (XIII→ spores) groups was 84.4% and 70%, respectively. No statistically significant difference was observed between the negative control larval survival and F→ spores and WA→ spores larval survival ($P = 0.785$, $P = 0.958$); however, the difference between the negative control and XIII → spores treatment groups was only slightly significant ($P = 0.047$).

Survival of spores→phage treatments are presented in Fig. 3 along with negative control and spore-infected control groups. Percent survival for Spores→ F, Spores→ WA and Spores→ XIII treatments at the end of each experiment was 55%, 56.6%, and 68.3%, respectively. No statistically significant difference was observed between larvae fed only spores on day 1 and spores→ F larvae ($P = 0.2082$). The same was true for the spores→ WA treatment group ($P = 0.1776$), although the difference between spores control and spores→ XIII treatment group was significant ($P = 0.0093$). When comparing the order of administration of phages and spores, a significant difference was observed between F→ spores and spores→ F treatment ($P = 0.0003$). WA→ spores vs. spores→ WA was also statistically significant ($P = 0.0005$), yet there was no difference between XIII→ spores vs. spores→ XIII treatment ($P = 0.9098$).

A phage cocktail consisting of equal parts of each of the three phages but totaling $10^7$ pfu (F, WA, and XIII) similar to the concentration of each phage alone in the other phage treatments was also administered to the larvae on day 0 followed by spores on day 1 and vice versa. The percent survival of the larvae was not different than the value of any of the phages individually (data not shown).

Tables 3 and 4 list the number and percentage of "PCR positive" larvae that contained confirmed *P. larvae* DNA in each of the control and treatment groups. None of the negative or phage control groups were positive for *P. larvae* DNA. *P. larvae* DNA was recovered from 86 to 100% of dead larvae in spore-infected control groups depending upon the day. Interestingly, when spore-infected survivors were tested on day 8, only 25% of them were *P. larvae*-PCR positive.

PCR analysis of phage→spores and spores→phage-treated larvae varied depending upon the type of phage and order of administration of phages and spores. No phage only or negative control larvae contained PCR products demonstrating that there was no cross contamination between controls and spore treatments. The percentage of PCR positive samples from spores→phage treatments indicated that the larvae treated with spores→ XIII, spores → WA, and spores → F had 50, 100, and 100% positive results for presence of *P. larvae* DNA, respectively. However, when XIII and WA phages were applied before spores, the results were less consistent at 75 and 0%, respectively. The high value for phage treatment with XIII may be in part due to the small sample size for this treatment.

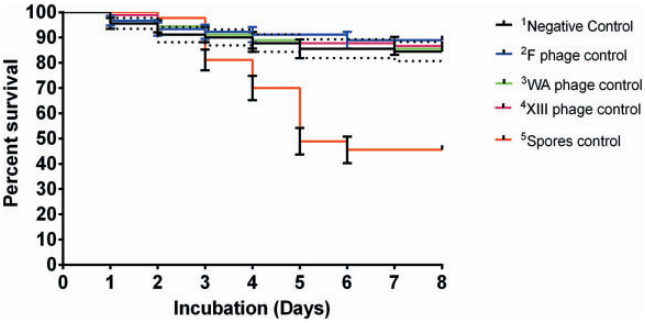


**Fig. 1.** Experimental controls for phage therapy experiments. Errors bars represent standard error of the mean

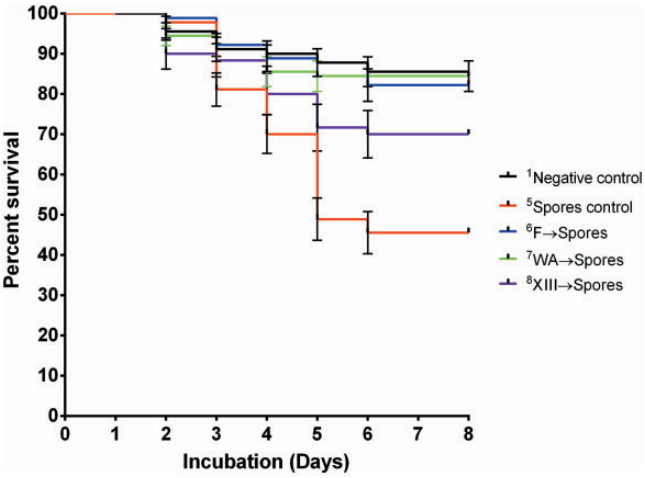


**Fig. 2.** Phage prophylaxis compared with spores treatment alone. Phages were administered on day 0 (grafting day) followed by administration of spores on day 1 (24 h later). Error bars indicate standard error of the mean

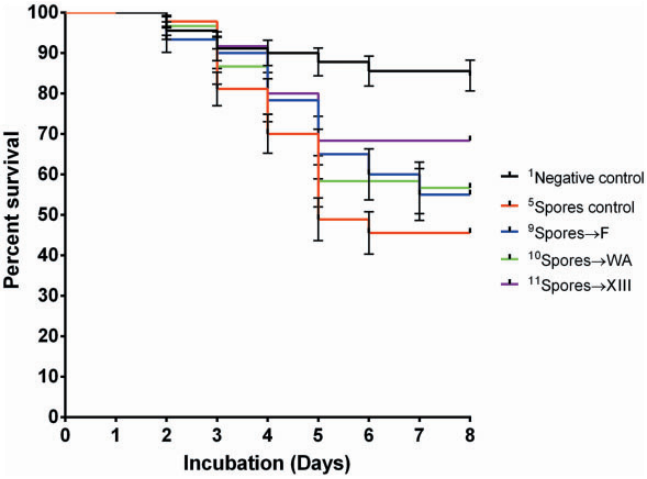


**Fig. 3.** Phage treatment of existing disease in honeybee larvae. Spores were administered on day 0 (grafting day) followed by administration of phages on day 1 (24 h later). Error bars indicate standard error of the mean

## Discussion

In the past few years, a renewed interest has been expressed for bacteriophage therapy mainly due to problems associated with antibiotic resistance and also due to inherent phage advantages such as high specificity to their hosts (Matsuzaki et al. 2014). Phage therapy has been used as an alternative to antibiotics in humans (Fischetti et al. 2006) and has also been proposed as an alternative treatment for animal disease, such as in poultry and other food animals (Valerianov et al. 1976, Dingman et al. 1984, Bakheit and Stahly 1988, Campana et al. 1991, Doyle and Erickson 2006). Phage therapy in mice models and food animals as well as fresh food products has demonstrated their capability to act against a variety of pathogens (Smith et al. 1987, Soothill 1992, Barrow et al. 1998, Leverentz et al. 2003, Matsuzaki et al. 2003). In one study, bacteriophage demonstrated the ability to reduce mortality due to *Escherichia coli* infection by 50% when a high titer of the phage was administered in poultry (Huff et al. 2005). Very few studies have been carried out on bacteriophage therapy in invertebrates, most of which have used invertebrate models to study phages on pathogens originally affecting mammals (Seed and Dennis 2009, Takemura-Uchiyama et al. 2013, Matsuzaki et al. 2014). One exception is a study by Lindberg et al. (2014) where bacteriophages were used to successfully treat *Pseudomonas aeruginosa* infections in *Drosophila melanogaster* (fruit flies). Here, we investigate if honeybee larvae infected with *P. larvae* can be rescued by phages and whether bacteriophages can serve as a preventive measure to *P. larvae* infection.

Negative control experiments consisted of diluting basic larval diet in Gm-BHI broth, the results of which demonstrated that Gm-BHI broth does not result in a significant decrease in survival of larvae as average negative control survival was 84% (Fig. 1). Larvae fed only phages in Gm-BHI also survived well and to approximately the same level as negative control larvae (∼85%). Survival percentage of negative control and phage control experiments are consistent with previous reports indicating that a 15% mortality rate is expected while rearing honeybee larvae under laboratory conditions (Crailsheim et al. 2012). The results also indicate that administering each of the phages to larvae does not pose any adverse effect to larval survival since the survival rates of all phage control groups were slightly higher than the negative control group (Fig. 1).

Previous reports indicate that as low as 10 *P. larvae* spores can be infective (Genersch 2010) yet when the larvae used here were infected with an initial dose of 300 spores, a very low mortality rate occurred, therefore a higher number of spores was used in the study (∼1,000 spores/treatment). *P. larvae* have varying degrees of virulence depending upon strain and genotype (Genersch et al. 2005); therefore, it is possible for the *P. larvae* NRRL B-3650 strain to possess a lower virulence compared with others. However, a significant decrease in larval survival rate infected with spores compared with the negative control was observed, and this is in agreement with previous reports (Tarr 1938, Woodrow 1943).

A comparison between survival rates of larvae treated with phages before *P. larvae* infection and the negative control larvae indicates that administering F or WA phage before an infection can increase survival rates equal to larvae which had never been infected with spores (Fig. 2).

By comparing survival rates in Fig. 3, it appears that administering phage post-infection also increases the larval survival compared with infected larvae, although administering phage prior to spores administration results in a significantly higher survival from administering spores prior to phage. Survival of larvae treated with F or WA phages 1 d after administering spores resulted in approximately 55% survival compared with 45% survival with spores alone. Treatment with phage XIII resulted in a slightly better survival (70%) compared with spore treatment alone under these experimental conditions.

Administering the phages whether prior to or following spore infection has beneficial effects on larvae survival. Overall, the results indicate that administering phages prior to infection with *P. larvae* spores decreases mortality of infected larvae and can potentially be used as a prophylactic treatment for AFB.

PCR results from dead larvae post-infection demonstrated that no cross contamination of samples occurred during preparation or incubation since none of the negative control treatment larvae and phage control samples showed evidence of bacterial DNA (Tables 3 and 4). The presence of bacterial DNA in dead larvae spore treatments indicates that at least some of the larvae died due to a *P. larvae* infection. *P. larvae* DNA was recovered as soon as 2 d after spore ingestion (day 2), showing that the bacteria can quickly cause larval death. DNA recovery from phage→ spore-treated larvae differed significantly with the type of phage, indicating that some of the phages (in this case WA) might be more efficient in eliminating the bacteria yet only when administered prior to an established infection (Tables 3 and 4). A low percentage of *P. larvae* DNA recovery from larvae surviving spore infection alone, spores→ phage and phage→ spores treatment groups on day 8, is demonstrative of an ability to eliminate the bacteria and survive the deadly infection.

In conclusion, our results indicate that a prophylactic treatment with each of three phages is effective in increasing survival rates of larvae infected with *P. larvae* spores. Even though the survival percentage is lower when phages are administered after *P. larvae* infection, they may still result in an effective treatment. Phage therapy can be used as a potential treatment for AFB, although use of a combination of phages that can effectively lyse the broadest range of *P. larvae* strains is required, as well as more testing, to refine techniques for application directly to infected honeybee hives.

**Table 3. Percentage of dead larvae positive for *P. larvae* by PCR amplification by each control and treatment group**

| Treatment | Day | No. tested[a] | PCR positive (%) |
|---|---|---|---|
| [5]Spores control | 2 | 2 | 100 |
| | 3 | 7 | 86 |
| | 4 | 10 | 100 |
| | 5 | 6 | 100 |
| | 8 | 18 | 89 |
| [1]Negative control | 5 | 1 | 0 |
| | 6 | 2 | 0 |
| | 7 | 4 | 0 |
| [2]F Phage control | 7 | 3 | 0 |
| [3]WA Phage control | 7 | 5 | 0 |
| [4]XIII Phage control | 7 | 4 | 0 |
| [8]XIII →Spores | 4 | 4 | 75 |
| | 6 | 4 | 75 |
| [11]Spores→XIII | 5 | 2 | 50 |
| [9]Spores→F | 6 | 2 | 100 |
| [7]WA→Spores | 6 | 2 | 0 |
| [10]Spores→WA | 6 | 2 | 100 |
| Total | | 87 | |

Superscripts refer to experimental results in Figs. 1–3.

[a]Number of tested samples indicates the number of tubes containing dead larvae from each experiment stored at −20°C before testing. Each tube contained a pooled sample of four larvae stored in 1 ml of water.

**Table 4. Percentage of surviving larvae positive for *P. larvae* by PCR amplification by each control and treatment group**

| Surviving larvae | Day | No. tested[a] | PCR positive (%) |
|---|---|---|---|
| [5]Spores control | 8 | 4 | 25 |
| [11]Spores→XIII | 8 | 1 | 0 |
| [6]F→Spores | 8 | 3 | 33 |

Superscripts refer to experimental results in Figs. 1–3.

[a]Number of tested samples indicates the number of tubes containing alive larvae from each experiment stored at −20°C before testing. Each tube contained a pooled sample of four larvae stored in 1 ml of water.

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