Evaluation of Lysozyme-HCl for the Treatment of Chalkbrood Disease in Honey Bee Colonies

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J. Econ. Entomol. 105(6): 1878–1889 (2012); DOI: http://dx.doi.org/10.1603/EC12227

ABSTRACT Chalkbrood, caused by *Ascosphaera apis* (Maassen and Claussen) Olive and Spiltor, is a cosmopolitan fungal disease of honey bee larvae (*Apis mellifera* L.) for which there is no chemotherapeutic control. We evaluated the efficacy of lysozyme-HCl, an inexpensive food-grade antimicrobial extracted from hen egg white, for the treatment of chalkbrood disease in honey bee colonies. Our study compared three doses of lysozyme-HCl in sugar syrup (600, 3,000, and 6,000 mg) administered weekly for 3 wk among chalkbrood-inoculated colonies, colonies that were inoculated but remained untreated, and colonies neither inoculated or treated. Lysozyme-HCl at the highest dose evaluated was found to suppress development of chalkbrood disease in inoculated colonies to levels observed in uninoculated, untreated colonies, and did not adversely affect adult bee survival or brood production. Honey production was significantly negatively correlated with increased disease severity but there were no significant differences in winter survival among treatment groups. Based on our results, lysozyme-HCl appears to be a promising, safe therapeutic agent for the control of chalkbrood in honey bee colonies.

KEY WORDS *Apis mellifera*, honey bee, *Ascosphaera apis*, chalkbrood, lysozyme

Chalkbrood, a fungal disease of honey bee brood caused by *Ascosphaera apis* (Maassen and Claussen) Olive and Spiltoir (Spiltoir and Olive 1955) occurs worldwide (Ellis and Munn 2005). Identified by the sporulated black or nonsporulated white chalk-like larval cadavers it produces, the spores of *A. apis* are spread within and between colonies via adult worker bees who transmit spores to honey bee larvae during feeding (Heath 1982). Chalkbrood epizootics are variable and unpredictable (Befus–Nogel et al. 1992) but generally increase in incidence in early spring (Heath 1982). Cool temperatures, high humidity, inadequate nutrition, stress, and genetic factors have all been implicated as predisposing conditions in the development of chalkbrood disease (Aronstein and Murray 2010).

Although an extensive range of available natural compounds and fungicides has been investigated for the treatment of chalkbrood both in the laboratory and in the field (Hornitzky 2001, Davis and Ward 2003), there is at present no registered chemotherapeutic treatment. Widespread acceptance of a chemical for the treatment of chalkbrood requires that it must be effective, easy to use, and economical (Menapace and Hale 1981). Moreover, it must not compromise the safety and quality of the honey produced for human consumption. Contamination of honey by drug residues used in the treatment of honey bee diseases is an important public safety issue and can affect the import and export of hive products between countries (McKee 2003).

Lysozyme-HCl is an inexpensive, food-grade antimicrobial enzyme used in cheese and winemaking processes in the European Union, United States, and Canada to inhibit the growth of damaging gram-positive bacteria (Johnson and Larson 2005). Gram-positive bacteria with cell walls rich in peptidoglycan are particularly susceptible to lysozyme-HCl as the main mode of action is the hydrolysis of the ß(1–4) linkages in the polymers that make up peptidoglycan causing the cell to lyse (Strominger and Tipper 1974). In addition, lysozyme-HCl is also capable of breaking down chitin (Berger and Weiser 1957), a component of most fungal cell walls (Griffin 1994). The antimicrobial activity of lysozyme-HCl is not solely dependent on its enzymatic activity. Lysozyme has been shown to cause cell death through cell membrane disruption by distorting lipid-lipid interactions and increasing membrane permeability (Ibrahim et al. 1996, Düring et al. 1999) and because lysozyme is highly cationic, it may be also capable of inducing autolysis in bacteria (Ginsburg and Koren 2008). Using in vitro larval rearing assays (Van Haga 2010), it has been shown that lysozyme-HCl is active against the fungus *A. apis*, the causative agent of chalkbrood disease in honey bees, although the mode of action is unknown.

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Safe for both honey bee and human consumption (Johnson and Larson 2005, Van Haga 2010), lysozyme-HCl shows great potential as a control for chalkbrood disease at the colony level. Its high solubility in water and sucrose solutions (Johnson and Larson 2005) allows easy integration into current management practices such as fall or spring feeding. Beekeepers routinely supplement colony diets both in the spring and fall with sugar syrup or high fructose corn syrup to boost colony growth. Lysozyme-HCl is also resistant to changes in temperature and pH. As a result, it is an extremely stable compound and will remain active during prolonged exposure at colony temperatures of 33–35°C (Johnson and Larson 2005). Safe, soluble, and stable, lysozyme-HCl is an ideal candidate for colony-level treatment of chalkbrood disease.

Although widespread, the severity of infection and subsequent economic impact of chalkbrood on beekeeping operations worldwide are highly variable. Considered by some beekeepers to be the most important brood disease encountered in their apiaries (Jākobsons 2005), it is only a mild nuisance for others (Ileana 2007). Even though chalkbrood is rarely lethal to the colony, larval mortality as a result of chalkbrood infection can have a serious impact on the buildup of adult bee populations. It has been suggested that 100 infected larvae is equivalent to a 5% loss in potential adult populations (Taber et al. 1975). Significant reductions in the worker bee population can translate into decreases in foraging resulting in both decreased honey production and pollination efficacy. Estimates of losses attributed to chalkbrood range from 1 to 37% of honey yields (Heath 1982, Yakobson et al. 1991) and up to 49% of foraging capacity (Heath 1982). Experimental assessments of yield losses in honey from clover (*Trifolium alexandrinum* L.) in Egypt were reported to be 18.4 ± 0.7% in naturally infected colonies and as high as 30.1 ± 1.8% in colonies artificially infected with black mummies (Zaghloul et al. 2005). In Beaverlodge, Alberta, Canada, where this study was conducted, previous experiments did not establish a significant relationship between chalkbrood infection levels and honey production (Nelson and Gochnauer 1982).

In this study, therapeutic doses of lysozyme-HCl for the treatment of chalkbrood disease in honey bee colonies were evaluated and the impact of chalkbrood disease on: 1) colony population, 2) honey production, and 3) winter survival in newly established package colonies was assessed.

Materials and Methods

Colony Establishment and Management. Forty colonies were established 24 April 2007 at the AAFC Beaverlodge Research Farm (55° 18′ N; 119° 17′ W). One-kilogram packaged bees with queens (mixed race) were imported from New Zealand and placed in irradiated (10 kGy, Iotron Industries Canada Inc., Port Coquitlam, BC) single brood chambers (nine frames, full depth Langstroth supers). Each colony was equipped with a dead bee trap, modified from Illies et al. 2002, which was attached below the front edge of the bottom board. Bottom boards were modified by removing the rim along the rear of the colonies to accommodate the removal of corrugated plastic sheets (Tenplast 37 × 46 cm) that lay flat beneath the brood chamber. These sheets facilitated the counting of chalkbrood mummies removed from the comb by bees. Colonies were managed as single brood chambers throughout the experiment. Before or during the experiment, colonies were requeened as required (e.g., replacing queens that had perished) with New Zealand queens of similar age to those established with the packages.

At the time of establishment, all colonies were treated with Fumagilin-B (Medivet Pharmaceuticals High River, AB, Canada) in sucrose syrup according to label directions for package colonies (100 mg active ingredient [AI]/colony) for the control of *Nosema apis* Zander. Each colony also received a 454 g pollen supplement patty (Global Patties, Airdrie, Alberta, Canada). At no time in the experiment were varroa mites (*Varroa destructor* Anderson and Truemann) detected.

Colony population (adult bees, sealed and unsealed brood cells) was assessed before start of experiment on 11 May 2007 using a Plexiglas grid (2.5 cm^2) and grouped into eight strength categories (strongest to weakest) based on adult bee and brood population size. One colony per group was randomly selected from within each of the eight strength categories and assigned to each treatment group.

Colony Inoculation. Thirty-two colonies were inoculated on 15 May 2007 (day 1) with chalkbrood spores according to the method outlined by Gilliam et al. (1988). Each colony received a 113 g pollen patty [40% (wt:wt) irradiated pollen, 35% (wt:wt) commercial table grade sucrose, 5% (wt:wt) Brewer's yeast, 20% (wt:wt) sterile distilled water] containing chalkbrood mummies. Mummies were collected from diseased apiaries in the Beaverlodge area and homogenized in 5 ml sterile distilled water using a glass/glass tissue homogenizer (Kontes, Vineyard, NJ). For a batch of 32 pollen patties, a combination of five black and five white mummies (approximately double the inoculation dose used by Gilliam et al. [1988]) to ensure prolonged infection), were used; each pollen patty contained ≈1.56×10^7 spores. Control colonies received a similar pollen patty without the addition of homogenized mummies. Pollen patties were placed on the top bars in the center of the colony. The viability and uniformity of *A. apis* distribution in the patty mixture was determined by plating six pollen patty samples collected from throughout the patty mixture on PDY (Potato Dextrose Agar [Difco, Detroit, MI] + 0.4% (wt:vol) Yeast Extract) media (Shimanuki and Knox 2000). The plated samples were incubated at 30°C for 120 h and examined for growth of *A. apis.* Additionally, two pollen patty samples were collected from each colony if available, 3 and 10 d postinoculation and plated on PDY as previously described to confirm viability of *A. apis* spores.

Colony Treatment. Lysozyme-HCl (inovapure 300, Neova Technologies, Abbotsford, BC, Canada) was mixed in 1:1 (wt:vol) sucrose syrup prepared with table grade sucrose and applied to colonies using frame feeders. Inoculated colonies were untreated or given weekly applications of 600, 3,000, or 6,000 mg lysozyme-HCl for 3 wk. The first treatments for all doses were dissolved in 2 liters of syrup, while in subsequent treatments only 1 liter of syrup was used. The dates of application were 15, 22, and 29 May 2007 (days 1, 8, and 15). The uninoculated and inoculated untreated colonies were fed syrup without lysozyme-HCl. At the fourth weekly assessment the volume of any unconsumed syrup was measured and recorded.

In total, there were five treatment groups containing eight colonies each: uninoculated (uninoculated and untreated); inoculated (inoculated and untreated); low (inoculated and treated with 600 mg × 3 doses of lysozyme-HCl); medium (inoculated and treated with 3,000 mg × 3 doses of lysozyme-HCl); high (inoculated and treated with 6,000 mg × 3 doses of lysozyme-HCl).

Colony Assessments. *Disease Severity and Bee Mortality.* Black and white chalkbrood mummies and dead adult bees were collected and counted from 1) dead bee traps and 2) bottom board sheets on weekdays from 14 May (day 0) until 28 August 2007 (day 106). Chalkbrood mummies and bees collected on Mondays were averaged over 3 d (Saturday, Sunday, and Monday). The frames in the colony were inspected and the numbers of black and white chalkbrood mummies in uncapped cells were counted on days 1, 4, 8, 11, 15, 22, 29, 36, 43, 50, 57, 64, 78, 92, and 106.

Colony Strength. Colony strength was estimated on days 5, 15, 29, 43, and 57. The areas of sealed brood, unsealed brood, and adult bee populations were estimated using a Plexiglas grid (2.5 cm^2). Grids were placed over all the frames in the colony and the number of squares of brood cells and worker bees were counted on both sides of each frame. A conversion factor of 1.5188 bees/cm^2 was used to estimate adult bee populations (Westcott and Winston 1999) and 3.9 worker cells/cm^2 to estimate absolute numbers of sealed and unsealed worker brood cells (Harbo 1986). Adult bee population estimates were performed during early morning hours before bees began to fly.

Honey Production. Honey supers were weighed before placement on colonies and after removal to measure net honey yield. All colonies were provided one honey super on 13 June 2007 and two additional honey supers on 4 July 2007 after which time colonies were provided additional honey supers as needed. Honey was harvested from colonies on 17 July, 31 July, 14 August 2007; supers were stripped from the brood chambers on 27 August 2007.

Stability and Persistence of Lysozyme-HCl. Stored food samples (≈1.5 ml) from each colony were collected from the outer edges of three brood frames to assay for lysozyme-HCl activity 7 d after the first treatment application and then weekly for three additional weeks. A sample from each colony was also collected in the same manner 3 d before the start of the experiment before treatments were applied. Samples were stored at 5°C in 1.5 ml microfuge tubes until analysis of lysozyme-HCl concentrations by the manufacturer, Neova Technologies (Abbotsford, BC, Canada).

Presence of Spores. Five larvae were sampled from each colony on days 1, 8, 15, 22, and 29. Larvae were collected singly into sterile 1.5 ml microcentrifuge tubes using sterile forceps and stored at −20°C until time of plating. Larvae were surface sterilized by wrapping them in sterilized cheesecloth, dipping in 75% ethanol and immediately transferring for 60 s to a 0.5% solution of sodium hypochlorite. Samples were rinsed twice with sterile distilled water then immediately homogenized in 1.0 ml sterile distilled water. The homogenate was vortexed with 15 ml YGSPA + 0.01% chloramphenicol (10 g yeast, 10 g dextrose, 13.5 g KH_2PO_4, 10 g soluble starch, and 20 g agar in 1 liter H_2O) and poured onto plates containing a 7 ml solid media layer. Plates were incubated at 37°C, 10% CO_2 for 24 h to stimulate germination and then at 37°C, 0% CO_2 to establish mycelial growth according to the methods of Nelson and Gochnauer (1982) and Anderson et al. (1997).

Adult nurse bees (30–100) were collected in 50 ml centrifuge tubes with caps and held overnight at −4°C. The digestive tract or gut from five adult bees was removed and rinsed three times with distilled water to remove extraneous debris and microorganisms. After rinsing, each gut was placed into a sterile 1.5 ml microcentrifuge tube and stored at −20°C until time of plating. To withdraw digestive tracts, the stinger was grasped and gently pulled until the entire tract (honey stomach, midgut, hindgut, and rectum) was removed (Shimanuki and Knox 2000). After excising the stinger, each digestive tract was homogenized in 1.0 ml sterile distilled water and cultured according to the methods of Anderson et al. (1997).

Winter Survival. Colonies were assessed for survival (presence of queen and brood) the following spring on 14 May 2008. Frames in the colony were inspected and number of black and white chalkbrood mummies in uncapped cells counted. Colony population (unsealed brood, sealed brood, and adult bees) was estimated as previously described.

Statistical Analysis. Colonies were excluded from the experiment when chronic queenlessness or diseases other than chalkbrood resulted in less than one frame of healthy unsealed brood at any time before over-wintering. The numbers of colonies included in the final analysis for each of the treatment groups were: uninoculated (7); inoculated (6); low (8); medium (5); and high (7).

Differences among treatment groups for the number of average mummies (total, black, and white) collected daily from traps, average weekly frame mummy counts, adult mortality, honey yield, accumulation of lysozyme-HCl in stored food and differences in the number of white and black mummies collected were compared using one-way analysis of variance (ANOVA) and a posteriori comparisons

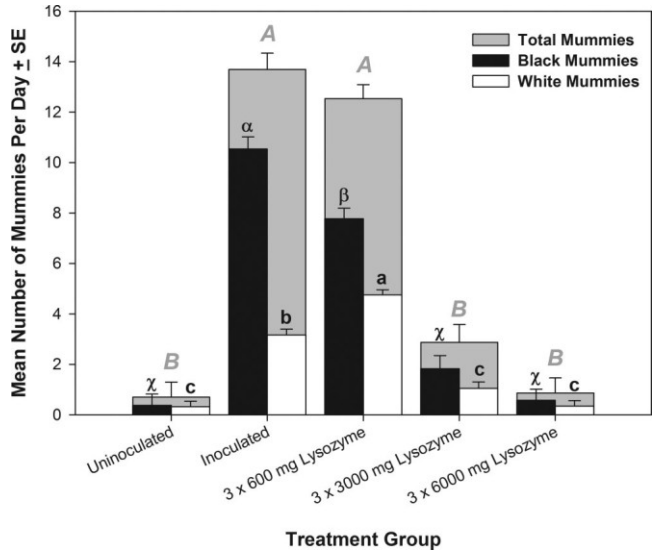

Fig. 1. The mean daily number of mummies (total, black, and white) collected on weekdays from traps and bottom boards for each treatment group from 14 May to 28 August 2007; collections on Monday were averaged over Saturday, Sunday, and Monday. The numbers of total, black, and white mummies collected were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey–Kramer HSD). For each category different letter styles were used to indicate significance: total mummy production (gray, uppercase, italicized letters, ***A***); black mummy production (black, lowercase, Greek letters, α); white mummy production (black, lowercase letters, a).

(Tukey–Kramer honestly significant difference [HSD]; $\alpha = 0.05$).

Winter survival between treatment groups was compared using a χ^2 test ($\alpha = 0.05$). Differences in colony strength (adult bee population, sealed, and unsealed brood cells) among over-wintered colonies were compared using ANOVA and a posteriori comparisons (Tukey–Kramer HSD; $\alpha = 0.05$).

The relationship between disease severity and honey yield was modeled using linear regression. Honey yield was transformed using a Log_{10} transformation to stabilize the variance and normalize the data.

The effect of time on disease severity (square root transformed to stabilize variances in the sample data), adult bee mortality, adult bee populations, sealed, and unsealed brood cells between treatment groups was compared using repeated measures ANOVA. The Huynh–Feldt correction was used, as assumptions of sphericity were not met. Contrast analysis was used to compare the differences within treatment groups for disease severity. All analyses were performed using JMP (7.01, SAS Institute, Gary, NC).

Results

Disease Severity and Adult Bee Mortality. The mean number of total chalkbrood mummies collected daily from the traps and bottom boards (Fig. 1) differed significantly between the low and inoculated treatment groups and the uninoculated, medium, and high treatment groups ($F = 112.5413$; df = 4, 3526; $P < 0.0001$). The inoculated and low treatment groups produced between 12.5 and 13.7 mummies daily, over 16 times more than the uninoculated and high treatment groups and four times more than the medium treatment group. Significantly higher numbers of black mummies were collected ($F = 102.48$; df = 4, 3526; $P < 0.0001$) from colonies in the inoculated treatment group than the low treatment group but significantly higher numbers of white mummies were collected ($F = 87.75511$; df = 4, 3526; $P < 0.0001$) from the low treatment colonies than the inoculated treatment group. Both the low and inoculated treatment groups produced significantly more black and white mummies than the uninoculated, medium, and high treatment groups. Numbers of both black and white mummies collected daily did not differ significantly between the uninoculated, medium, and high treatments and ranged between 0.38–1.8 and 0.32–1.04 mummies, respectively.

Similar to the daily collections, the total numbers of chalkbrood mummies counted in the brood frames during the weekly colony inspections ($F = 14.2929$; df = 4, 489; $P < 0.0001$) were significantly higher in the inoculated and low treatment group brood frames than in the uninoculated, medium, and high treatment groups (Fig. 2). On average, 34–37 mummies per week were counted in the inoculated and low treatment brood frames compared with the 3–8 mummies in the brood frames in the other treatment groups. Similar to the total chalkbrood counts, the numbers of both black and white mummies counted separately in the uninoculated, medium, and high treatment groups were similar and significantly lower than the inoculated and low treatment groups ($F_{\text{Black Mummies}} = 12.34$, df = 4, 489, $P < 0.0001$; $F_{\text{White Mummies}} = 9.63$, df = 4, 489, $P < 0.0001$). The numbers of white mummies in the inoculated and low

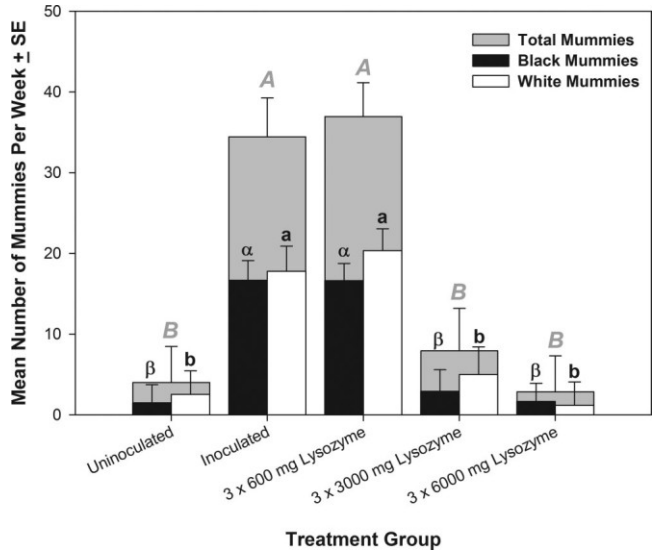

Fig. 2. The mean number of visible chalkbrood mummies (black and white) in brood frames counted weekly for each treatment group from 14 May to 28 August 2007. The numbers of total, black, and white mummies counted were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey–Kramer HSD). For each category different letter styles were used to indicate significance: total mummy production (gray, uppercase, italicized letters, *A*); black mummy production (black, lowercase, Greek letters, α); white mummy production (black, lowercase letters, a).

treatment groups averaged 17.8–20.3 per week and were higher than the number of black mummies counted. In comparison, the uninoculated, medium, and high treatment groups had extremely low levels of mummies in the brood frames ranging from 1.5 to 2.9 black mummies and 1.2–5.0 white mummies per week.

Numerical differences in the number of black mummies visible in the brood frames compared with the number of visible white mummies was not significant among treatment groups ($F = 0.35$; df = 4, 489; $P = 0.84$) over the entire duration of the experiment ($F = 0.7615$; df = 1, 986; $P = 0.3831$) but the differences in the number of black mummies compared with the number of white mummies collected daily from the traps and bottom boards were significant ($F = 88.3566$; df = 1, 7060; $P < 0.0001$) (Fig. 3). In the daily counts significantly more black than white mummies, 7.4 more per day, were collected from the inoculated colonies compared with the other treatment groups ($F = 65.85$; df = 4, 3526; $P < 0.0001$).

A closer examination of the nontransformed daily average of total mummies collected in each treatment

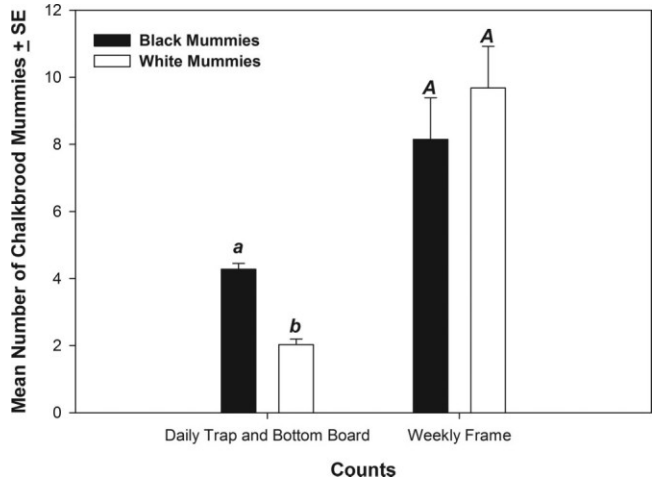

Fig. 3. The mean number of black and white chalkbrood mummies collected daily from the traps and bottom boards or counted weekly in brood frames for all colonies in the experiment ($n = 33$) from 14 May to 28 August 2007. Daily and weekly counts were analyzed separately by ANOVA. Letters denote a significant difference ($\alpha = 0.05$) in the number of black mummies compared with the number of white mummies counted within each category.

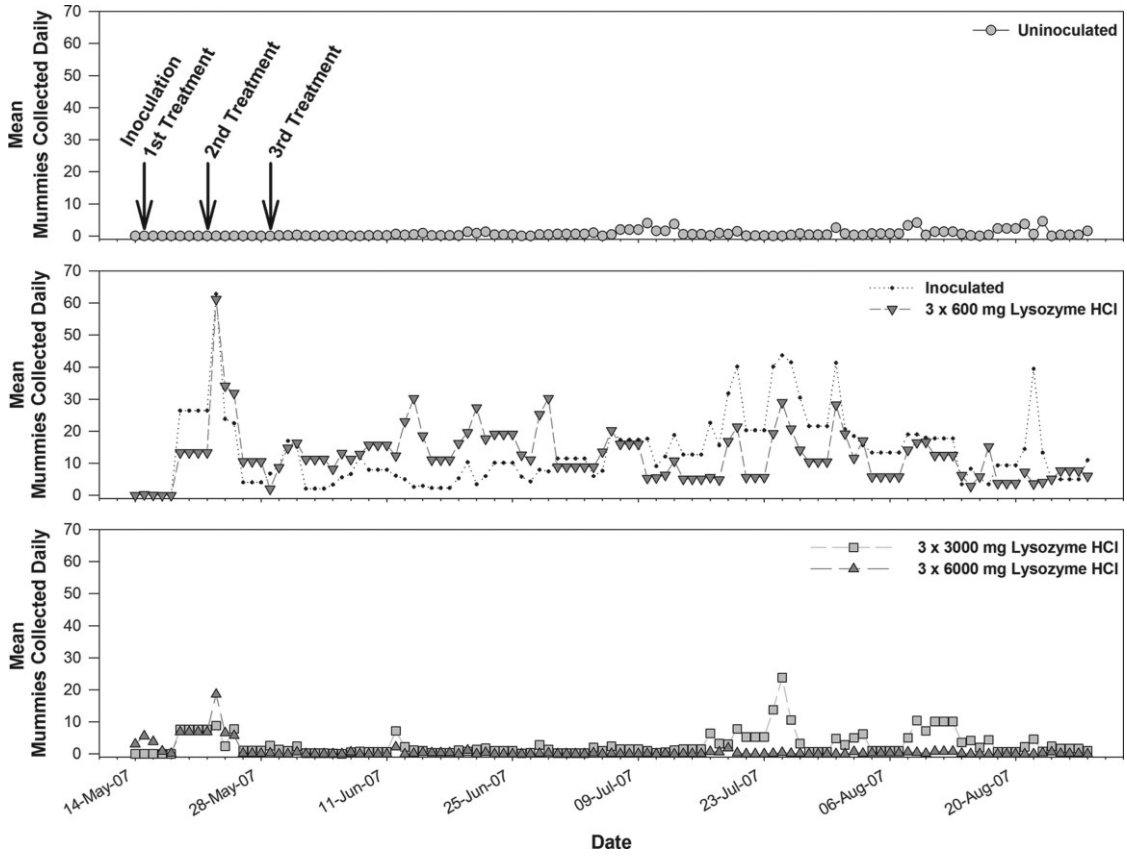

Fig. 4. Mean number of chalkbrood mummies collected daily from traps and bottom boards for each treatment group from 14 May through 28 August 2007. Pollen patties (113 g) containing homogenized chalkbrood mummies were applied to all inoculated treatment groups (Inoculated, Low, Medium, High) and clean patties to the uninoculated treatment group (Uninoculated) simultaneously with first treatment of lysozyme-HCl on 15 May 2007. All treatment groups received three weekly treatments of 50% (wt:vol) sucrose syrup containing 0 (Uninoculated, Inoculated), 600 (Low), 3,000 (Medium), or 6,000 mg (High) of lysozyme-HCl.

group over the period of 14 May through 28 August 2007 reveals some general trends (Fig. 4). Five days postinoculation chalkbrood mummies appeared in the traps and bottom boards of all inoculated treatment groups and peaked in number 9 d after inoculation. Mummy count decreased almost immediately and 12 d postinoculation, the medium and high dose treatment groups were producing extremely low levels of mummies until late July when an increase and fluctuation in mummy counts was recorded for the medium treatment group. Mummy counts in the inoculated and low treatment colonies also decreased 12 d postinoculation but mummy levels fluctuated in both treatment groups for the remainder of the experiment. The peak in mummy production at the end of July, 10 wk postinoculation, seen in the medium treatment group was also seen in the low and inoculated treatment groups. The uninoculated colonies produced minimal chalkbrood mummies for the entirety of the experiment but 5 wk postinoculation small increases in mummy count began and occurred sporadically for the remainder of the experiment. Although repeated measures analysis of daily mummy counts (square root transformed data) over the duration of the experiment (106 d) shows a marginally nonsignificant treatment effect ($F = 2.63$; df = 4,28; $P = 0.0552$), it does reveal a significant time effect ($F = 6.43$; df = 7.12, 199.43; $P < 0.0001$) and significant time by treatment interaction ($F = 1.57$; df = 28.48, 199.43; $P < 0.0406$). Posthoc comparisons reveal a significant difference in the effect of the treatment over time ($F = 3.05$; df = 7.12, 199.43; $P = 0.0043$) when the uninoculated, medium, and high dose treatment groups are contrasted with the inoculated and low treatment groups.

Within each treatment group the cumulative number of mummies collected daily varied among colonies. Regardless of scale, there were one or two colonies in each treatment group that produced at least two or three times more mummies than the other colonies. In each of the inoculated and low treatment groups there were colonies that produced >4,000 mummies over the duration of the experiment, 10 times the maximum amount produced by any colony in the uninoculated or high treatment groups.

There were no significant differences in the mean adult bee mortality among treatment groups ($F = 1.65$;

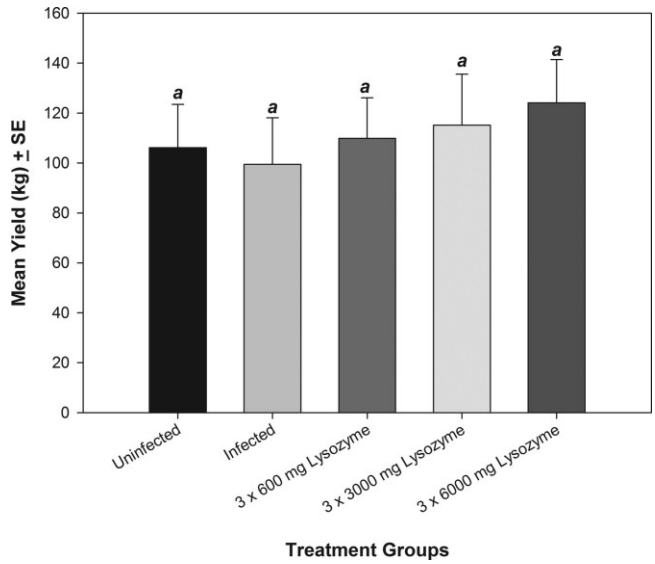

Fig. 5. Mean honey production (kilogram) per colony compared among treatment groups. Honey was collected for the duration of the trial (14 May through 28 August 2007). Treatments were not significantly different at $\alpha = 0.05$ (Tukey–Kramer HSD).

df = 4, 3526; $P = 0.1597$). Adult bee mortality ranged from 8.7 bees/d in the inoculated treatment group to 10.5 bees/d in the uninoculated colonies. Repeated measure analysis of adult bee mortality over time (14 May through 28 August 2007) did not show a significant treatment effect ($F = 0.5639$; df = 4, 28; $P = 0.6908$) or time by treatment interaction ($F = 0.8136$; df = 68.885, 482.2; $P = 0.8547$). There was a significant time effect ($F = 20.4678$; df = 17.221, 482.2; $P < 0.0001$) corresponding to increased adult bee mortality across all treatment groups on the day after colony inspections and assessments.

Colony Strength. Repeated measures analysis on number of adult bees, number of sealed brood cells, and number of unsealed brood cells counted every 2 wk from the start of the experiment until 10 July 2007 showed a significant effect of time for all three measures of the population ($F_{\text{Adult}} = 160.27$, df = 3.68, 102.91, $P < 0.0001$; $F_{\text{Sealed}} = 56.78$, df = 3.87, 108.31, $P < 0.0001$; $F_{\text{Unsealed}} = 35.89$, df = 2.82, 78.87, $P < 0.0001$). In general, all measures of population increased in number over time except adult bee populations, which increased until 26 June and decreased 10 July. There was no significant treatment effect ($F_{\text{Adult}} = 0.4732$, df = 4,28, $P = 0.7550$; $F_{\text{Sealed}} = 0.0350$, df = 4, 28, $P = 0.9975$; $F_{\text{Unsealed}} = 0.2296$, df = 4, 28, $P = 0.9195$) or time by treatment interaction for adult bee, sealed brood, or unsealed brood numbers ($F_{\text{Adult}} = 0.7467$, df = 14.70, 102.91, $P = 0.7292$; $F_{\text{Sealed}} = 0.8458$, df = 15.47, 108.31, $P = 0.6283$; $F_{\text{Unsealed}} = 1.10$, df = 11.27, 78.87, $P = 0.3738$).

Honey Production. Although mean honey yield did not differ significantly among treatment groups ($F = 0.2703$; df = 4,28; $P = 0.8946$), there was a mean increase in honey yield as the amount of lysozyme-HCl applied to the colonies increased (Fig. 5). Honey yields ranged from 99.5 kg in the inoculated untreated treatment group to 124.2 kg in the inoculated high dose treatment group. Nevertheless, there was a significant ($F = 16.03$; df = 1,31; $P = 0.0004$) and correlated ($R^2 = 0.34$) relationship between the total mummies collected from traps and bottom boards and honey yield. As the number of mummies collected increased, honey yield decreased (Fig. 6).

Stability and Persistence of Lysozyme-HCl. Lysozyme-HCl activity was detected in all treatment groups given lysozyme-HCl (Fig. 7) and not found in the treatment groups given only sugar syrup. The amount of lysozyme-HCl detected in the stored food samples increased over time in a dose-dependent manner. Repeated measures analysis on the amount of lysozyme-HCl detected over time showed a significant treatment effect ($F = 45.88$; df = 4,28; $P < 0.0001$), time effect ($F = 5.81$; df = 2.36,66; $P = 0.003$), and treatment by time interaction ($F = 3.63$; df = 9.43,66; $P = 0.0008$). Posthoc comparisons revealed a significant difference in the effect of treatment over time when the high dose treatment group was contrasted with both of the medium and low dose groups ($F = 9.07$; df = 2.36,66; $P = 0.0002$).

Presence of Spores. Spores were present in all treatment groups in either the larvae or adult bee digestive tracts over all dates sampled.

Winter Survival. There were no significant differences in the percentage of colonies surviving the winter among treatment groups ($\chi^2 = 6.18$; df = 4,33; $P = 0.1502$); winter survival ranged from 57% in the uninoculated treatment group to 100% in the inoculated and medium dose treatment groups. Survival in the low and high treatment groups was 88 and 86%, respectively.

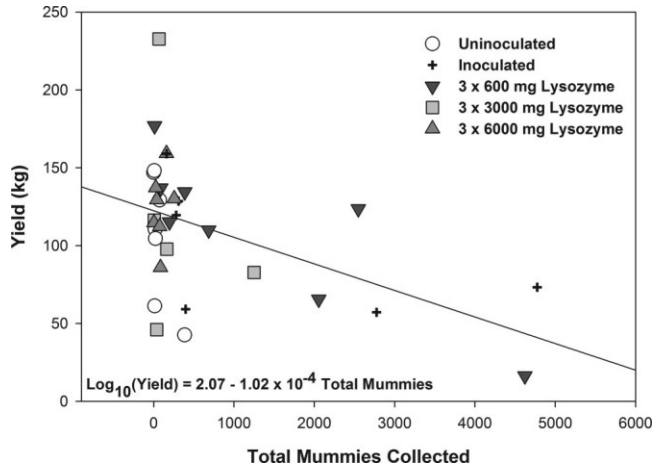

Fig. 6. The relationship between total mummies collected daily from traps and bottom boards and honey yield (kilogram). Each data point represents an individual colony (n = 33). Data presented in the figure is untransformed.

At the time of inspection, 14 May 2008, there were no significant differences in the adult bee populations (F = 0.4643; df = 4, 23; P = 0.7612), number of sealed brood cells (F = 0.4497; df = 4, 23; P = 0.7715), or unsealed brood cells (F = 0.5731; df = 4, 23; P = 0.6849). There were no significant differences among treatment groups in the number of total (F = 1.088; df = 4, 23; P = 0.3857), black (F = 0.8952; df = 4, 23; P = 0.4828), or white (F = 1.279; df = 4,23; P = 0.3071) chalkbrood mummies visible in the brood frames when inspected on 14 May 2008 (Fig. 8). However, in the inoculated and low treatment groups total mummy counts visible in the brood frame averaged between 9.5 and 26.7, higher than the other treatment groups that averaged 0.5–1.8 chalkbrood mummies per colony.

Discussion

Lysozyme-HCl in the medium (3,000 mg × 3 doses) and highest (6,000 mg × 3 doses) doses tested was capable of suppressing mummy production in artificially inoculated colonies to levels similar to that of uninoculated untreated colonies. Mean daily mummy production was 15 times lower in the inoculated high dose treatment group than in the inoculated untreated colonies. Although all inoculated treatment groups saw a dramatic increase in mean mummy production 5 d postinoculation and a sharp decrease 1 wk later, it was only the highest dose treatment group that suppressed mummy production until the end of the summer. Long-term suppression in the high dose treatment group may be the result of the significantly

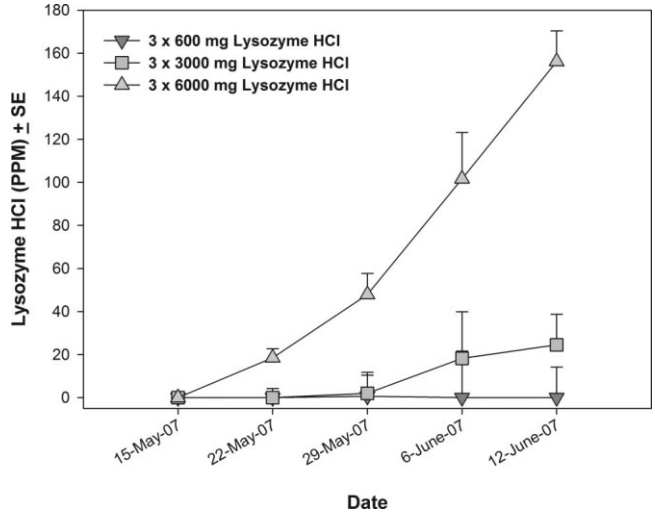

Fig. 7. The mean amount (parts per million, ppm) of lysozyme-HCl detected in the stored food collected from the outer edges of three brood frames of each colony compared among treatment groups that were administered three dosages of lysozyme-HCl. Colonies were sampled before the first treatment was applied and then weekly for 4 wk afterwards.

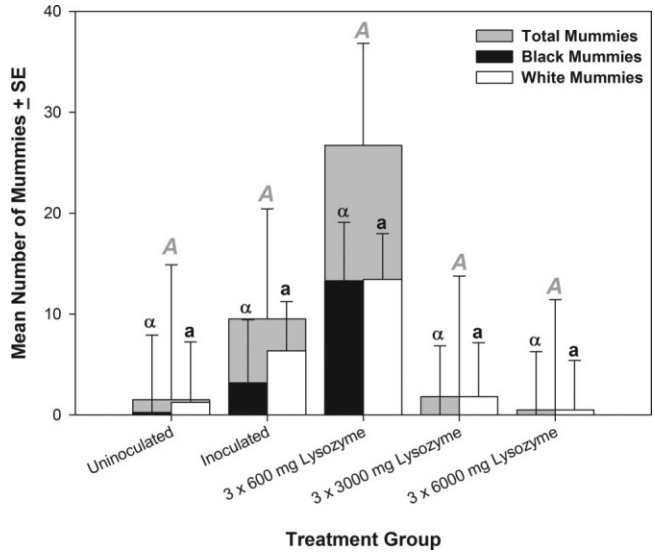

Fig. 8. The average number of visible chalkbrood mummies (black and white) in brood frames counted at the end of the experiment (14 May 2008) for each treatment group. Total, black, and white mummies from each treatment group were analyzed separately. Treatments with different letters are significantly different at $\alpha = 0.05$ (Tukey–Kramer HSD). For each category different letter styles were used to indicate significance: total mummy production (gray, uppercase, italicized letters, ***A***); black mummy production (black, lowercase, Greek letters, α); white mummy production (black, lowercase letters, a). For total (***A***), black ($\boldsymbol{\alpha}$), and white (a) mummy counts.

higher levels of lysozyme-HCl built up in the stored food cells surrounding the brood comb. The lowest dose (600 mg × 3 doses) of lysozyme-HCl assessed was ineffective and produced high numbers of chalkbrood mummies in artificially inoculated colonies throughout the entirety of the experiment.

Mummy numbers in the uninoculated untreated colonies were low throughout the experiment but did show slight increases in July and August. Drifting, the movement of adult bees from one colony into another, could be one explanation of how *A. apis* spores were transmitted into uninoculated colonies. Similarly, spores could have been transferred at forage sites common to the apiary (Heath 1982). However, spores were detected at the start of the experiment in the larvae and worker bee guts before inoculation indicating a preexisting chalkbrood infection. It is common for low levels of *A. apis* spores to be detected in colonies asymptomatic for chalkbrood disease (Gilliam 1986). The source of *A. apis* spores in the colonies preinoculation was likely the package bees imported from New Zealand as the equipment the bees were established in was disinfected by irradiation.

Although predominantly black mummies were collected from the dead bee traps and bottom boards, similar numbers of black and white mummies were counted in the weekly frame inspections. Additionally, the number of black to white mummies collected daily was significantly higher in the inoculated untreated and low dose colonies compared with the other treatment groups. The color variation in chalkbrood mummies is because of the presence of spore cysts that are brown to black when mature; mummies remain white if the fungus does not mate and produce spores (Gilliam et al. 1988). Diseased larvae left to sporulate and harden into black mummies contain as many as 1×10^8 *A. apis* spores (Nelson and Gochnauer 1982), and if not removed provide a constant source of spores for reinfection. The higher level of black mummies and spores in the colony feeds back into and intensifies the natural disease cycle, and may cause the fluctuating levels of mummies seen in the inoculated untreated and low dose treatment groups throughout the experiment. The natural infection cycle may have been interrupted as the chalkbrood mummies that were collected daily were removed and discarded. Mummies left on the bottom boards and at the entrance of colonies provide a reservoir of spores that left uncollected could have increased disease severity in infected colonies or increased transmission of chalkbrood disease in uninoculated colonies.

Because the frame inspections involved counting only what was visible, chalkbrood mummies in capped cells were not counted. The differences between the daily collections and weekly frame counts may mean that once detected and uncapped, black mummies are removed faster than white mummies. The detection and removal of diseased larvae or hygienic behavior is a heritable colony-level trait important in disease resistance (Rothenbuhler 1964) and studies have shown that colonies that exhibit this behavior are more resistant to chalkbrood (Gilliam et al. 1983, Spivak and Reuter 1998). Hygienic behavior is a quantitative trait influenced by multiple loci (Lapidge et al. 2002) and individual worker bees within a colony have different response thresholds to stimuli that trigger the uncapping of the brood cell and the removal of the larva (Oxley et al. 2010). Olfactory sensitivity to chalkbrood

odors is higher in honey bees bred for hygienic behavior than in bees from nonhygienic lines (Masterman et al. 2001, Gramacho and Spivak 2003) and recently, it was shown that the volatile compound phenethyl acetate isolated from larvae infected with *A. apis* (presporulation) was capable of inducing hygienic behavior in the field (Swanson et al. 2009). If infected larvae are removed before *A. apis* sporulates, the disease cycle is interrupted. However, it is not known if black sporulated mummies have other volatiles not found in infected nonsporulated larvae or if they have a greater quantity of the volatile phenethyl acetate. The colonies in this experiment were not assayed for hygienic behavior and it was not known which colonies were naturally resistant to chalkbrood disease. Although the queens were all imported from the same source, the genetic background and relatedness of the queens used in the field trial was unknown. However, it was noted that within all treatment groups, one or two colonies appeared to be more susceptible to chalkbrood disease than the others regardless of treatment that may be a result of genetic variability in the population.

Genetic variation in honey production among colonies is also well documented (Guzmán–Novoa and Gary 1993) and may be one reason why there was high variability in honey production within treatment groups. There were no significant differences among treatment groups in mean honey production even though the inoculated high dose treatment group produced on average 24 kg more than the inoculated untreated group and surprisingly, 18 kg more than the uninoculated untreated colonies. As the dose of lysozyme-HCl applied to the colony increased, so did mean honey production. It may be that lysozyme-HCl is having a positive effect on colony disease suppression and production unrelated to chalkbrood suppression. Although not significant, from a management perspective an 18–24 kg increase in mean honey yield per colony is economically important and reflects a $60–90 increase per colony at current honey values (U.S. Department of Agriculture [USDA] 2012) especially when the cost of the highest treatment administered (6,000 mg $\times$ 3 doses lysozyme-HCl) is less than $0.50.

Although there were no significant differences in mean honey production among treatment groups there was a moderately correlated significant negative relationship between total mummies collected and honey production. As chalkbrood disease increases in severity, increased larval death should translate into fewer adult worker bees and overall lower colony productivity but the consequences of larval death may not be immediate or straightforward. The honey bee colony can be considered a superorganism where individual actions are determined by colony needs (Seeley 1989). Division of labor in honey bee colonies is age-related and different age castes task specialize. However, worker bees demonstrate great flexibility in performing age-related tasks important to colony fitness throughout their lifetime (Winston 1987). Foraging is generally the final task a worker bee performs in her lifetime but in the absence of an older aged cohort, younger bees will become precocious foragers. Conversely, in the absence of young worker bees, older aged workers will switch to brood tending and other tasks typically performed by younger bees (Seeley 1989). The flexibility of the worker bee population is one factor that contributes to colony resilience when dealing with disturbance. Combined with the ability of the queen bee to lay 1,000–2,000 eggs per day (Bodenheimer 1937) it may mean that the impact of chalkbrood disease is minimal unless severe and prolonged. Additionally, if there is a lag between larval death and adult worker bee reduction, it may be that in the Peace River region where the honey flow is brief but intense (Pankiw 1968), that chalkbrood disease severity will not be a good predictor of honey production. This is illustrated by the comparison of two colonies within the same treatment group; one colony that produced 2,548 mummies over the entire experiment yielded 38 kg more honey than a colony that produced only 84 chalkbrood mummies.

Nelson and Gochnauer (1982) hypothesized that in years of high honey production, infection levels required to cause economic loss must be higher than in years with low honey production. Honey yields in the province of Alberta and specifically the Peace region of northern Alberta where this study was conducted have been historically high. In the last decade (2001–2011) Alberta has produced 40% of Canadian honey yields (Statistics Canada 2002–2005, 2006, 2007–2011) and the Peace region alone produced 11% of total Canadian honey production between 2007–2010 (Alberta Agriculture and Rural Development 2007–2010). The mean honey yield per colony in the Peace region during that time period was 65 kg per colony, 10 kg more than the overall Albertan average, and 12 kg more than the average Canadian yield. It may be that in northern Alberta where honey production is high, chalkbrood disease will have minimal economic impact.

There were no significant differences in adult bee populations, sealed or unsealed brood cells between treatment groups at any of the dates measured. The buildup of adult bee populations peaked 26 June ($\approx$14,500 bees) coinciding with the start of major nectar flow in the Peace River region (Pankiw 1968) and decreased on 10 July. A previous study on package bee colonies in Manitoba (Nelson and Jay 1972) showed similar numbers of adult bees on 21 June 1969 and 22 June 1970 (15,000–20,000 bees) but the populations continued to increase throughout the summer and did not experience the decline observed in this experiment. Package colonies in the Canadian Prairies take 110 d postestablishment to reach maximum population levels of 45,000–50,000 bees (Nelson and Jay 1982). Population measurements ended 10 July before colonies reached maximum population levels and the effects of chalkbrood disease on colony strength for the entire season was not assessed. However, colony populations were assessed one year later on 14 May 2008 and there were no significant differences among treatment groups. Additionally, winter survival of col-

onies did not differ significantly among treatment groups and although the mean number of chalkbrood mummies counted in the brood frames of the colonies were higher in the inoculated untreated and low dose treatment groups, it was not significant.

Trends in the pathology of the disease in the colony mirrored the results of Taber (1986) who used the same method of artificial inoculation and observed emergence of chalkbrood mummies 3 d postinoculation and complete removal by 3 wk in chalkbrood-susceptible colonies. Although there was a decrease in the number of mummies collected in this experiment 2 wk postinoculation, unlike Taber (1986) and Gilliam et al. (1988), some colonies never fully recovered. The use of chalkbrood mummies as an inoculant in field studies is problematic as mummies are not a pure source of *A. apis* spores and can contain other molds, yeast, and bacteria (Johnson et al. 2005). The response of the colony to inoculation by homogenized mummies may not only be to chalkbrood infection but other microbes as well. It has been shown that inoculation of colonies with chalkbrood mummies is more infective than by *A. apis* spores alone (Jākobsons 2005). However, it is unlikely that colonies under natural conditions would encounter uncontaminated *A. apis* spores. In this experiment, the pathology of the disease may have also been affected by the presence of preexisting *A. apis* spores from another source. Heterogeneity in the biochemistry and virulence of different *A. apis* strains has been reported (Gilliam and Lorenz 1993, Jākobsons 2005) and it is not known if different strains also vary in their susceptibility to lysozyme-HCl.

The impact of chalkbrood disease on package colonies is variable and individual colony response to artificial inoculation with *A. apis* spores is influenced by both environmental and genetic factors. Despite the highly variable response to chalkbrood infection, lysozyme-HCl at the medium and highest doses tested significantly reduced disease severity to levels similar to that of uninoculated colonies. The highest dose (6,000 mg × 3 doses) evaluated suppressed mummy production for the entire summer. Inexpensive and easily integrated into established colony management practices, lysozyme-HCl at the highest dose evaluated is an effective control for chalkbrood disease.

Acknowledgments

The authors thank John Zhang and Neova Technologies Inc. for supplying the lysozyme-HCl and performing detections of lysozyme-HCl in the stored food samples. Special thanks are owed to Andony Melathopoulos, Wendy Walter, Peter Mills, and Sterling Smith for their technical assistance. We also thank Devon Coupland, Robert Annett, and Brandon Smith, our summer students, for their contributions to field experiments. Funding was provided by Neova Technologies Inc., the Canadian Bee Research Fund, the Alberta Beekeepers' Commission, Bee Maid Honey, and the Matching Investment Initiative of AAFC.

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Received 5 June 2012; accepted 31 August 2012.